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**The Optimisation, Characterisation and Platelet
Compatibility of Polymeric
Nanocarriers Loaded with NSAIDs**

by

Joanne Crean, B.Sc. (Pharm.), MPSI, Dip. Stat. Q.I.

**being a thesis submitted for the degree of
Doctor of Philosophy in Pharmacy and Pharmaceutical Sciences**

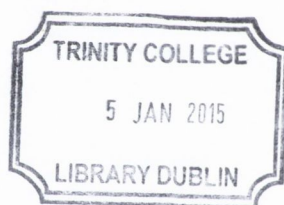
at

University of Dublin, Trinity College

**under the supervision and direction of
Lidia Tajber, M. Sc. (Pharm.), Ph.D., Dip. Stat. Q.I.**

January 2014

To my parents Edmund and Geraldine Crean



Thesis 10764

Declaration

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Joanne Crean

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Abbreviations and Symbols

CS	chitosan chloride CL113
HA	hyaluronic acid
PLGA	poly(lactic-co-glycolic acid)
TPP	tripolyphosphate
NP	nanoparticles
PECNs	polyelectrolyte complex nanoparticles
IND	Indometacin
KET	Ketoprofen
PDI	polydispersity Index
ZP	zeta-potential
pl	isoelectric Point
w/w	weight per weight
w/v	weight per volume
v/v	volume per volues
nm	nanometres
ul	microliters
ADP	adenosine diphosphate
DL	drug loading
EE	encapsulation efficiency
SEM	scanning electron microscopy
TEM	transmission electron microscopy
PVA	polyvinyl acetate
Aq	aqueous
mV	millivolts
pDNA	plasmid deoxyribonucleic acid
Caco-2	
kDa	Kilo daltons
Da	daltons
RES	reticuloendothelial system
Au	gold
BBB	blood-brain barrier
%DA	% deacytlation
DSC	differential scanning calorimetry
PXRD	powder x-ray diffraction

FTIR	Fourier transform infrared spectroscopy
DLS	dynamic light scattering
NC	nanocarrier
PALS	phase analysis light scattering
HF-DSC	heat flux differential scanning calorimetry
PC-DSC	power compensation differential scanning calorimetry
PRP	platelet rich plasma
PPP	platelet poor plasma
CL113	Protasan chitosan chloride
DLS	dynamic light scattering
HPLC	high performance liquid chromatography
°C	degrees celcius
%DA	degree of deacytlation
NMR	nuclear magnetic resonance
PE	polyelectrolyte
VA	van der Waals attractive forces
VR	electrical double layer repulsive forces
G	glutamate
Neg	negative charge
Pos	positive charge
MR	mass ratio
CR	charge ratio
pH	$pK_a - \log (A^-)/(salt)$
pKa	$-\log_{10}K_a$

Summary

There is a constant drive towards improving global health by continuously enhancing the quality of drugs available and the systems to deliver these drugs efficiently in order to treat disease. This thesis focused on the development of two types of biodegradable polymeric nanocarriers (NCs), poly(lactic-co-glycolic) acid (PLGA) nanoparticles (NPs) and hyaluronic acid:chitosan (HA:CS) polyelectrolyte complex nanoparticles (PECNs) loaded with two hydrophobic model drugs with anti-platelet action, indometacin (IND) and ketoprofen (KET). PLGA NPs were a hydrophobic system formed by an emulsion–solvent diffusion technique and HA:CS PECNs, which were hydrophilic systems formed based on electrostatic interactions between two polymers.

Firstly, the polymeric NC systems were optimised without the presence of drug before loading them with KET and IND at different concentrations (0.1 mg/mg, 0.2 mg/mg and 0.4 mg/mg). Both, formulation and process parameters involved in the NC formation were optimised. Post-optimisation physical characterisation of the polymeric systems was conducted using dynamic light scattering, laser doppler velocimetry followed by high performance liquid chromatography, which was used to measure drug loading (DL) and release. The solid-state properties of these systems were investigated by differential scanning calorimetry (DSC) and powder X-ray diffraction. Further investigations were carried out for HA:CS PECNs using optical microscopy to look at polymer/drug miscibility as well as using DLS to investigate the particle/crystal growth. Particle morphology was visualised by scanning electron microscopy (SEM) and transmission electron microscopy (TEM). Interactions between these NCs, without or with loaded drug, and human platelets were studied using light transmission aggregometry and flow cytometry.

PLGA NPs were successfully optimised using PLGA RG 504H as the polymer, ethyl acetate as the solvent, 45 ml of Pluronic F68 1% w/v solution as the surfactant phase and two washing steps involving centrifugation at 13,000 rpm for 15 mins. PLGA NPs were loaded with IND and KET and the highest DL values achieved were $19.0 \pm 0.4\%$ for IND at 0.2 mg/mg and $12.0 \pm 3.0\%$ for KET at 0.2 mg/mg. PLGA NPs loaded with IND and KET had a higher particle size than unloaded NPs with a maximum size of 344 ± 2 nm being reached, the

ZP also increased when drug loaded to a maximum value of -29 ± 0.3 mV. SEM showed that the PLGA NPs were spherical, non-porous, smooth spheres and the shape did not change when the drug was loaded. Release studies showed a burst release within 24 hours followed by slow release of the remaining drug over a 24 day period for KET and IND. Stability studies over 14 days showed some drug release and a small reduction in particle size. All IND and KET systems were amorphous with no melting points seen on DSC or Bragg peaks visible on powder X-ray diffractograms.

The production of HA:CS PECNs was optimised and characterised to develop a positively charged HA:CS PECN formulation at a HA:CS 7.5:6.5 weight ratio and a negatively charged HA:CS PECN formulation at a HA:CS 10:4 weight ratio. The particle size regardless of charge was approximately 160 – 173 nm showing slight increases with DL and the ZP varied with charge. The optimised positively charged and negatively charged HA:CS PECNs were loaded with IND and KET at 0.1 mg/mg and 0.2 mg/mg. 0.4 mg/mg DL was possible for KET but not for IND due to poor IND solubility. Higher DL was obtained using negatively charged HA:CS PECNs for KET and positively charged HA:CS PECNs for IND. KET present in HA:CS PECNs was mainly in the amorphous/complex form but for IND it was loaded as a combination of amorphous and crystalline drug of different polymorphic forms. Loading was higher for KET than IND as the HA:CS PECNs showed a preference for hydrophilic drug. These HA:CS PECNs were quick release formulations regardless of the drug, charge or release medium used (PBS, 0.01M HCl or deionised water). Stability studies over 14 days showed a greater reduction in the particle size for positively charged HA:CS PECNs than for negatively charged PECNs.

None of the empty NCs induced platelet aggregation at a concentration of 80 ug/ml over 25 minutes. KET and IND effectively inhibited collagen (2 ug/ml) stimulated platelet aggregation when loaded into all the NC types. The inhibition was not significantly different to the free IND and KET not associated with NCs. As PLGA NPs showed a higher DL capacity than the HA:CS PLGA NPs they appeared to be more efficient NCs to target platelets. KET or IND loaded NCs incubated with platelets did not show up regulation of P-selectin expression induced by collagen. The number of P-selectin copies on platelets observed for KET and IND loaded NCs was not significantly different to the number of copies observed for free IND and KET.

Introduction

Origin and Scope

Polymeric nanocarriers (NCs) are important to consider in the progression of current drug delivery systems. There is a growing number of approved NC based products on the market (Dobrovolskaia and McNeil, 2007) and research groups internationally are investigating the potential of polymeric NCs for a diverse range of medical purposes (Panyam and Labhasetwar, 2003; de la Fuente et al. 2008 and Chan et al. 2009).

The origin of this work is in studies conducted by Li et al. (2009). Li et al. (2009) investigated the compatibility of NCs prepared from PLGA, CS and PLGA-CS with blood platelets. It has been previously reported that nanoparticles (NPs) prepared from carbon nanotubes (Radomski et al. 2005), silver (Shrivastava et al. 2009), and silica (Lin et al. 2006) induced platelet aggregation, while Miller et al. (2009) found that NPs derived from two different mammalian sources (bovine blood and human tissue homogenates) containing calcium phosphate and complexed with protein inhibited platelet aggregation. Li et al. (2009) used aggregometry and flow cytometry to test the compatibility of PLGA, CS and PLGA-CS NPs with platelets and found that the NPs did not induce platelet aggregation. In fact, they found that at high concentrations of NPs (100 ug/ml), weak inhibition (10-40%) of collagen(2 ug/ml) induced aggregation was observed.

As NCs are currently under global investigation for use in medicine, it was seen as the next step to load these NCs with anti-platelet drugs to test if the compatibility of the NCs with platelets would change following drug loading and if it would affect the action of the drugs. Two anti-platelet drugs, indometacin (IND) (Leach and Thorburn 1982) and ketoprofen (KET) (Gandini et al. 1983), were chosen as they are low molecular weight, organic, hydrophobic drugs (Bodmeier and Chen, 1989) and so were used as models to represent this class of drugs. KET and IND are poorly water soluble with the latter showing rich solid-state polymorphism (Surwase et al. 2013).

The first type of NC used in this current work was PLGA based NPs. These were chosen following on from the work of Corrigan and Li (2009) in which PLGA (50:50 RG 504 H) NPs were developed and loaded with the small, organic

molecules KET and IND. Corrigan and Li (2009) studied the mechanism of release from these PLGA NPs. They found that the PLGA NPs were acting as slow release drug delivery dosage forms in which the initial drug load located at the surface was rapidly released by burst release and then the rest of the drug load was released slowly over 20 days. This work showed that IND and KET could be loaded into PLGA NPs, which then successfully released the drug in a predictable manner. Corrigan's work did not include the optimisation steps involved in preparing their PLGA NPs and so the current studies aimed to optimise these PLGA NPs loaded with IND and KET and investigate their physicochemical properties before using them to represent a slower release formulation for the platelet aggregation studies.

The hyaluronic acid (HA):chitosan (CS) polyelectrolyte complex nanoparticles (PECNs) used for this study were developed as a progression from those prepared by Umerska et al. (2009). The HA:CS PECNs used by Umerska et al. (2009) were prepared without the use of cross-linkers and it was shown that the HA:CS PECNs could be prepared with good physical stability at specific ratios of HA:CS. Umerska et al. proved in their work that these HA:CS PECNs were compatible with Caco-2 cells as no cytotoxicity was observed at concentrations of CS below 40 ug/ml. The current studies attempted to load these HA:CS PECNs with IND and KET. These were prepared with both, negative and positive surface charges. These HA:CS PECNs were expected to be a fast release formulation and thus were considered as suitable NCs to test for the platelet aggregation experiments in contrast to the slow release PLGA NPs.

The scope of this thesis was:

- To formulate and optimise PLGA NPs loaded with IND and KET.
- To formulate and optimise HA:CS PECNs loaded with IND and KET.
- To investigate the effect of NP composition on the ability of HA:CS PECNs to associate with IND and KET.
- To analyse the solid-state form of IND and KET present in HA:CS PECNs, with particular emphasis on IND as it is less soluble than KET and exhibits solid-state polymorphism.
- To study the outcome of incubating blood platelets, pre- and post-stimulation with collagen, with PLGA NPs and HA:CS PECNs loaded with IND and KET.

Introduction

Chapter 1

1.1 Introduction to Nanomedicines

Nanocarriers (NCs) for medicinal use can vary in size from 10 to 1000 nm. Nanomedicines are usually prepared as NCs consisting of a drug and a medically approved carrier material forming drug delivery structures in the nano size range. There are many different types of NCs being developed; liposomes, polymeric nanoparticles (NPs), polyelectrolyte complex nanoparticles (PECNs), nanocapsules, nanocrystals, dendrimers, quantum dots and metallic NPs are examples. The chosen drug for association is dissolved, entrapped, encapsulated or attached to a polymeric NC (Soppimath et al. 2001). Biodegradable NCs are being heavily researched as a means to improve the therapeutic value by improving bioavailability, solubility and retention time of many water soluble/insoluble medicinal drugs and bioactive molecules (Schroeder et al. 1998, Raghuvanshi et al. 2002, Kreuter et al. 1997, Kumari et al. 2010). Kumari et al. (2010) also stated that the advantages of NCs could include protection against premature degradation and interaction with the biological environment or the enhancement of absorption into a selected tissue, bioavailability, retention time and improvement of intracellular penetration (Alexis et al. 2008). Many properties of nanomedicines can be altered to make the nanomedicine suit its purpose, for example particle size, surface charge, surface modification, and hydrophobicity. These will also influence the biological efficacy and compatibility of these nanomedicines (Brannon-Peppas et al. 2004).

Some examples of diseases that NCs have been developed to treat include cancer (Mu and Feng 2003), AIDS (Shah and Amiji 2006), diabetes (Sarmiento et al. 2007), malaria (Date et al. 2007) and tuberculosis (Ahmad et al. 2006). New types of NCs are continuously being developed by researchers to suit the needs of modern drug delivery and thus the breadth of research on NCs is rapidly expanding. Methods of preparation are very varied and the characterisation of nanomedicines has yet to be standardised. This lack of boundaries and standards means that there is a lot of creativity to be observed in the research of scientific groups working with NCs. It appears much more investigation is still required into nanomedicines in order to build up reliable evidence to govern their most suitable uses, physical stability, their toxicity and efficacy. This work aimed to investigate new types of NCs using the small hydrophobic drugs indometacin (IND) and ketoprofen (KET) in polymeric hyaluronic acid (HA): chitosan (CS)

PECNs and also to observe and compare their compatibility with blood platelets with poly(lactic-co-glycolic acid) (PLGA) NPs loaded with the same drugs. This is the first study, to the best of knowledge, looking directly at the effects of drug loaded polymeric carriers on blood platelets.

1.2 Background to the Polymers Used

1.2.1 PLGA

Poly(lactic-co-glycolic acid) (PLGA) is a biocompatible, biodegradable, aliphatic polyester that has been US Food and Drug Administration (FDA) and European Medicine Agency (EMA) approved for medical use (Danhier et al. 2012). PLGA is composed of lactide and glycolide and on degradation hydrolyses to the monomers, lactic acid and glycolic acid (**Figure 1.1**). These two monomers are endogenous and easily metabolised by the body via the Krebs cycle and thus PLGA used for drug delivery is associated with low toxicity (Kumari et al. 2010). PLGA is available in different forms, which are usually defined by the molecular weight, copolymer composition and the PLGA end-groups used (**Table 1.1**). The difference in composition affects the degradation time, which can vary from several months to several years, depending on the molecular weight and copolymer ratio (Prokop and Davidson 2008, Vert et al. 1994)

The different ratios of lactide and glycolide monomers, PLA stereochemistries, and end-group functionalisation means that different types of PLGA display different properties ranging from stiff, semi-crystalline materials with long degradation times, to softer, amorphous materials with faster degradation rates (Danhier et al. 2012).

PGA is a highly crystalline material with a melting point at 225 - 230 °C and low solubility in organic solvents which depends on polymer molecular weight. It is still susceptible to hydrolysis due to the ester bond in the polymer backbone (Middleton et al. 1998). PLA is a chiral molecule with an L and a D form. Polymerisation of these individual monomers results in semi-crystalline monomers but a racemic mix of D- and L- lactide results in poly-D,L-lactide (PDLLA), which is amorphous with a glass transition temperature (T_g) of

approximately $42.5 \pm 0.5^{\circ}\text{C}$ (Passerini and Craig 2001). This means that the PLA stereochemistry can have a major effect on the polymer properties, processability and biodegradability (Athanasίου et al. 1996).

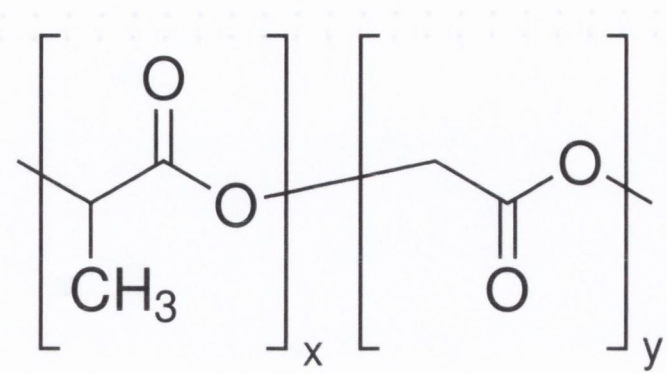


Figure 1.1 PLGA structure and its component lactic acid and glycolic acid monomers. x = number of units of lactide acid, y = number of units of glycolic acid.a

Table 1.1 Key parameters and corresponding effects on PLGA properties

Parameter	Influence	Reference
Molecular Weight	High M_w increases the degradation time	Vert et al. 1994
Ratio Lactide/Glycolide	Polymers with one monomer degrade more slowly. Degradation times: PLA > PGA > PLGA 50:50	Cohn et al. 1987
Stereochemistry	L-PLA: semicrystalline D,L-PLA: amorphous	Athanasίου et al. 1996
Blockage of Acidic Endgroups	Polymers with free -COOH groups are more hydrophilic (e.g. R503H compared to R503)	Houchin 2008
PEGylation	Increase in hydrophilicity, change of degradation and release behavior	Lochmann et al. 2008

Degradation of PLGA occurs by ester hydrolysis. Initially water starts to diffuse into the polymer triggering ester linkage hydrolysis, which cleaves the PLGA chain into smaller chain lengths. This degradation eventually produces smaller chain segments (<100 g/mole), which can dissolve resulting in polymer erosion (Mäder et al. 1997a). Finally excretion of the solubilised monomers/oligomers takes place via the kidney or else they are metabolised into carbon dioxide and water. This means at the end of the degradation process PLGA will have been completely absorbed and eliminated from the body, which is a highly desired feature for polymers used in drug delivery (Mäder et al. 1997b). Another method of degradation observed for PLGA is when there is an associated material, often encapsulated, which is acidic in nature. This can create an acidic microclimate within the PLGA which autocatalyses degradation from within the PLGA polymer. This has also been observed if alkaline drugs are associated (Brunner et al. 1999, Siepmann et al. 2005, Sophocleous et al. 2009). The ratio of glycolide to lactide at different compositions allows control of the degree of crystallinity of the polymers (Cohn et al. 1987). This ratio determines the degradation time of the copolymer. When the glycolide content is higher, the rate of degradation is faster except at the 50:50 ratio of PGA: PLA, which exhibits the fastest degradation, although the reason is not fully understood (Park et al. 1995, Miller et al. 1977). The end-group of PLGA is varied between a free carboxylic acid groups an ester terminated group and an alkyl ester group with the free carboxylic analogues showing the fastest degradation rates (Houchin 2008).

PLGA has been used in the medical industry often in implants or micro/nanoparticles for drug delivery. An example of the implants developed is a dexamethasone-loaded PLGA intravitreal implant (Ozurdex) prepared by Boyer et al. (2011) which has reached the market to treat macular oedema in the eye. Micro/nano particle development also heavily uses PLGA, mostly for the parenteral delivery of hydrophilic or hydrophobic drugs (Corrigan et al. 2009).

1.2.2 Chitosan (CS)

Chitosan (CS) is a polysaccharide formed by deacetylation of the natural polymer chitin to a degree of 40%-98% (Illum et al. 1994). It is a linear monosaccharide consisting of 2-amino-2-deoxy- β -D-glucan units joined by glycosidic linkages (**Figure 1.2**).

CS production begins by extracting chitin from crab or shrimp by the processing of crustaceans shells. The extracted chitin is deacetylated in 40% sodium hydroxide at 120°C for 1–3 hrs to produce chitosan, which is approximately 70% deacetylated (Madhavan and Nair 1974). The degree of deacetylation (%DA) can be determined by nuclear magnetic resonance (NMR) spectroscopy and the %DA in commercial CS products is in the range 60-100%. CS stands out from most naturally occurring polysaccharides because it is a basic polysaccharide (pKa=6.5) while the majority are acidic (Di Martino et al. 2005). As a result of its basic nature CS is positively charged, soluble in acidic to neutral solution and has a charge density dependent on pH and the %DA value (Hench 1998). CS derivatives are easily obtained under mild conditions. CS chloride salt was used in the present work, as it is water-soluble.

Degradation of CS *in vivo* is often due to enzymatic hydrolysis of the acetylated residues primarily by lysozyme (Di Martino et al. 2005). This implies that higher levels of deacetylation will increase the resistance to degradation. Degradation results in CS oligosaccharides.

CS has been used for a wide range of biomedical applications. Some examples of the uses of CS include tissue engineering and tissue repair, wound healing, and the delivery of therapeutic molecules e.g. cyclosporine (Shabouri et al. 2002) and insulin (Mao et al. 2005). Currently, nasal delivery technology with chitosan shows great potential, with formulations even being tested in humans e.g. morphine hydrochloride in chitosan microspheres as a powder formulation for nasal delivery (Illum et al. 2002).

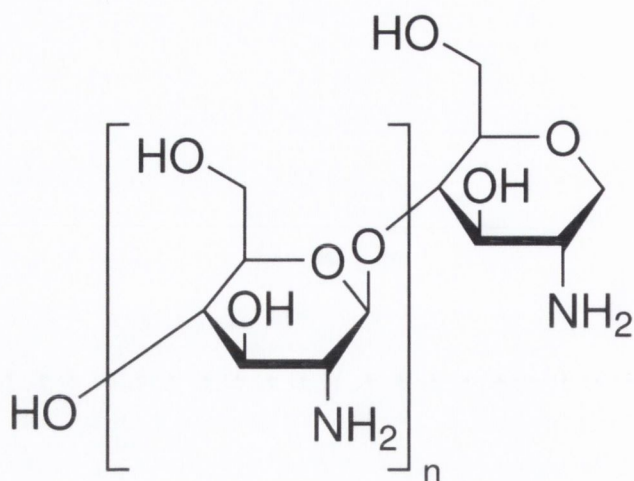


Figure 1.2. Chemical structure of deacetylated chitosan.

1.2.3 Hyaluronic Acid (HA)

Hyaluronic acid (HA) is a linear, unbranched polysaccharide consisting of glucuronic acid (GlcUA) and N-acetyl glucosamine (GlcNAc) units linked by a β 1-4 glycosidic bond (**Figure 1.3**). The disaccharides are linked by β 1-3 bonds to form the HA chain (Weigel et al. 1997 and Weissmann, 1954). HA is present in the extracellular matrix (ECM), connective tissues and synovial fluid, being discovered by Meyer and Palmer in 1934 in the vitreous humour of cattle eyes. It has a pKa of 2.9 and is negatively charged above this pH due to ionisation of the carboxylic acid groups on the polymer chain. Its hydrophilic chemical nature means that HA molecules can expand in volume up to 1000 times and form loose hydrated matrices in water (Lapcik and Lapcik 1998, Brown and Jones 2005). Another positive aspect to using HA is that HA molecules present the same structure in all species and all tissues and so exhibit low immunogenicity (Matarasso 2004).

The isolation and purification of nearly pure HA was originally conducted by freezing umbilical cords and rooster combs to destroy the cell membranes at which point HA was extracted with water and precipitated in organic solvents (Balazs 1974 and 1979). Since then the bacterial production of HA by *Streptococcus equi* (Mashimoto et al. 1988) and *Streptococcus zooepidemicus* (Akasaka et al. 1988) enabled it to be produced in larger quantities. This current

work used HA produced by *S. equi* which has a lower molecular weight (MW) than that produced by *S. zooepidemicus*.

The *in vivo* degradation of HA is caused by three different enzymes; hyaluronidase (hyase), β -D-glucuronidase, and β -N-acetyl-hexosaminidase which occur intracellularly and in serum. Leach and Schmidt (1994) reported that hyase generally breaks down higher molecular weight HA into smaller oligosaccharides and then the other two enzymes further degrade these oligosaccharides by removing non-reducing terminal sugars. It has been estimated that the half-life of HA in the skin is about 24 h, in the eye 24-36 h, in the cartilage 1-3 weeks and 70 days in the vitreous humour (Laurent & Reed 1991, Stern et al. 2007, Murray et al. 2005).

Stern et al. (2006) explained how chain length affects the biological functions of HA. Longer HA chains are capable of suppressing angiogenesis and inhibiting phagocytosis, while oligomeric HA has angiogenic, immuno-stimulatory and inflammatory properties. HA has been shown to play a role in maintaining the water balance in tissues, in wound healing and in nutrient transfer (Goa et al. 1994).

As HA is found naturally in the ECM of the body, it displays viscoelastic properties (Gibbs et al. 2004), which can be very useful in drug delivery as it mimics the properties of certain body tissues. HA solutions behave as pseudo-plastic materials decreasing their viscosity as the shear rate increases. Many of the HA medical uses are based on this shear thinning behaviour. Research groups have been investigating this property to use HA in arthritis (Balazs et al, 1967), dermal tissue restoration (Park et al., 2003) and ocular delivery (Awesh et al. 2008). In some interesting work HA was shown to specifically interact with cell-surface receptors, e.g. CD44 and thus had been investigated for the treatment of cancer cells over-expressing CD44 (Choi et al. 2010).

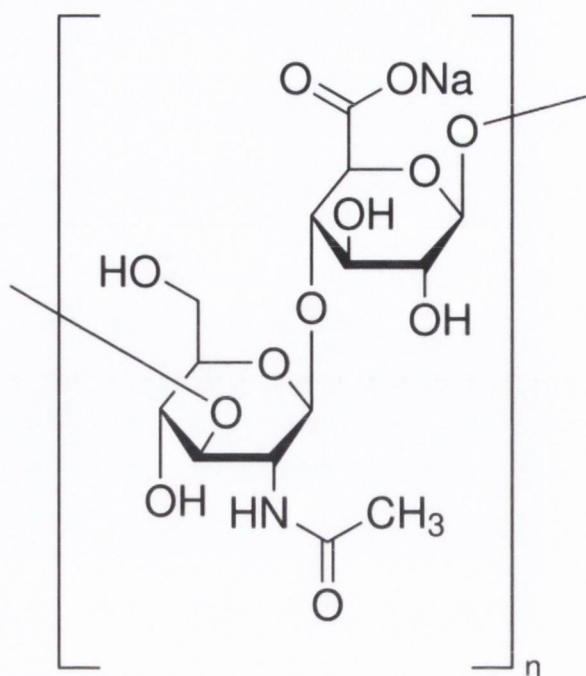


Figure 1.3 Disaccharide repeating unit of HA comprising GlcNAc and GlcUA.

1.3 Background to the drugs used

1.3.1 Ketoprofen (KET)

Ketoprofen (KET) is a propionic acid derivative - 2-(3-benzoylphenyl) propionic acid - which was synthesised in France by Rhône-Poulenc chemists in 1967 and has been used since 1973 for anti-inflammatory use (Kantor 1987) (**Figure 1.4**). KET was rated as one of the safest non-steroidal anti-inflammatory drugs (NSAIDs) available in the United Kingdom by the Committee on the Safety of Medicine in 1986 and it has been associated with a very low incidence of serious renal, hepatic or cutaneous reactions (Avouac and Teule 1988). The brand of KET Orudis was well tolerated by patients and has a very low incidence of side effects and toxic reactions (Cathcart et al. 1972, Gomez 1972, Mason et al. 1972).

KET is a non-selective COX inhibitor with a usual adult dose of 300 mg/day in 3-4 divided doses. It is 99% bound to plasma proteins post-absorption (Martindale, 2013). KET is classified as group II in the Biopharmaceutics Classification System, having very low aqueous solubility and high permeability. KET has a

short half-life (approximately 2 hrs), a simple metabolism, a broad therapeutic window and does not accumulate with multiple doses (Kantor 1987).

KET is available in a wide range of formulations, each designed to provide appropriate therapy in specific clinical situations: oral capsules for short term therapy; sustained release forms for chronic therapy and once-daily administration; suppositories to avoid possible gastrointestinal disturbances in susceptible patients; intramuscular preparations for rapid action and a gel formulation for topical treatment (Veys 1991). KET is used for the treatment of osteoarthritis having therapeutic equivalence with aspirin, indomethacin and ibuprofen in rheumatoid arthritis (Petrusewicz et al. 1995, Ali et al. 1962, Roth et al. 1975).

Aspirin is the most widely used anti-aggregation agent for secondary prevention of cardiac events; however, problems have arisen with its GIT irritation side effects and aspirin resistance (Hankey et al. 2006). KET has been shown to be more potent than aspirin at inhibiting collagen induced platelet aggregation (Petrusewicz et al. 1995, Leech et al. 1979) with IC_{50} values of KET 5.3×10^{-6} M and aspirin 1.53×10^{-4} M.

In its solid-state KET may be present in a crystalline or amorphous form. Bosselmann et al. (2011) published SEM pictures of bulk crystalline KET, which revealed crystalline KET to consist of rod-shaped crystals ranging in size from 5 to 10 μ m. Amorphous KET is reported to be highly unstable especially in the presence of water and crystallises spontaneously at room conditions after 2-3 days (Di Martino et al. 2004). Investigations into the available literature did not reveal any commonly documented polymorphic forms of KET. When spray drying KET microcrystals consisting of KET in chloroform solution, Dixit et al. (2012) did not produce different crystalline forms of KET by changing the conditions for preparation. The melting point of KET which Dixit et al. (2012) reported had an onset temperature at 94.17 °C followed by no decomposition. Its glass transition temperature (T_g) was observed at an onset temperature of approximately -1.99 °C.

KET has been previously investigated for use in NPs by many other research groups (Hannele et al. 2004, Kim et al. 2008, Tuan Anh et al. 2012, Dixit et al. 2012, Sugita et al. 2013). The research most linked to this current work is the

KET loaded PLGA NPs prepared by Corrigan et al. (2009). Corrigan et al. outlined the mechanism of KET release from PLGA NPs loaded with an initial loading of 0.2 mg/mg of KET. These current studies aimed to refine the PLGA KET loaded NPs described by Corrigan et al. (2009) and to move forward by attempting to load KET into hydrophilic HA:CS PECN systems which, to the best of knowledge, has not been done previously.

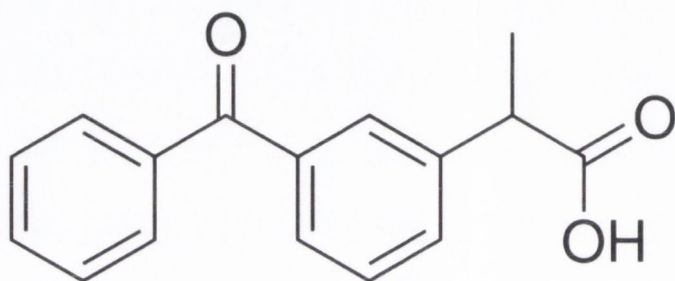


Figure 1.4 Chemical structure of ketoprofen.

1.3.2 Indometacin (IND)

IND is an indole acetic acid derivative, 2-[1-[(4-chlorophenyl)carbonyl]-5-methoxy-2-methyl-1H-indol-3-yl]acetic acid. **Figure 1.5** shows the chemical structure of IND. IND was discovered in 1963. It is used as an anti-inflammatory analgesic and has been heavily investigated for use in neonatal angiogenesis (Hart and Boardman 1963). Like KET, IND is a non-selective COX inhibitor and the usual adult dose of IND is up to 200 mg daily in 2-3 divided doses. IND is 99% bound to plasma proteins post absorption (Martindale, 2013). IND is classified as group II in the Biopharmaceutics Classification System, having very low aqueous solubility and high permeability with lower aqueous solubility than KET. Like KET and aspirin, IND has been proved to be as an anti-aggregation agent (Petrusewicz et al. 1995, Ali et al. 1962 and Roth et al. 1975). IND has been show to be more potent than aspirin at inhibiting collagen induced platelet aggregation (Leach and Thorburn, 1982) with IC_{50} values for IND at 1×10^{-7} M and aspirin 1.53×10^{-4} M.

The solid-state of IND is complex and there are on-going investigations into its crystalline polymorphic forms despite the fact that it was discovered in 1963. Surwase et al. (2013) reported the newest polymorphic forms recently in 2013. Polymorphism is an important factor to be considered when formulating drug product as the drugs' different metastable polymorphic forms may have different dissolution rates, thermodynamic properties, as well as the *in vivo* drug kinetics (Brittain 2005, Lancaster et al. 2007, Bauer et al. 2001). Five different polymorphic forms of IND have traditionally been recognised; α , β , γ and δ as well as an unnamed crystal form (Borka 1974 and Lin 1992). Of these the γ form is the most thermodynamically stable form and the α form is the most commonly observed metastable form (Savolainen et al. 2007, Andronis et al. 1997). It has been documented that when amorphous IND crystallises it is usually to either the metastable α or thermodynamically stable γ forms, depending on amorphous form preparation and storage conditions. The δ and β forms have been reported to occur less often and only under specific conditions. The δ form was reported to crystallise from IND–poly(vinyl pyrrolidone) solid dispersions at 94% relative humidity (Crowley and Zografi 2002 and Rumondor 2009). The δ form has also been reported to crystallise from a methanolic solution on drying, the β form crystallised from dioxane solution and the unnamed form crystallised from a cyclodextrin solutions (Borka 1974, Lin, 1992).

Surwase et al in 2013 identified three new polymorphic forms of IND. These were named ϵ , ζ and η . The appearance of new polymorphic forms was connected to both the pH and temperature at which amorphous suspensions of IND crystallised. Surwase et al. found that at 25 °C the amorphous form crystallised directly to the α form, except at pH 6.8, where it initially converted into the ϵ form. At 5 °C, all three new polymorphic forms were observed sequentially in the order ϵ , ζ and then η with the number of these forms observed increasing sequentially with decreasing pH. Each of the polymorphic forms of IND has a unique X-ray powder diffraction (PXRD), differential scanning calorimetry (DSC) and Fourier transformation infra-red (FTIR) profile. While DSC is suitable for identifying polymorphs of IND, if the aim is quantifying IND polymorphs Atef et al. (2012) reported that Raman spectroscopy was found to be more reliable than DSC. The partial conversion of the α to γ polymorphic form during the DSC measurement was the major limitation for the use of DSC as a quantitative method for IND.

Table 1.2 Unique PXRD peaks and thermal properties of indomethacin solid state forms. NA = Not Applicable , ND = Not Detected. Taken from Surwase et al. (2013).

solid state form	PXRD			DSC		
	unique positions (°2θ)	diffraction peak	T_g (°C)	onset temperature (°C)		
amorphous	NA		43.3 ± 0.9	exothermic event	101.2 ± 2.2	endothermic event
						153.8 ± 0.4, 158.1 ± 1.1
α	7.0, 8.5, 11.6, 12.0, 14.0, 8.0		NA	ND		154.1 ± 0.6
γ	10.2, 11.8, 17.0, 19.9, 21.9		NA	ND		159.2 ± 0.1
δ	9.6, 10.5, 11.3, 13.0, 14.9		NA	134.1 ± 0.2		129.2 ± 0.4, 158.1 ± 0.2
ζ	6.5, 11.0, 11.8, 12.8, 14.4, 16.4		NA	ND		142.2 ± 0.4, 157.0 ± 0.3
η	9.1, 9.3, 12.2, 18.2, 20.5		NA	114.1 ± 0.6		154.2 ± 0.2

IND has been loaded into many types on NCs most likely due to the fact that it is a very hydrophobic drug and has been extensively studied over the past 40 years. Some examples of IND loaded NPs are as recent as 2013 when Andonova et al. prepared IND loaded delayed release poly(vinyl acetate) NCs for ocular delivery. Nita et al. (2010) coupled IND onto macromolecular nanostructures based on methyl methacrylate copolymers with glycidyl methacrylate. Debrassi et al. (2011) prepared magnetic N-benzyl-O-carboxymethylchitosan NPs loaded using a solvent evaporation method with IND loading between 60.8 and 74.8% w/w. IND loaded PLGA NPs have previously been prepared by Tomoda et al. 2011. These PLGA NPs had an average diameter of 100 nm. They studied the permeability of IND-loaded PLGA NPs through rat skin and found that a greater amount of IND was delivered through skin when IND was loaded in PLGA NPs than free IND. Corrigan et al. (2009) also prepared IND loaded PLGA NPs but these NPs were larger in size at 300 – 500 nm; they were prepared to investigate the release of IND. The current studies sought to optimise these IND loaded PLGA NPs to see what role the different formulation variables were playing in the production of PLGA NPs. Like for KET the aim was also to attempt to load IND into hydrophilic PECN systems consisting of HA:CS as described by Umerska et al. (2012).

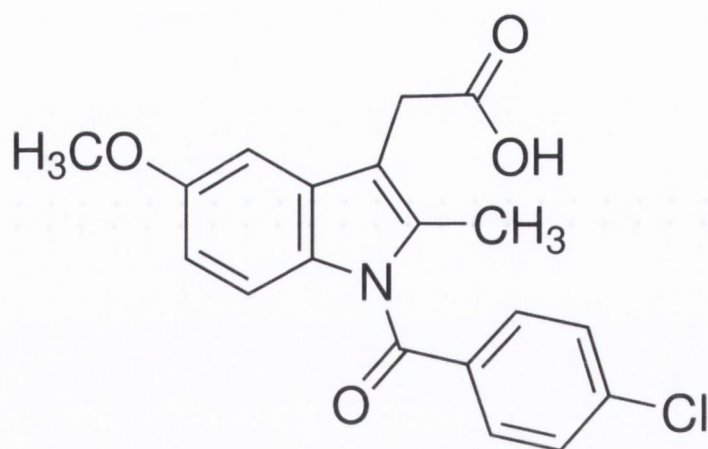


Figure 1.5 Chemical structure of indometacin.

1.3.3 Solid-State of Pharmaceuticals in Nanoparticles

The drug and polymer solid-state is important to consider in the development of drug delivery dosage forms as it will impact upon the physical stability and biological effectiveness of the drug (Calcagno and Siahaan 2005). The solid-state of loaded drugs can be altered during the processing of drugs in delivery systems, during storage at different temperature and humidity conditions and by interacting with the formulation excipients. These changes during processing are undesirable and could impact strongly upon the formulation physical stability, dissolution rate and effectiveness. From an economical point of view polymorphic drugs can have a negative effect on budgets as deviations are usually only realised late in the processing (Lennernas 1998).

1.4 PLGA NPs

1.4.1 Methods of Preparation

PLGA NPs have been widely prepared using different methods of formation. They are prepared using techniques such as salting-out, emulsification/diffusion, solvent-displacement/nanoprecipitation, emulsion/diffusion/evaporation, single emulsion/evaporation and double-emulsion/evaporation. Single emulsion/evaporation is suited best to hydrophobic drugs as the polymer and drug are dissolved in a water-immiscible organic solvent, which is then

emulsified in an aqueous solution containing a stabiliser. If the drug is more hydrophilic, such as a protein, then the double-emulsion/evaporation technique is used; this first involves the addition of aqueous drug solution to organic polymer solution to form a w/o emulsion which is then added into a second aqueous phase containing stabiliser with stirring to form a w/o/w emulsion (Vandervoort and Ludwig 2002). In both types of emulsion the emulsion is subjected to homogenisation before the organic phase is evaporated (Guarrero et al. 1998 and Song et al. 1997).

Salting-out, emulsification/diffusion and emulsion/diffusion/evaporation have similarities in their methods. Firstly, salting-out involves the addition of polymer and drug solution in a slightly water-miscible solvent, such as acetone, to an aqueous solution containing the salting-out agent and a colloidal stabiliser under vigorous mechanical stirring. When this o/w emulsion is diluted with a sufficient volume of water, it induces the formation of NPs by enhancing the diffusion of acetone into the aqueous phase. The remaining solvent and salting-out agent are eliminated by cross-flow filtration (Allemann et al. 1992). This is the same process as for emulsification/diffusion except that the salting out agent is not required because the solvent chosen for drug and polymer is a partially water-miscible solvent e.g. ethyl acetate, benzyl alcohol (Takeuchi et al. 2001). This can be further modified to call it the emulsion/diffusion/evaporation method, when used to prepare cationic surface modified PLGA NPs using a blend of PVA and CS as stabiliser (Kumar, 2004)

Solvent-displacement/nanoprecipitation is usually employed to incorporate lipophilic drugs by dissolving the polymer, drug and lipophilic surfactant (e.g., phospholipids) in a semipolar water-miscible solvent, such as acetone, which is then poured into an aqueous solution containing a stabiliser (Molpeceres et al. 1996, Govender et al. 1999). NPs are formed instantaneously by rapid solvent diffusion and removal.

The method used in this work was initially referred to as a single emulsion/evaporation method by Corrigan et al. (2009) but this method was further refined and optimised in the current work when loaded with KET and IND. The opinion held in this current work is that the method used is an emulsion/diffusion method as the solvent used (ethyl acetate) is partially miscible with water and this ethyl acetate was removed by slow diffusion and evaporation

as opposed to quick, mechanical evaporation. It should be noted that the method definitions are informal as there are many variations in the current literature.

1.4.2 Uses and physical characteristics

PLGA NPs have been studied for approximately 20 years and consequently there is a great body of research on their possible uses in nanomedicine. Some examples of this include their use for the delivery of protein, peptides and small drug molecules for the treatment of cancer, inflammatory diseases, in vaccinations, for diabetes, for infection control and for gene delivery (Kumari et al. 2010).

One of the main purposes of using PLGA NPs has been as a controlled release vehicle for drugs. The release from the PLGA NPs is most often due to slow hydrolysis and degradation of the PLGA in a triphasic manner (Corrigan et al. 2009). This may have many benefits, as sustained drug release provides a method for maintaining plasma concentrations of drugs over a longer period of time and makes dosing less frequent for the patient. Other advantages of these PLGA NPs are that they are stable to a range conditions *in vivo* and have a high loading capacity especially for hydrophobic drugs. Disadvantages are that, since these particles are of negative charge, they may not have the same affinity for negatively charged endothelial cells and tumour cells (Nafee et al. 2007).

Having being loaded with other NSAIDs, PLGA NPs have been shown to have a high loading capacity and slow release properties (Tomoda et al. 2011). PLGA NPs show the ability to protect drugs from rapid degradation *in vivo* and the small NPs size means that PLGA NPs can penetrate capillaries, undergo cellular internalization and endosomal escape (Feng 2004). For the treatment of cancer many research groups have encapsulated cancer related drugs; paclitaxel (Fonseca et al. 2002), taxol (Wang et al. 1996), doxorubicin (Gulyaev et al. 1999), 9-nitrocamptothecin (Derakhshandeh et al. 2007) and cisplatin (Avgoustakis et al. 2002). For diabetes 1.6% zinc insulin in PLGA NPs with fumaric anhydride oligomer and iron oxide additives has been found effective for oral administration (Kumar 2006). PLGA maintains the integrity of insulin during formulation and delivery. PLGA has been used for encapsulation and for extended controlled release of haloperidol and has successfully crossed the

blood brain barrier (Budhian et al. 2005). PLGA entrapped tetanus toxoid NPs have been prepared for vaccination immunisation with these NPs resulting in a very high and early immune response (Soppimath et al. 2001).

After their systemic administration, PLGA-based drug delivery systems are preferentially taken up by the reticuloendothelial system (RES) and present a high and selective uptake in inflamed areas (Danhier et al. 2012). This phenomenon might be explained by the increased presence of immune-related cells like macrophages, lymphocytes or by disruption of epithelium in these inflamed sites, resulting in a preferential accumulation of these NPs (Ulbrich and Lamprecht 2010). This has led to the development of PLGA NPs for arthritis (Higaki et al. 2005), inflammatory bowel disease (Schmidt et al. 2010) and ophthalmic inflammation (Agnihoti and Vavia 2009). Rifampicin and azithromycin were encapsulated in PLGA NPs (Toti et al. 2011) and the NPs enhanced the effectiveness of the antibiotics by reducing microbial burden.

The platelet compatibility of PLGA NPs has also been investigated and they have been shown not to induce platelet aggregation *in vitro* (Li et al. 2010, Ramtoola et al. 2011). In fact Li et al. (2010) have suggested that these NPs have the ability to inhibit collagen (2 ug/ml) induced platelet aggregation by $21.3 \pm 3.3\%$ at 100 ug/ml of particles either due to steric effects or the blocking of collagen binding sites on platelets. This makes them an exciting possibility for therapeutic use in drug delivery.

The properties of PLGA NPs which can be altered to make them suitable nanomedicines are particle size, size distribution, surface morphology, surface chemistry, surface charge, surface adhesion, surface erosion, interior porosity, drug diffusivity, drug encapsulation efficiency, drug stability and drug release kinetics (Kumari et al. 2010). The surface charge of the NPs is important for the cellular internalization of the NPs, clustering in blood flow, adherence and interaction with oppositely charged cells membrane (Feng 2004). There is still the requirement to improve these parameters and understand them more clearly to make PLGA NPs therapeutically relevant. For this study it was intended to take the NPs prepared by Corrigan et al. (2009) and instead of modifying them to work on refining and optimising them more completely so that they could be used for comparison with HA:CS PECNs as described in **Section 1.5**.

1.5 HA:CS PECNs

1.5.1. Methods of Preparation

Water-soluble, biodegradable, polymeric polyelectrolyte complex NPs (PECNs) are a popular choice in current drug delivery research (Hartig et al. 2007a). These PECNs use water as a solvent, do not require surfactants and can be used with biodegradable, natural polymers such as CS and HA. PECNs are rapidly formed based on electrostatic interactions between charged ionic groups of oppositely charged polyelectrolytes (Schatz et al. 2004). Alongside the electrostatic interactions some hydrogen bonding, hydrophobic interactions and van der Waals forces may be present (Dragan et al. 1999).

The literature describes three different types of aqueous PECs which have been prepared: 1) soluble PECN which are macroscopically homogeneous systems containing small PECN aggregates 2) turbid, colloidal PECN systems which have an observable light scattering or Tyndall effect and 3) a two-phase system of supernatant liquid and precipitated PECN, which are readily separated as a solid after washing and drying. Of these number 3) shows the least physical stability and is not desirable (Webster et al. 1997).

There are two PECNs structures described by Hartig et al. (2007a): 1) a ladder-like structure and 2) a scrambled structure. In the ladder-like structure complex formation takes place on a molecular level via conformational adaptation (**Figure 1.6**). The oppositely charged ions having either having weak ionic groups and large differences in molecular dimensions complex or high molecular weight polyions with weak charge density are titrated into low molecular weight counterions nonstoichiometrically in excess to form the insoluble PECNs (Kabanov and Kabanov 1995). This ladder type results in sections of double-stranded hydrophobic segments and single-stranded hydrophilic segments. The scrambled-egg type is formed when polyions with strong ionic groups and comparable molar masses combine to form insoluble and highly aggregated complexes under a strict 1:1 stoichiometry. The stoichiometry is adjusted to form colloidal PECNs consisting of a neutral and stoichiometric core surrounded by excess binding polyelectrolytes to stabilise (Schatz et al. 2004). The excess polyelectrolyte provides stability in different solvent conditions (Dautzenberg and Kriz 2003). The conditions for formation dictate the structures assembled.

The steps in PECN complexation involve a rapid (5 ms, Dautzenberg 2000) kinetic diffusion process of mutual entanglement between polymers depending on molar size differences, followed by a thermodynamic rearrangement of the PECN due to conformational changes and disentanglement over a long period of time (Hartig et al 2007b).

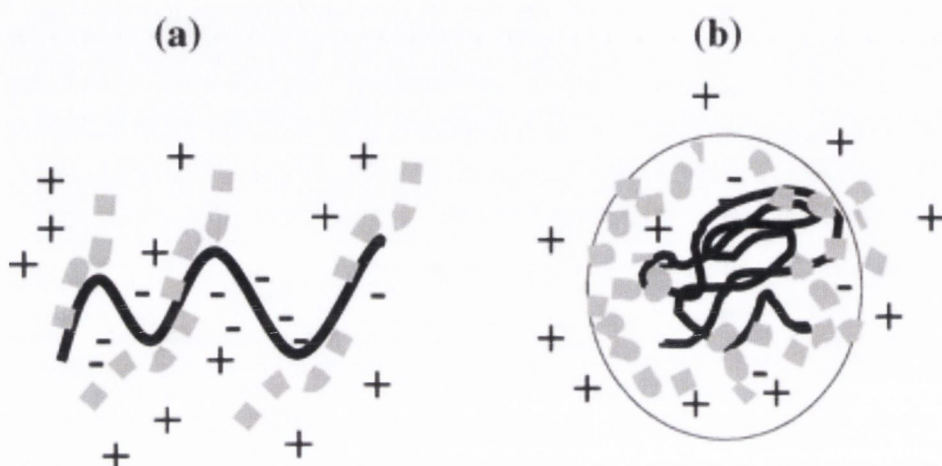


Figure 1.6 **a)** The ladder representation where insufficient ion pairing occurs under certain stoichiometric conditions leading to macromolecular aggregates, **b)** The scrambled egg model where polymers of comparable size complex yield insoluble PECNs under certain conditions. Black represents the large polyion (negative) while grey represents a polyion of opposite charge (positive). Taken from Hartig et al. (2007b).

Formation of PECNs is mainly determined by a combination of the strength and location of ionic sites, polymer chain rigidity, precursor chemistries, pH, temperature and salt concentration. The best combination has been found to be polyelectrolytes having significantly different molecular weights, weak ionic groups or nonstoichiometric mixing ratios leading to the formation of water-insoluble aggregates (Dautzenberg 1997, Reihs et al. 2004, Schatz et al. 2004).

It is proposed that hydrophobic drugs could be incorporated into PECN systems by two mechanisms. Firstly, they could be incorporated into the structure based on the charge (**Figure 1.7**) and secondly, it is possible that some amorphous drug may have been dispersed in amorphous HA during precipitation (**Figure 1.8a and 1.8b**).

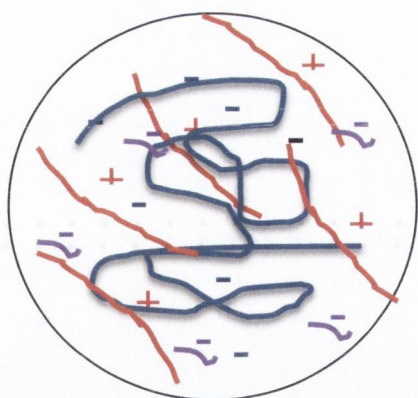


Figure 1.7 The scrambled-egg model for polyelectrolyte complex nanoparticle formation including the addition of negatively charged drug. Blue line = HA, red line = CS, purple line = drug (IND or KET). Formation based on charge interaction.



Figure 1.8a Amorphous drug (IND or KET) may be dispersed in amorphous HA during formation. Blue line = HA, Purple line = free drug (IND or KET), Green line = HA with dispersed drug (IND or KET)

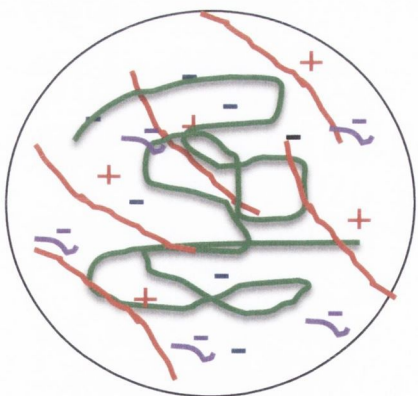


Figure 1.8b The scrambled-egg model for polyelectrolyte complex nanoparticle formation including the addition of negatively charged drug. Green line = HA and dissolved drug (IND or KET), red line = CS, purple line = drug (IND or KET). Formation based on charge and also dispersion of amorphous drug in HA.

1.5.2 Uses and physical Characteristics

The ideal physical properties desired for newly formed PECNs are a hydrodynamic diameter less than 200 nm (Panyam and Labhasetwar 2003, Carlesso et al. 2005), empirical surface charge of greater than 30 mV or less than -30 mV, spherical morphology and a low polydispersity index (PDI) indicative of a homogeneous distribution (Chern et al. 2004, Fatouros et al. 1992, Sugrue 1992). Maintenance of these properties, particularly size and shape, is critical for cellular uptake (Chithrani et al. 2006, Desai et al. 1997).

In the present work, the focus was on HA:CS PECNs which are hydrophilic PECNs formed due to electrostatic interactions between the oppositely charged polymers (Thunemann, et al. 2004). Preparation of HA:CS PECNs by ionotropic gelation has been reported by de la Fuente et al. (2008a). In this method solutions of HA with tripolyphosphate (TPP) and CS were mixed and PECNs were formed due to electrostatic interactions and ionic crosslinking with TPP. These PECNs were used to load bovine serum albumin and cyclosporine (de la Fuente et al. 2008b) and were demonstrated to successfully deliver pDNA (plasmid DNA) when used for ocular gene therapy (de la Fuente et al. 2010). These were further developed by Umerska et al. (2012) who prepared HA:CS PECNs without the use of crosslinkers such as TPP and found them to be physically stable and non-toxic to Caco-2 cells. Umerska et al. (2012) made it possible to prepare HA:CS PECNs without cross-linkers by reducing the molecular weight of HA by sonication of the HA solution. Sonication for at least 30 minutes resulted in the formation of HA:CS PECNs which were physically stable and were not observed to aggregate on preparation. The PDI values and ZP also decreased with the increase of the duration of sonication of HA. When the duration of sonication was increased to 30 minutes or more, it was possible to obtain PECNs without the tendency to sediment for 24 hours. HA with a molecular weight of 257 kDa, obtained by sonication of native HA for 2h, was used in the current experiments as this molecular weight provided the optimum balance between the viscosity of HA solution, leading to the production of non-sedimenting NPs and a reasonable sonication time. HA fragments were found to remain structurally intact despite the sonication process.

Cheow and Hadinoto (2012a) have combined hydrophobic drugs by electrostatic interactions, without cross-linkers, individually with the polymer poly(allylamine

hydrochloride) or with the polymer dextran sulphate. The drugs used in these studies were poorly soluble acidic (i.e., ibuprofen and curcumin) and basic (i.e., ciprofloxacin) drugs. They referred to their NCs as nanoplexes which were held together primarily by drug-polyelectrolyte electrostatic interactions along with inter-drug hydrophobic interactions. They were prepared by the mixing of drug and polyelectrolyte (PE) solutions in the presence of salt and surfactant. The drug's charge density and hydrophobicity and the PE ionisation at different pH values were important factors for formation. The drug loading (DL) for these nanoplexes was 80-85% and the particle sizes were ~300-500 nm depending on the drugs hydrophobicity. While these nanoplexes had a high DL and complexation efficiency the size was greater than 300 nm and there were no PDI values given in the publication. Also it was noted that Cheow and Hadinoto (2012b), in their specific study looking at ciprofloxacin-dextran PECNs, claimed these nanoplexes were observed immediately upon addition of the ciprofloxacin solution into the DXT solution as a white precipitate. If these particles were formed without aggregation, given the size range, it would not be expected that they would have observed precipitates. This precipitate raised the question as to whether their nanoplexes had started to aggregate into larger insoluble complexes.

The current studies were designed to progress from previous studies by preparing drug loaded HA:CS PECNs loaded with hydrophobic small drug molecules to see if they would successfully form. Progressing even more, this present work aimed to prepare HA:CS PECNs loaded with hydrophobic IND and KET without the need for surfactants, salt and also with a reliably low particle size and PDI. It was thought that adding a second polymer may help with this by stabilising the system. Also, it has been noted that HA acts in synergy with CS to enhance mucoadhesion (Wadhwa et al. 2009).

1.5.3 Surface charge influence

NCs can be manipulated by choosing different polymer or polymer combinations to create negatively or positively charged NCs. If the ZP is ± 30 mV (Hunter 1988) it is assumed that the NC dispersions will be stable. In between these values the ability of the individual particles to repel each other lessens as the ZP approaches zero at which point aggregation may be observed. The stability of

charged NCs will also depend on the medium in which they are dispersed, particularly if the NCs are PECNs where electrostatic charges are the dominant uniting interaction (Dautzenberg and Kriz 2003).

As these NCs will be used medicinally it is important to know how they interact with the body cells. There is a growing amount of information available on the interactions of NCs based on charge and many different types of NCs have been tested for their biological effect based on the ZP. A diverse selection of these studies will be mentioned in this section.

Kai et al. (2011) researched the effect of surface charge on the cellular uptake and *in vivo* fate of PEG-oligocholeic acid based micellar NPs, which were each derivatised with anionic aspartic acids (negative charge) or cationic lysines (positive charge). Their work delivered anti-cancer drugs to tumour sites. They concluded that NPs with high surface charge, either positive or negative, were taken up more efficiently by endocytic pathways into RAW 264.7 murine macrophages after opsonization in fresh mouse serum. After their cellular uptake, the majority of NPs were found to localise in the lysosomes. Positively charged NPs exhibited dose-dependent haemolytic activities and cytotoxicity against RAW 264.7 cells proportional to the positive surface charge densities whereas negatively charged NPs did not show obvious haemolytic and cytotoxic properties. The liver uptake was very high for highly positively or negatively charged NPs but when the surface charge of NPs was slightly negative the liver uptake was very low and tumour uptake was very high. Their final recommendation was for slightly negatively charged NPs to be used to reduce the clearance by the RES such as liver and improve the blood compatibility.

Asati et al. (2010) engineered polymer-coated cerium oxide NPs with different surface charges (positive, negative, and neutral) and studied their internalisation and toxicity in normal and cancer cell lines. The results showed that NPs with a positive or neutral charge enters most of the cell lines studied, while NPs with a negative charge internalises mostly in the cancer cell lines.

The cell lines used were cardiac myocytes (H9c2) and human embryonic kidney (HEK293) cells as nontransformed (normal) cells, while lung (A549) and breast (MCF-7) carcinomas were used as transformed (cancer) cell lines. They also concluded that the coating on NPs could be engineered in order to modulate its differential cytotoxicity behaviour in cancer versus normal cells.

Chunbai et al. (2010) prepared polymeric NPs from rhodamine B labelled carboxymethyl CS grafted NPs and CS hydrochloride grafted NPs as the model negatively and positively charged polymeric NPs, respectively. Like Kai et al. (2011) they reported that NPs with a high surface charge and large particle size were phagocytised more efficiently by murine macrophages. Both particle size and surface charge had significant implications in the cellular uptake of NPs and various mechanisms were involved in the uptake process. They found that NPs with slight negative charges (below 15 mV) and particle size of 150 nm tended to accumulate in tumours more efficiently. Small physicochemical differences such as 10 mV alternation of ZP played a vital role in non-phagocytic cell uptake.

Arvizo et al. (2010) stated that the cellular membrane potential plays a prominent role in intracellular uptake of gold (Au) NPs. Thus both malignant cells (ovarian cancer CP70 and A2780 cells) and non-malignant cells (human bronchial epithelial cells and human airway smooth muscle cells) were used to investigate whether cellular membrane potential plays a role in the uptake of gold NPs of different charges (positive, negative and neutral and zwitterionic). Their results showed that penetration of the membrane potential was dependent on surface charge of the NPs; positively charged NPs depolarised the membrane to the greatest extent with NPs of other charges having negligible effect.

Lockman et al. (2004) evaluated the effect of charged NPs on the blood-brain barrier (BBB) integrity and NP brain permeability using emulsifying wax NPs with neutral, anionic or cationic surfactants to provide the corresponding NP surface charge. Neutral NPs and low concentrations of anionic NPs were found to have no effect on BBB integrity, whereas high concentrations of anionic NPs and cationic NPs disrupted the BBB. The brain uptake rates of anionic NPs at lower concentrations were superior to neutral or cationic formulations at the same concentrations. They felt that NP surface charge must be considered for toxicity and brain distribution profiles.

Lundqvist et al. (2008) investigated the effect of NP surface charge on the uptake of proteins onto the NP surface as most NPs *in vivo* are found to have a protein corona. They found that many of the most abundant proteins in the corona are present independent of the particle size and ZP; however, some proteins with biological roles form part of the corona is the nature of which may

be determined by the local chemical properties of the nanomaterial, such as size, charge and changed surface properties.

Most relevant to this current work, Umerska et al. (2012) tested HA:CS PECNs of positive and negative charge with Caco-2 cells for cytotoxicity and found that a concentration-dependent cytotoxicity was observed for positively charged CS solution on its own and for positively charged HA:CS PECNs. There was a significant difference between the IC_{50} values calculated from the cell viability-concentration graph ($p < 0.0001$) for the CS solutions and the HA:CS PECNs with a positive charge. This suggested that positively charged HA/CL113 PECNs were a less toxic alternative to CS formulations. HA/CL113 with a negative charge were found to be non-toxic in the whole range of concentration studied and furthermore, cytoprotective effects were seen at higher HA concentrations.

When these studies are compared it can be seen that investigations into the charge effect of NCs showed the most toxicity for positively charged NCs regardless of the cell type and that neutral and negatively charged NCs show better cell compatibility. The strength of the charge also appears to be an important factor and most research groups agreed that NCs with a slight negative charge showed the best uptake along with low toxicity. There is still plenty of room for experimentation on this and most studies agree that the charge of the NCs could be designed for the purpose of use.

1.6 Platelet Aggregation

1.6.1 Platelet Activation and Aggregation

Human platelets are the smallest formed elements of blood that play a crucial role in haemostasis, inflammation, thrombosis, wound healing and host defence. They are 2 to 4 μm in diameter and have an average volume of 6–8 femtoliter (10^{-15} L). Platelets contain the normal complement of cellular organelles, but also contain 2 unique organelles, which are the alpha and dense granules. The majority of platelets circulate for 7-10 days until phagocytic cells remove them and the normal circulating blood platelet count is 150,000 to 450,000 cells/ mm^3 (Chaer et al. 2006).

Platelet activation is initiated on vascular injury by the adhesion of platelets to the sub-endothelial matrix. Platelet surface-expressed integrins such as GP Ia/IIa and the immunoglobulin-like receptor GP VI interact with collagen to activate platelets and induce a conformational change of the GP IIb/IIIa receptor on the platelet surface to a higher energy state (**Figure 1.7**). This conformational shift mediates platelet aggregation (Denisa et al. 2003) by promoting the binding of both fibrinogen and von Willebrand factor to this receptor with high affinity. GP IIb/IIIa is the most abundant cell surface protein of platelets, accounting for almost 15 % of the protein mass of the platelet membrane (Chaer et al. 2006).

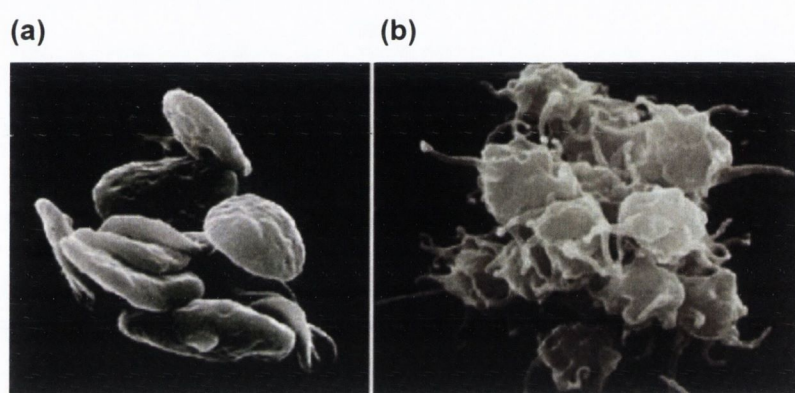


Figure 1.7 (a) Resting platelets are disc-shaped and smooth whereas (b) activated platelets are irregularly shaped with projecting pseudopodia (Chaer et al. 2006).

The platelet agonists adenosine diphosphate (ADP) and thromboxane A₂ (TxA₂) are released in response to collagen binding which amplifies integrin adhesiveness and thereby increases thrombus growth (Savage et al. 1998, Watson et al. 2000, Nieswandt and Watson, 2000). Given that this current work involves the use of IND and KET, which are cyclooxygenase (COX) inhibitors it is worth outlining that TxA₂ is converted from arachidonic acid mediated by the COX enzymes, therefore, inhibiting these enzymes inhibits the conversion and prevents further thrombus formation. With the activation of platelets, the contents of alpha and dense granules are secreted, recruiting additional platelets, which then aggregate into a platelet “plug” (**Figure 1.8**). Stabilisation of the platelet plug occurs via cross-linkage of platelets across fibrin (Chaer et al. 2006). The glycoprotein P-selectin is exclusively located in the α -granule membrane of resting platelets. Upon activation, the α -granule membrane merges with the plasma membrane and releases P-selectin onto the platelet surface (Jurk and

Kehrel 2005). For this reason, it can be used as a marker of platelet activation through the binding of monoclonal antibodies to these receptors (Griesshammer et al. 2009).

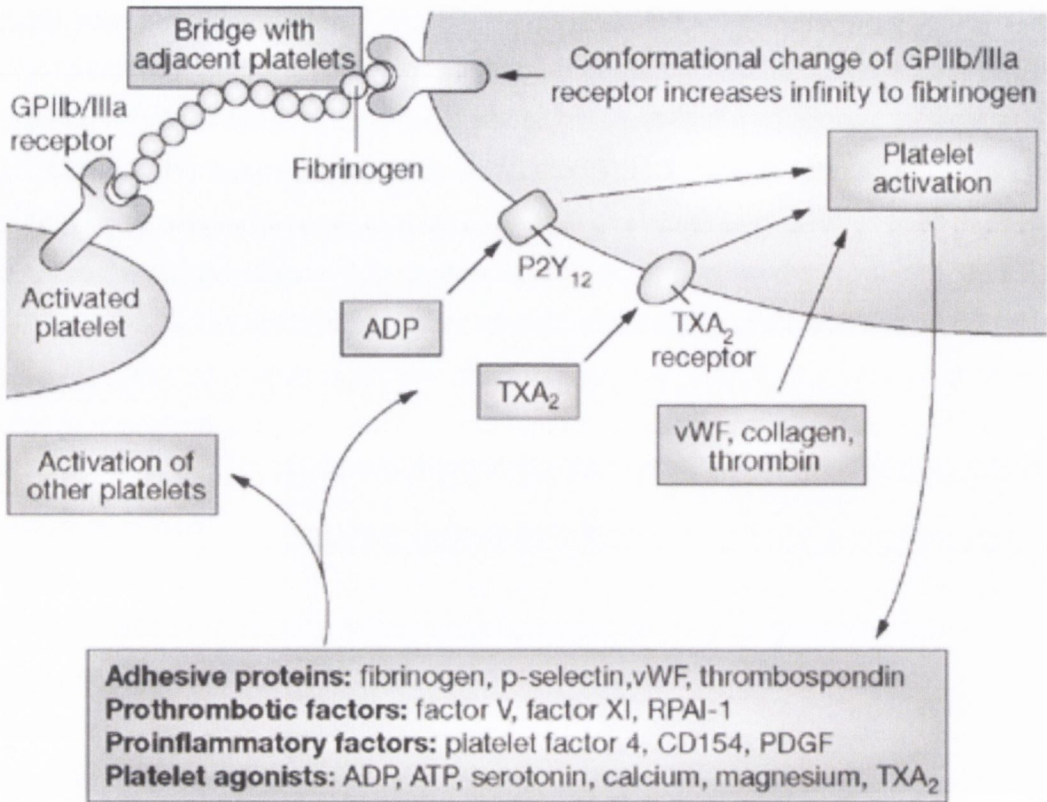


Figure 1.8 Formation of a platelet plug (Denisa et al. 2003).

A third mechanism for platelet aggregation involves matrix metalloproteinases (MMPs), which are proteolytic enzymes in platelets. Sawicki et al (1999) showed that one way that platelet aggregation may be mediated is via the release of MMP-2 from platelets. During platelet aggregation MMP-2 is translocated to the platelet surface where it may interact with cell surface proteins and receptors. MMP-2 seems to amplify the pro-aggregatory effects of different agonists showing that the enzyme may be involved in facilitation of platelet activation at the level of the second messenger system generated during phosphoinositide breakdown. In humans, MMP-2 is also released from platelets *in vivo* at a localized site of vessel wall damage in amounts sufficient to potentiate platelet aggregation (Falcinelli et al. 2007).

1.6.2 Interaction between Platelets and Nanomedicines

The compatibility of nanomedicines with platelets is important to research for two reasons: 1) there is an ongoing effort to improve anti-platelet therapies as our knowledge of platelet coagulation expands (Chaer et al. 2006) and 2) NCs intended for *in vivo* use intravenously will come into contact with platelets. Some examples of incompatibility between platelets and NPs, noted throughout the literature, include: Radomski et al. (2005), who revealed that single walled nanotubes C60 and mixed carbon black NPs can induce platelet aggregation; Corbalan et al. (2010), who showed a link between silica NPs and platelet aggregation; and studies by Glynn et al. (1965) and Bloom et al. (1995), which observed that platelets were aggregated by latex NPs at 200 – 400 nm and titanium dioxide submicroscopic particles at < 5 μ m respectively. Given this bleak picture it was interesting when Li et al. (2010) studied the effect of PLGA and CS NPs on platelet aggregation and found there to be no induced aggregation. These findings were further confirmed by Ramtoola et al. (2011) who also studied the effect of PLGA NPs and CS NPs on platelet aggregation and found that they did not induce platelet aggregation at a concentration up to 500 μ g/ml. Given that many nanomedicines being investigated are prepared from biocompatible and biodegradable polymers it was considered necessary to further the work carried out by Li et al. (2010) by adding model drugs with anti-platelet action. The role of platelets in chronic inflammatory diseases such as atherosclerosis has now been convincingly demonstrated and thus, platelets are central to both thrombosis and inflammation (Denisa and Burger 2003). IND and KET were selected for this work, two NSAIDs exhibiting anti-platelet aggregation, due to the inhibition of the COX enzyme involved in platelet aggregation. Peripheral arterial disease is most commonly caused by atherosclerosis and is treated with anti-platelet drugs, as they are effective in the arterial circulation while anti-coagulants are not. The most common anti-platelet drug in use is aspirin (Warfarin Antiplatelet Vascular Evaluation Trial Investigators, 1997) but its effectiveness has come under question and so newer anti-platelet drugs are being developed (McNicol and Israels 2003). It was considered a useful step to look into the effects NPs could have on the actions of anti-platelet drugs *in vitro*.

1.7 Physical Characterisation Techniques for NPs

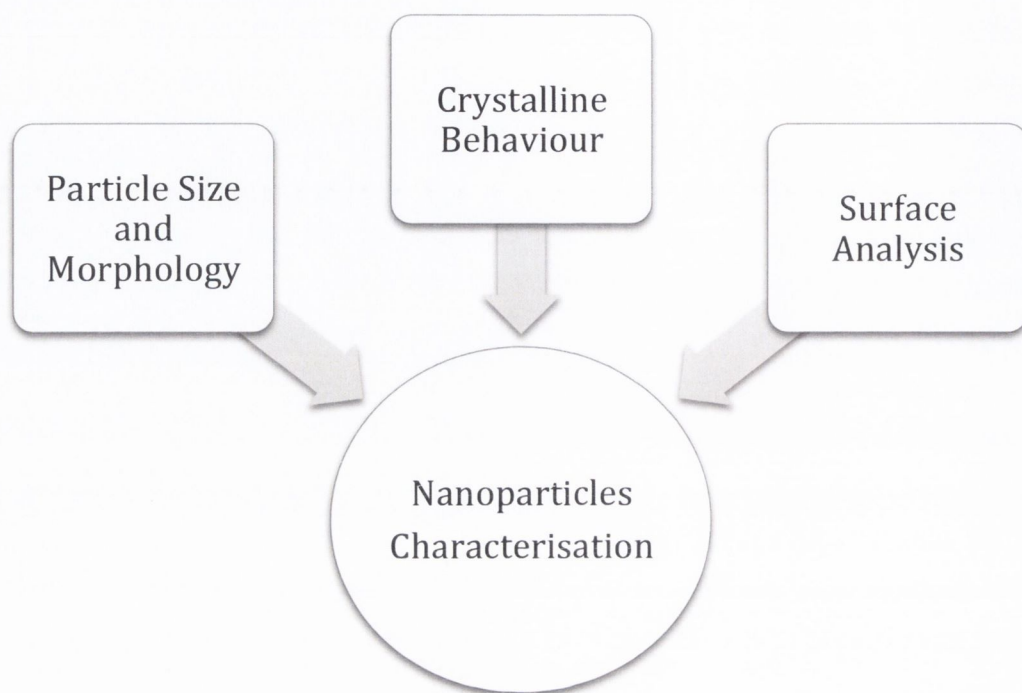


Figure 1.9 Nanoparticle characterisation investigation areas.

Figure 1.9 highlights the characterisation techniques used in the studies, which will be explained in this section.

1.7.1 Particle Size and Zeta Potential

The particle size of a NC is very important as the physical stability, fate and biological effect of the NC will be influenced by its particle size (Bertrand and Leroux 2012). Two of the most common methods used to determine the particle size of NCs are dynamic light scattering (DLS) and electron microscopy (Bootz et al. 2004). Microscopy is the preferred technique for some irregular shaped NC types as DLS makes some assumptions about the particle shape i.e. that they are spherical, which can make the particle size difficult to estimate. It may also take longer to acquire data for microscopy as individual particles have to be manually counted and measured before statistical analysis (Allen 1997). For that reason DLS was used for the current experiments as it was capable of

measuring large numbers of particles quickly. Microscopy was used to verify the physical particle size.

DLS is used for measuring the size of particles typically in the sub-micron region, dispersed in a liquid. It does this by measuring Brownian motion, which relates to the size of the particles. Brownian motion is the random movement of particles due to bombardment by the solvent molecules that surround them and is slower for larger particles. The translational diffusion coefficient (D) describes the velocity of the Brownian motion and is used in the Stokes-Einstein equation to calculate particle size (**Equation 1.1**). Essentially, Brownian motion is measured by optically detecting the rate at which the intensity of the scattered light fluctuates due to the speed of diffusion of the NPs.

$$D = \frac{KT}{6\pi\eta R_h}$$

Eqn. 1.1 Stokes-Einstein equation where: R_h = hydrodynamic diameter, D = translational diffusion coefficient, K = Boltzmann's constant, T = absolute temperature, η = viscosity.

Two types of light scattering can be observed by the particles. Rayleigh scattering is observed when the particle size being measured is approximately

$D = \lambda/10$ where λ = the wavelength of the laser (633 nm). When the particle size (nm) reaches the wavelength of the laser then the Mie theory is applied to estimate the particle size.

It is important to note that DLS measures the hydrodynamic diameter, which is the diameter of a sphere that has the same translational diffusion coefficient as the particle. D includes the particle itself but it is also influenced by the particles surface structures affecting its diffusion speed, the ionic strength of the medium and the type of ions in the medium. The ionic strength affects the diffusion speed by influencing the thickness of the electric double layer surrounding the particle, which is called the Debye length (K^{-1}).

The surface charge of NCs is crucial to measure as it will determine the purpose that the NCs are most suited to and help predict the physical stability of the sample (Dautzenber and Kriz 2003). The parameter used to define the surface charges is the zeta potential (ZP). The ZP is a very good index of the magnitude

of the interaction between colloidal particles and measurements of ZP are commonly used to assess the stability of colloidal systems.

Particles suspended in liquid will have a layer of ions in their surrounding interfacial region. These ions are of opposite charge to that of the particle and are found close to the surface of the particle. This means that an electrical double layer exists around each particle, which exists as two parts: an inner region (Stern layer) where the ions are strongly bound and an outer (diffuse) region where they are less firmly associated. The diffuse region can be further divided into those ions that are more closely associated with the particle and will move with it, and those ions that will stay with the bulk dispersant as the particles move. This boundary is called the surface of hydrodynamic shear and the potential at this boundary is the ZP.

If all the particles dispersed have a large negative or positive ZP then they will tend to repel each other and resist aggregation. Often the dividing line between physically stable and unstable suspensions is +30 or -30 mV (Dautzenberg and Kriz 2003).

The stability of NCs can be explained by the Derjaguin, Verwey, Landau and Overbeek (Verwey and Overbeek 1948) (DLVO) theory, which suggests that the stability of a colloidal system is determined by the sum of the van der Waals attractive (VA) and electrical double layer repulsive (VR) forces that exist between particles as they approach each other due to the Brownian motion. The DLVO theory suggests that particles that are initially separated are prevented from approaching each other because of the repulsive force but that the attractive forces can overcome this force so repulsive forces need to be the dominant force to ensure longer physical stability.

The ZP is measured by electrophoresis: the movement of a charged particle relative to the liquid it is suspended in under the influence of an applied electric field. The velocity of the NPs in a unit electric field is referred to as its electrophoretic mobility and the Henry equation is used to relate the electrophoretic mobility to the zeta potential (**Equation 1.2**).

$$UE = 2\varepsilon Zf(\kappa a)3\eta$$

Eqn. 1.2 Henry Equation where UE = electrophoretic mobility, z = zeta potential, ϵ = dielectric constant, η = viscosity and $f(\kappa a)$ = Henry's function. For particles with a size greater than 200 nm in aqueous media with moderate electrolyte concentration ($> 10^{-3}\text{M}$ salt) $F(\kappa a)$ is 1.5, is referred to as the Smoluchowski approximation.

The Malvern Zetasizer Nano Series uses a combination of laser Doppler velocimetry and phase analysis light scattering (PALS) in a patented technique called M3-PALS to measure particle electrophoretic mobility.

1.7.2 Particle Shape

Scanning electron microscopy (SEM) works on the principle that primary electrons penetrate the solid specimen and are deflected by a large number of elastic scattering processes (Egerton 1986, 1996 and 2011). The signals that derive from electron-sample interactions reveal information about the sample including external morphology (texture), chemical composition and crystalline structure and orientation of materials making up the sample. Accelerated electrons in SEM carry significant amounts of kinetic energy and this energy is dissipated as a variety of signals produced by electron-sample interactions when the incident electrons are decelerated in the solid sample. These signals can be secondary electrons, backscattered electrons, diffracted backscattered electrons, photons or visible light and heat (Brundle et al. 1992). An image detailing morphology and topography is formed by the secondary electrons that bounce off the surface of the specimen and are then collected onto the imaging screen, the contrast of the sample is formed by the backscattered electrons. The observer therefore sees a picture of the surface of the sample, without any internal information (Klang et al. 2012).

Polymers do not readily reflect electrons, and so for these experiments a gold coating was used. This coating provided a conducting surface for electrons to avoid charging of the sample (Newbury et al. 1986).

Transmission electron microscopy (TEM) differs from SEM as it looks through the sample while SEM only focuses on surface morphology. In TEM analysis an electron source (tungsten fibre) produces a beam of monochromatic electrons

that are transmitted through a sample. The stream is focused by the use of two condenser lenses; the first determines the size of the focus spot and the second changes the size of the spot the electrons pass through. As the electron beam passes through it is affected by the sample (Egerton 2005). As a result only certain parts of the electron beam are transmitted through the sample. Projector lenses then project the transmitted beam onto a phosphor image screen, forming an enlarged image of the sample. Darker areas of the image represent areas that fewer electrons were transmitted through (they are thicker or denser). The lighter areas of the image represent those areas of the sample that more electrons were transmitted through (they are thinner or less dense) (Rose 2008).

TEM was used for HA and CS samples in these experiments while SEM were used for PLGA samples. This was because the PLGA has a low T_g ($\sim 42.5 \pm 0.5^\circ\text{C}$) (Passerini and Craig 2001) and is susceptible to heat damage from the electron beam, which is more likely with TEM as the beam passes through the sample.

1.7.3 Thermal Analysis

Differential scanning calorimetry (DSC) monitors the heat changes associated with physical transformations which includes transformation from amorphous to crystalline form, from crystalline drug to a different polymorph form, and from crystalline drug to an amorphous solid-state mixture (Lefort et al. 2009, Ahmed et al. 1996, Nemet et al. 2009). It can be used for qualitative or quantitative purposes although Atef et al. (2012) did not recommend it as very accurate qualitative technique for IND because it has multiple polymorphic forms. If the aim is the quantification of one polymorphic form it can be difficult to predict if the heat will cause a transformation of that polymorph before quantification. For that reason it was only used for qualitative purposes in the current experiments.

DSC works by monitoring the heat transfer (flow) within a material (solid or liquid) relative to an applied temperature program (heating, cooling, isothermal analysis) in reference to the control sample (Hohne et al. 1996). With temperature change, the detector records the difference in heat output between the sample heater and the reference heater. The heat absorbed or released by the sample is then plotted versus temperature. The heat flow (ΔH) defined as

heat change (H) per unit of time (t). The heating rate (ΔT) is temperature increase (T) per a unit of time (t). Heat capacity (C_p) can be therefore defined as **Equation 1.3**.

$$C_p = \frac{\Delta H}{\Delta T}$$

Eqn. 1.3 Heat capacity equation. C_p = heat capacity, ΔT = temperature increase, ΔH = heat flow.

The specific heat is the amount of work, in Joules (J), required to raise the temperature of 1 gram of material by 1 °C. From **Eqn. 1.3** it can be seen that key parameters that can affect heat flow (ΔH) in DSC are the heating rate (ΔT) and a change of specific heat capacity (ΔC_p). The ΔC_p occurs because of the change of thermal resistance between the sample and the reference. The reference is an empty sample holder which remains unaffected by the temperature change over time and thus, the change in heat flow is related to the thermal resistance of the sample material. Melting decreases this resistance showing an endothermic event in which the ΔH increases as the material absorbs energy while crystallisation of an amorphous material results in a decrease in ΔH showing as an exothermic event in which energy is released. Amorphous materials can undergo changes from a glassy material to a liquid at its glass transition temperature showing a broad endothermic event as ΔH increases and energy is absorbed (Moynihan et al. 1976).

There are two types of DSC machines that are commonly used. The first type is a heat flux DSC that has a common heater for both the sample and the reference, therefore, the measured signal is the difference in temperature between the sample and the reference upon controlled ΔT . The other type is the power compensation DSC in which the sample and the reference have separate heaters and separate temperatures sensors. The temperature control is more complex as it requires careful maintenance to ensure that the sample and reference furnaces are calibrated exactly the same. The heat flow difference is measured as the difference in power required to be delivered to the heaters to keep the sample and reference at the same temperature, which can give more precise results of measurement (Haines et al. 1998).

In this current work DSC was necessary to detect the solid-state of the drugs in the NCs and to determine which polymorphic forms of IND were present.

1.7.4 Powder X-ray Diffraction

Powder X-ray diffraction (PXRD) is a non-destructive method of structural analysis for powdered samples, but it is relatively low in sensitivity (Santiago et al. 2011). PXRD can be used for quantitative analysis or qualitative analysis to determine crystallinity, residual stress, crystallite size and texture (Araki 1989). PXRD is based upon the constructive interference of monochromatic X-rays (produced by a cathode ray tube) with a sample (Dutrow et al. 2012). Applied current heats a filament within the tube. The filament emits the electrons. A high voltage (15-60 kV) applied within the tube accelerates the electrons. The beam of electrons hits a copper target causing the production of X-rays. These X-rays are filtered, collimated and directed onto the sample. The detector collects the X-ray signals and converts them into a count rate (Blake et al. 2009).

The diffraction angle along with the wavelength of electromagnetic radiation and the lattice spacing in a crystalline sample are used to produce a unique fingerprint for each crystalline material according to Bragg's law. This law (Bragg 1913) states that X-ray waves scatter on the faces of crystalline materials (**Equation 1.4**).

$$n_1 \lambda = 2d_{hkl} \sin \theta$$

Eqn. 1.4 Bragg's equation where: n_1 = an order of reflection, λ = a wavelength of incident X-ray beam ($\lambda=1.542 \text{ \AA}$ for $\text{CuK}\alpha$ radiation), d_{hkl} is the interplanar distance between (hkl) lattice planes (hkl)₁ and (hkl)₂, θ = a diffraction angle.

This diffraction technique can only see order. Thus, disorder is only implied by the absence of order. A crystalline material has a long-range order and so it will diffract the X-rays resulting in an arrangement of clearly defined Bragg peaks (Perchasky et al. 2008). By scanning the sample through a range of angles, all possible diffraction directions of the lattice should be attained due to the random orientation of the powdered material. Conversion of these diffraction peaks to d-spacings allows identification of the material (because each material has a set of unique d-spacings) by comparison with standard reference patterns (Dutrow et al. 2012). The information contained in a PXRD diffraction pattern shows faces

present (peak positions), face concentration (peak height), amorphous content (background) and crystallite size/strain (peak widths) (Blake et al. 2009).

In this work, PXRD was used to determine the solid-state of the NPs, including the drug and polymer. PXRD was used in tandem with DSC to give an insight into how the drug was present in different NC types.

1.7.5 Aggregometry and Flow Cytometry

Aggregometry involves measuring the light transmittance through a sample of platelet rich plasma to measure the amount of aggregation of the platelets in response to stimuli (Knoefler et al. 2005). Aggregometry evaluates the aggregation or clumping of platelets in response to aggregating stimuli.

An aggregometer typically comprises two chambers for cuvettes: the sample (platelet rich plasma (PRP)) and the reference (platelet poor plasma (PPP) or washed platelets). A beam of light shines through both cuvettes and the light passing through is detected. As PRP is considered to be 0% light transmission (or 0% aggregation) and PPP assumed to be 100% light transmission (or 100% aggregation), the measured difference in light transmission between the PRP and PPP samples is proportional to the % aggregation (Knoefler et al. 2005).

The most common agents added to induce platelet aggregation are ADP, epinephrine and collagen. While ADP and epinephrine are contained in storage organelles within the platelets and are released during formation of the primary hemostatic plug to enhancing further platelet aggregation, collagen is found in the supporting connective tissue of the vessels and is considered to be the first procoagulant factor that the platelet encounters following the vessel's injury (Breddin 2005). Following the addition of a stimulus to the cuvette containing PRP, changes in light transmission occur as a consequence of platelet response and are recorded over time. Unfortunately, several procedural variables may account for poor reproducibility using this technique although it is still the most routinely used for testing platelet function (Breddin 2005).

Flow cytometry involves the transmission of a beam of laser light of a single wavelength directly onto a hydrodynamically focused stream of liquid. A number of detectors are aimed at the point where the stream passes through the light

beam: one in line with the light beam (forward scatter) and several perpendicular to it (side scatter) and one or more fluorescence detectors. As particles pass through the beam they scatter the light ray, and fluorescent chemicals, used to label the particles, which may be excited into emitting light at a longer wavelength than the light source. Detectors pick up a combination of scattered and fluorescent light and analyse the fluctuations in brightness at each detector. Flow cytometers are capable of analysing several thousand particles every second, and actively separate and isolate particles having specified properties. A flow cytometer is similar to a microscope, except that, instead of producing an image of the cell, flow cytometry offers "high-throughput" (for a large number of cells) automated quantification of set parameters (Shapiro 2003)

In this work P-selectin was chosen as the marker of platelet activation and its expression was monitored. In determining platelet activation, Ruf and Patscheke (1995) tested the concentration-effect relationships for: ADP and U46619-induced platelet shape change; fibrinogen binding; and expression of P-selectin. They concluded that, for detecting platelet activation *in vivo*, P-selectin expression had the highest diagnostic sensitivity.

Experimental

Materials & Methods

Chapter 2

2.1 Materials

Material	Supplier/Manufacturer
Acetone	Solvent stores, TCD
Acetonitrile > 99.9% HPLC grade	Fisher Scientific, Ireland
APC-conjugated monoclonal antibody against human platelet P-selectin (CD62P)	Becton Dickinson (BD) Biosciences, UK
BD FACS sheath solution with surfactant	Becton Dickinson (BD) Biosciences, Belgium
Citric Acid	
Collagen	CHRONO-LOG, USA
Dimethylsulphoxide (DMSO)	Aldrich, UK
Ethanol 96%	Lab Scan Analytical Sciences, Ireland
Ethyl acetate >99.8%, GC grade	Fluka, Germany
Hyaluronic acid from <i>Streptococcus equi</i> sp.	Sigma, Czech Republic
Indometacin	Sigma, USA
Liquid Nitrogen	BOC, Ireland
Nitrogen Gas	BOC, Ireland
Ortho-Phosphoric acid (85%)	Riedel-de Haën, Germany
Phosphate buffered saline	Sigma-Aldrich, Ireland
PLGA Resomer RG 752H (75:25)	Boehringer Ingelheim, Germany
PLGA Resomer RG 504H (50:50) Batch No. 640662	Boehringer Ingelheim, Germany

Pluronic F-68	Sigma, USA
Protasan Ultrapure Chitosan chloride salt CL113 (low molecular weight 50,000 – 150,000 Da)	FMC BioPolymer AS, NovaMatrix™, Norway
Sodium chloride	Merck, Germany
Sodium Hydroxide	Riedel-de Haën, Germany
Tween (Polysorbate) 80	Sigma, Ireland
Tyrode's Salt Solution (T2397)	Sigma, Ireland
Water, deionised	Purite Prestige Analyst HP, Purite Ltd, UK
Buffer solution pH 4.0 (20C) 33665	Fluka Analytical Sigma-Aldrich, Germany
Buffer solution pH 7.0 (20C) 33666	Fluka Analytical Sigma-Aldrich, Germany
Buffer solution pH 10.0 (20C) 33668	Fluka Analytical Sigma-Aldrich, Germany

2.2 Methods

2.2.1 Preparation and Optimisation of PLGA NPs

PLGA NPs were prepared using a modified version of the single emulsion-diffusion technique described by Corrigan and Li (2009). 200 mg of PLGA, either RG504H or RG752H, was dissolved in 2 ml of ethyl acetate. This solution was then added to 4 ml of Pluronic F-68 (0.8, 1.0 or 2.4% w/v) or Tween 80 1% w/v solution drop-wise with the use of syringe needle (25G) under stirring conditions. The resulting o/w emulsion was sonicated on ice (Sonics VC130PB, Sonics and Materials Inc., USA) for 10 minutes at amplitude 80, which corresponds to sonication intensity of approximately 99 W/cm^2 . This emulsion was diluted with 25, 45 or 90 ml of Pluronic F-68 solution (1% w/v) and the organic solvent was eliminated by stirring overnight using a magnetic stirrer. The samples were then centrifuged for 10, 15, 20 or 30 min at 8,000, 10,000, 13,000 or 15,000 rpm (Sorvall, RC 5B Plus, Thermo Fisher Scientific Inc. USA) and washed twice or thrice with water. After the last washing the samples were reconstituted in water. The details of method optimisation are described in **Section 3.2.1**.

2.2.2 Preparation of NSAID loaded PLGA NPs

NSAIDs (IND, KET) loaded PLGA NPs were prepared by a single emulsion-diffusion technique. 20 or 40 mg of drug was dissolved in a 2 ml solution of PLGA (RG 504H) in ethyl acetate (100 mg/ml) giving a ratio of 0.1 or 0.2 mg drug/1 mg of polymer. This solution was then added to 4 ml of Pluronic F-68 (1% w/v) solution drop-wise with the use of syringe needle (25G) under magnetic stirring conditions. The resulting o/w emulsion was sonicated on ice (Sonics VC130PB, Sonics and Materials Inc., USA) for 10 minutes at amplitude 80. The emulsion was diluted with 45 ml PVA solution (1% w/v) and stirred overnight to eliminate the organic solvent. Finally, the drug loaded PLGA NPs were isolated by centrifugation for 15 mins at 13,000 rpm (Sorvall, RC 5B Plus, Thermo Fisher Scientific Inc. USA) and washed twice with water.

2.2.3 Preparation of HA and CS Solutions

A 0.1% w/v HA solution was prepared by dissolving 100 mg of sodium hyaluronate powder in 100 ml deionised water and allowing it to stir until dissolved. This solution was then sonicated at amplitude 80 equivalent to 13 W using a 30 W ultrasonic processor (Sonics VC130PB, Sonics and Materials Inc., USA) equipped with a probe with a diameter of 3 mm for 120 minutes. The length of HA solution sonication was earlier optimised to yield HA fragments forming non-sedimenting NPs (Umerska et al. 2012). The molecular weight of these fragments was approximately 257kDa.

A 0.1% w/v CS solution was prepared by dissolving 100 mg of CS chloride (Protasan UP CL113) in 100 ml deionised water and allowing it to stir until dissolved. The degree of deacetylation was 83% according to manufacturer's data and the molecular weight was 110 ± 7 kDa (Umerska et al., 2012).

All solutions were prepared fresh for all experiments.

2.2.4 Preparation of HA:CS PECNs

A volume of HA solution (0.1% w/v), sonicated as described in **Section 2.2.3** was stirred at room temperature at 900 rpm using a magnetic stirrer. A volume of CS solution (0.1% w/v) was added by one-step addition and a dispersion of HA:CS PECNs was obtained instantly. The HA:CS PECN dispersion was left to stir for 5 minutes allowing stabilisation of the HA:CS PECNs. The HA:CS PECN dispersion was washed twice using centrifugal filter devices (Amicon Ultra-15, MWCO of 30 kDa; Millipore, USA). The devices containing PECN dispersions were centrifuged twice for 20 min at 4000 rpm using a Hettich Universal D7200 fixed rotor centrifuge (Germany). The filter was first centrifuged with 7 ml of deionised water alone under these conditions to ensure that the full volume of water passed through.

The ratio of the volume of HA solution to CS solution was varied to obtain NPs with different surface charge values. The total polymer concentration was always 0.1% w/v.

2.2.5 Preparation of Drug loaded HA:CS PECNs

The above method of HA:CS PECN production (**Section 2.2.4**) was modified to include KET or IND by first dissolving the drug in ethanol. To ensure complete drug solubility, the concentration of drug in ethanol solution was IND 1 mg / 98.6 μ l and KET 1 mg / 89.8 μ l. Depending on the final HA:CS PECN composition, the required quantity of HA solution (0.1% w/v) was placed in a glass vial and stirred magnetically at 900 rpm. The drug in ethanol solution was added simultaneously with a volume of CS solution (0.1% w/v). The volume of drug solution was varied to give a final drug concentration in the formulation of 0.01% w/v, 0.02% w/v and 0.04% w/v. A drug loaded HA:CS PECN dispersion was formed instantaneously and comprised HA, CS and the drug (IND or KET). This dispersion was left to stir for 5 min to stabilise. The final addition ratio was 0.1, 0.2 or 0.4 mg drug / 1mg of polymer.

To wash the drug loaded HA:CS PECNs, 7 ml of the dispersion was placed in a centrifugal filter device (Amicon Ultra-15, MWCO of 30 kDa; Millipore, USA) and centrifuged twice for 20 min at 4000 rpm using a Hettich Universal D7200 fixed rotor centrifuge (Hettich, Germany). The filter was first centrifuged with 7 ml of deionised water alone under these conditions to ensure that the full volume of water passed through.

2.2.6 Optimisation Studies on HA:CS PECN Formation

2.2.6.1 Varying Polymer Ratio

The following HA:CS volume ratios were tested with a total polymer concentration of 0.1% w/v: 10:4, 9:5, 8:6, 7:7, 7.5:6.5, 6:8 and 5:9. These samples were prepared unloaded or loaded with 0.2 mg/mg (0.02% w/v) KET or IND as described in **Section 2.2.4** and **Section 2.2.5**. Samples were stirred at 900 rpm for 5 minutes following addition of all components.

2.2.6.2 Varying Type of Solvent

Drug loaded (IND and KET) HA:CS PECNs were prepared as in **Section 2.2.5**, replacing ethanol by either acetone, ethyl acetate or dimethylsulphoxide

(DMSO). The same volume of each solvent was used (IND 1 mg / 98.6 μ l and KET 1 mg / 89.8 μ l) so that only the solvent was changing.

2.2.6.3 Varying pH of HA Solution

The pH of the HA 0.1% w/v solution (preparation described in **Section 2.2.3** was varied by dropwise addition of 0.1 mM NaOH or 0.1 M HCl. pH was altered to give HA solutions of pH 2, 4, 6, 8, and 10. These solutions were used to make unloaded and drug loaded HA:CS PECNs as described in **Section 2.2.4** and **Section 2.2.5** using 0.2 mg/ml of each drug (0.02% w/v).

2.2.6.3.1 pH measurements

A pH meter (Orion 420A+) with an Orion Ross™ 8103SC glass body pH semi-micro electrode was calibrated using Fluka standard analytical buffers at pH 4, 7 and 10 (± 0.01).

2.2.6.4 Investigating Stirring Speed and Time

HA:CS PECN samples were prepared as described in **Sections 2.2.4 and 2.2.5**. The following duration of stirring: 10 sec, 20 sec, 1 min, 10 min and 1 hr and stirring speeds: 0, 900 and 1200 rpm were investigated.

2.2.7 Precipitation of IND from Liquid Media for Solid-State Analysis

IND ethanolic solution (1 mg/98.2 μ l) was added to deionised water, 0.1% w/v HA solution, 0.1% w/v CS solution and 7.5:6.5 HA:CS NPs at drug concentration of 0.2 mg/ml and 0.4 mg/ml. The precipitate was isolated by decanting the liquid from the precipitate and the precipitate then was freeze dried as described in **Section 2.2.13**. The powder was characterised by PXRD (**Section 2.2.17**) and DSC (**Section 2.2.18**).

2.2.8 Solubility Studies for Solid-State Analysis

To measure the solubility of KET and IND in water, an excess of drug (0.05 g excess) was added to 5 mls of deionised water in a glass vial. When ethanol was the solvent the amount of drug required for excess was greater for KET (5.98 g) than IND (0.2 g). The amount of drug added varied because KET is freely soluble in ethanol while IND is sparingly soluble in ethanol. The samples were left for 24 hrs in a water bath (Precision scientific, Reciprocal Shaking Bath Model 25) at 25 °C shaking at 60 rpm. After this, 1.5 ml of sample was removed and centrifuged at 13,000 rpm for 30min in a micro-centrifuge to remove suspended material. The drug concentration in the supernatant was then tested using HPLC analysis as described in **Section 2.2.11**. pH of the samples was measured at 25 °C pre and post solubility study.

2.2.9 Particle Size, Polydispersity Index and Zeta Potential Analysis

The intensity-averaged mean particle size (mean particle size) and the polydispersity index (PDI) were determined by dynamic light scattering (DLS) with a Zetasizer Nano series Nano-ZS ZEN3600 (Malvern Instruments Ltd., UK) fitted with a 633 nm laser at 25°C using a 173° scattering angle. Three measurements were taken for each sample.

The zeta potential was an estimate of the surface charge based on the electrophoretic mobility measured by laser Doppler velocimetry (LDV), which was then converted to the zeta potential using the Henry equation.

0.9 ml of sample was placed into a clear disposable zeta cell for size and zeta potential measurement. The samples were undiluted on addition. The equilibration time was set to 5 min at 25 °C. Three repetitions were carried out and the results presented are the average values. The results were corrected for viscosity.

2.2.9.1 Viscosity measurements

The sample viscosity was measured using a low frequency vibration viscometer (SV-10 Vibro Viscometer, A&D Company, Limited). Samples were equilibrated at

25 °C in a water bath (Precision Scientific Reciprocal Shaking Bath Model 25) prior to measurement.

2.2.10 Drug Loading

Drug loading was measured by indirect and direct methods.

In the direct method 5 mg of freeze dried (as described in **Section 2.2.13**) NCs were added to 5 ml of the appropriate HPLC mobile phase and sonicated at amplitude 80 using an ultrasonic processor (Sonics VC130PB, Sonics and Materials Inc., USA), equipped with a probe with a diameter of 3 mm for 10 min, to ensure the NPs were completely disintegrated. The solution was centrifuged for 30 min at 13,000 rpm and the drug concentration in the supernatant was assayed using the HPLC method as described in **Section 2.2.11**.

The indirect method involved centrifugation of the PLGA NPs for 30 min at 13,000 rpm or in the case of PECNs - 5 ml of the dispersion was centrifuged for 40 min at 4,000 rpm using an Amicon Ultra-15 Centrifugal Filter Units 30 kDa. The drug content in the supernatant was measured using HPLC. For PECNs, NPs retained by the filter were reconstituted by adding the same volume of water as had been in the filtrate.

2.2.11 HPLC assay

The HPLC system was composed of a SCL-10A VP system controller, a LC-10 AT VD liquid chromatography pump system, SIL-10 AD VP autoinjector, FCV-10 AL VP low pressure gradient flow-control valve, DGU-14A degasser and a SPD-10A VP photodiode array UV-VIS detector (Shimadzu, Japan). The column used was Spherisorb S5 ODS2 14.6 mm × 125 mm (Waters, UK). The mobile phase for IND was acetonitrile:0.2% phosphoric acid at a 50:50 v/v ratio and for KET acetonitrile:0.2% phosphoric acid at a ratio of 40:60 v/v. A flow rate of 1 ml/min was used for 9 min for IND and 10 min for KET and the separation was carried out at room temperature. The UV wavelength used for IND was 237 nm and for KET was 233 nm. The calibration curve for IND covered the concentration range 0.001 – 0.05 mg/ml with an R^2 value of 0.999. The calibration curve for KET covered the concentration range 0.001 – 0.1 mg/ml had an R^2 value of 0.997.

2.2.12 Drug Release Studies

Drug release studies involved placing 5 mg of freeze dried NCs into 20 ml of release medium. The medium was either deionised water, 0.01M HCl or PBS buffer (pH of 7.4 and composed of 137 mM NaCl, 2.7 mM KCl, 1.4 mM NaH₂PO₄ and 1.3 mM Na₂HPO₄ as used by Oyarzun-Ampuero et al (2009)). The samples were placed in a water bath (Precision scientific, Reciprocal Shaking Bath Model 25) at 37 °C and shaken at 60 cpm (Corrigan and Li 2009). At specified time intervals 1 ml of sample was withdrawn and centrifuged at 13,000 rpm for 20 minutes using a micro-centrifuge. (Eppendorf Centrifuge 5415 D, USA) The supernatant was withdrawn for HPLC analysis (as described in **Section 2.2.11**). The pellet was reconstituted with the release medium to 1 ml and added back into the release dispersion.

2.2.13 Freeze-Drying of NCs

8 ml of NP dispersion was placed into a 15 ml plastic tube, frozen instantly in liquid nitrogen (-196 °C) and then placed into a wide mouth filter seal flask (VirTis SP Scientific, USA). The flask was attached to the vacuum chamber (VirTis 6K freeze-dryer model EL, SP Scientific, USA) and the freeze-drying process was continued for 48 hours. Primary drying was conducted at 5×10^{-2} mbar. Powders produced were stored at 4°C in a desiccator post production

2.2.14 Stability Studies

Empty and drug loaded PLGA and HA:CS PECN native dispersions were stored at 25 °C for 14 days. Particle size, PDI and ZP were measured as described in **Section 2.2.9** at predetermined intervals and visual observations were made.

2.2.15 Isoelectric Point Determination

The isoelectric point was determined using a Zetasizer Nano series Nano-ZS together with a MPT-2 autotitrator (Malvern Instruments Ltd., UK). The titrator

was calibrated at pH 4, 7 and 10 using Fluka analytical calibration buffers supplied. 0.1 M HCl and 0.1 M NaOH were used as titrants. The titrator was primed 10 times before experiments and the clear disposable zeta cell was filled twice to ensure the cell was filled without air. The 0.1M HCl or 0.1M NaOH was added at 0.5 pH unit increments to change the pH from the initial suspension pH to pH 11 or pH 2. Each analysis was carried out at room temperature in the automatic regime using a target pH tolerance of 0.2 units. Three particle size and three ZP measurements were carried out for each pH value and the sample was recirculated between repeat measurements.

2.2.16 Nanoparticle Imaging

HA:CS PECNs were viewed using Jeol 2100 Transmission Electron Microscope (TEM) (Jeol, Japan). It was operated at 200 kV with a lanthanum hexaboride emission source. The image was recorded digitally using an AMT digital camera. Samples were analysed by negative staining using 1% w/v uranyl acetate. Scanning Electron Microscope (SEM) analysis was performed on PLGA NPs using a Zeiss Supra Variable Pressure Field Emission Scanning Electron Microscope (Zeiss, Germany). Samples were prepared by affixing them onto aluminium stubs and sputter-coating with gold under vacuum prior to analysis.

2.2.17 Powder X-ray Diffraction Analysis (PXRD)

Samples were tested using a MiniflexII Desktop X-ray diffractometer, (Rigaku, Japan) with a Haskris cooling unit. The tube output voltage used was 30 kV and the tube output current was 15 mA. A copper tube with Ni-filter suppressing K β radiation was used. Measurements were taken from 5 to 40 on the 2-theta scale at a step size of 0.05° per second. Scans were performed using a low background silica sample holder at room temperature.

Freeze-dried NCs were analysed post-preparation as described in **section 2.2.13**.

2.2.18 Differential Scanning Calorimetry (DSC)

Differential Scanning Calorimetry (DSC) measurements were conducted using a Mettler Toledo DSC 821e module (Mettler Toledo, USA) with a refrigerated cooling system (LabPlant RP-100). Nitrogen was used as the purge gas. Top pierced 40 μ l aluminium pans were used. Samples were characterised by DSC in the range of 25 – 225 °C at a heating rate of 10 °C/ min. Samples were dried as described in **Section 2.2.13.**

2.2.19 Determination of Solubility Parameters

Hildebrand solubility parameters for CS (Ravindra et al. 1998), IND (Forster et al. 2001) and KET (Crowley et al. 2004) were obtained from literature. Ravindra, Forster and Crowley had calculated these Hildebrand solubility parameters from the chemical structures of CS, IND and KET by the Hoftyzer/Van Krevelen method (Van Krevelen and Nijenhuis 1990). The Hildebrand solubility parameter value for HA was calculated based on available literature values. The closer the Hildebrand solubility parameters for two substances the more miscible they are estimated to be. Madsen et al. (2013) reported that although the total Hansen solubility parameter is more accurate as they take into account polar and hydrogen bonds as well as dispersive interactions there was only minor differences observed for polymer solutions between the total Hansen solubility value and the Hildebrand solubility value.

2.2.20 Visual miscibility/solubility studies

Optical microscopy was used to visually assess miscibility/solubility of IND and KET with HA and CS. Unloaded and drug-loaded NPs as well as physical mixes of the individual polymers with IND and KET solutions were prepared and 1 ml of each was dropped onto glass slides and allowed to dry in nitrogen gas. They were then viewed using an upright Olympus BX51M optical microscope using a 10x magnification lens (Olympus, USA).

2.2.21 Aggregometry

Aggregometry studies were carried out as described by Radomski et al (2005). 35 ml of blood was collected into tri-sodium citrate (3.15% w/v) in a ratio of 9:1 v/v from healthy volunteers who had not taken any drugs known to affect platelet function for 2 weeks prior to the study. Platelet-rich plasma (PRP) was obtained by centrifugation at 240 g for 20 min at room temperature. Platelets were counted using a Coulter counter (Beckmann, USA) and the final platelet number (2.5×10^8 platelets per ml) was adjusted using Tyrode's solution. Platelet-poor plasma was obtained by centrifugation of PRP at 10,000 g for 5 minutes at room temperature. Aggregation was measured using a four-channel Chrono-log whole blood Lumi-Aggregometer (Chrono-log Corporation, USA) linked to Aggro-Link data-reduction. Collagen tests were performed to test the response of the platelets to Collagen at 1 ug/ml, 2 ug/ml, 3 ug/ml and 5 ug/ml to make sure that the platelets were sensitive enough to the selected 2 ug/ml concentration.

Preliminary studies on the ability of the empty NCs (PLGA NPs and HA:CS positively and negatively charged PECNs) to stimulate aggregation of resting platelets without collagen stimulation were conducted. To investigate this platelet samples were incubated in the presence or absence of NCs (80 µg/ml) for 24 min recording the effects.

To study whether either type of NP (PLGA and HA:CS) loaded with KET or IND influences collagen-induced platelet aggregation further aggregation studies were carried out. Platelet aggregation was initiated by the addition of collagen (2 µg/ml). Each type of NC loaded with IND or KET at 0.1 mg/mg or 0.2 mg/mg was pre-incubated with platelet samples for 1 min or 10 min before the addition of the collagen (2ug/ml) and the extent of aggregation was recorded until the aggregation had plateaued. Empty NCs were used in the blank resting platelet sample so as not to interfere with light transmission.

The results were calculated as an expression of the percentage of light transmission where 100% transmission (platelet-poor plasma) was taken as maximal aggregation. The mean value of three replicates was taken and 3 donors were used for each particle type.

2.2.22 Flow Cytometry

The abundance of P-selectin on the surface of platelets both in the presence and absence of NCs was measured using flow cytometry. Platelet aggregation was

measured using the PAP-8E Platelet Aggregation Profiler (Bio/Data Corporation, USA) which consists of eight channels. Platelet samples were pre-incubated with the loaded NCs at a preincubation time of 10 minutes. The concentrations that were used were decided based on the minimum concentration at which collagen-induced platelet aggregation was found to be statistically significant in the previous aggregometry studies (**Section 2.2.21**). Using the Chrono-log whole blood Lumi-Aggregometer (Chrono-log Corporation, USA), the platelet samples were activated with collagen (2µg/ml) at the chosen concentration for each sample. The reaction was ceased when platelet aggregation reached 50% maximal light transmission and 10 µl of each sample were extracted and placed in labelled tubes. Resting platelets were used as the negative control in this experiment and empty NCs added to resting platelets without collagen addition was used to ensure the empty carriers were not affecting the platelet aggregation. 10 µl of anti-P-selectin antibody was added to all tubes followed by 80 µl of sheath solution with surfactant. The platelet samples were then incubated in the dark for 5 minutes at room temperature. Following incubation, the samples were diluted with 300 µl of sheath solution with surfactant and 350 µl was loaded into the appropriate wells of the plate. The samples were then analysed using the BD FACS Array™ instrument (BD, USA), which was programmed to measure size, granularity and cell fluorescence. The individual platelets were analysed for fluorescence in order to determine antibody binding to the P-selectin antigen. The platelets were recognised by forward and side scatter signals, and 10,000 platelet-specific events were analysed by the flow cytometer for fluorescence.

2.2.23 Statistical Analysis

Statistical analysis for **Chapters 3-5** was carried out using Minitab™ software (version 13.32). Descriptive statistics (median and standard deviation), regression analysis and ANOVA were performed. Parameters found to be significant at the 95% confidence level were considered to have an effect or to be different.

Statistical analysis for **Chapter 6** was carried out using Graph Pad Prism 5. The results were analysed using a one-way ANOVA throughout the studies and the

data was expressed as mean $\pm$ standard deviation. Tukey post hoc tests were also performed using the software. A 95% confidence interval was used. Statistical significance was considered when $p < 0.05$ and highly significant when $p < 0.001$.

The Optimisation and Characterisation of Poly(lactic-co-glycolic) Acid Nanoparticles Loaded with Indometacin and Ketoprofen

Chapter 3

3.1 Introduction

Poly(lactic-co-glycolic) (PLGA) nanoparticles (NPs) have been prepared by many research groups for drug delivery purposes (Soppimath et al. 2001, Fonseca et al. 2002, Kumari et al. 2010 and Acharya et al. 2011) as PLGA is an FDA approved biodegradable and biocompatible polymer (Danhier et al. 2012). PLGA has a long degradation time of several months to years depending on its type and is degraded by hydrolysis (Prokop et al. 2008, Vert et al. 1994). Prior to the studies described in this thesis, Corrigan and Li (2009) prepared PLGA NPs and loaded them with drugs from different classes (the small organic molecules: ketoprofen, indometacin, coumarin-6 and the macromolecules: human serum albumin and ovalbumin). While Corrigan and Li (2009) focused on elucidating the release mechanism for the drug loaded PLGA NPs, current studies focused on a detailed characterisation of the PLGA NPs loaded with small organic molecules from the non-steroidal anti-inflammatory drug (NSAID) class. The drugs used were indometacin (IND) and ketoprofen (KET).

The first aim of this chapter was to optimise and characterise empty PLGA NP carriers to create a stable NP prototype into which drugs could be loaded. This was done by systematically working through the variables that influence formation of these PLGA NPs to develop a preparation method that would give PLGA NPs with the most desirable properties for loading KET and IND. Once the preparation method had been optimised, the empty PLGA carriers were physically characterised to establish the characteristics of these PLGA NPs for future experiments. The second aim of this chapter was to load the optimised and characterised empty PLGA NPs with IND and KET and then to characterise these drug loaded NPs. These drugs were loaded at two different concentrations 0.1 mg of drug/mg of polymer and 0.2 mg of drug/mg of polymer. The drug loaded PLGA NPs were characterised to understand the physical properties of these drug loaded PLGA NPs and to determine the amount of drug incorporated and the drug release profile.

PLGA NPs were formed for these current studies using an emulsification/diffusion method as described by Bala et al. (2004). This method involves the formation of an emulsion with the drug and PLGA in the oil phase added to a surfactant solution to act as the continuous phase for the emulsion. After this the

sample is sonicated to reduce the emulsion droplet size. This emulsion is then added to more of the surfactant solution, which causes the solvent to diffuse out of the nanodroplets in the emulsion resulting in the precipitation of solid PLGA-drug nanoparticles. The excess solvent evaporates and the nanoparticles are washed by centrifugation to remove excess surfactant and disassociated drug and surfactant (Song et al. 1997 and Guarrero et al. 1998).

3.2 Empty PLGA Nanoparticles – Optimisation and Characterisation

3.2.1 Optimisation of the Formulation Variables

3.2.1.1 Type of PLGA used to form PLGA NPs

PLGA is available with different compositions. The properties of PLGA are varied by varying the ratio of lactide:glycolide, by changing the molecular weight and by altering the PLGA end group (Houchin and Topp 2008). As there are many types of PLGA available it is important to consider the desired properties of the final PLGA NPs when selecting which PLGA type to choose (Makadia and Siegel 2011). Two types of PLGA copolymer were tested in the current study both of which have been used successfully by other research groups to produce PLGA NPs. Resomer RG504H is a 50:50 mix (% w/w) of lactide:glycolide, it has the fastest degradation rate of all PLGA polymer blends and is used frequently in the formation of nanoparticles (Govender et al. 1999, Barichello et al. 1999 and Mua and Feng 2003). PLGA RG752H has a ratio of 75:25 lactide:glycolide, and it has a longer degradation time due to increased lactic acid content (Park 1995) and like the 50:50 mix it is commonly used in the preparation of PLGA NPs (Sarti et al. 2011, Nair et al. 2012). The Mw of these Resomers are 38,000-54,000 g/mol for RG504H and 4,000-15,000 g/mol RG752H (Park 1995). The viscosity values are 0.45 – 0.60 dl/g for RG504H and 0.14 – 0.22 dl/g for RG752H (Park 1995). To compare the RG504H PLGA with the RG752H PLGA, both were used to make independent samples of PLGA nanoparticles using the emulsification-diffusion method (**Section 3.1**). Once prepared, the samples were characterised and the size (nm), PDI, and ZP (mV) were compared. The results (**Table 3.1**) showed that RG504H offered smaller size (nm) (RG504 H = 269 ± 33 nm and

RG752H = 484 ± 24 nm) and a more stable ZP (mV) lower than -30 mV (RG504H = -41 ± 1 mV) meaning that it was predicted that these NPs will be more stable than those made of RG752H, which had a ZP value close to neutral ($+9 \pm 2$ mV). The PDI was also a major factor in deciding which PLGA type to use as for RG504H the PDI was very low at 0.08 ± 0.02 but for RG752H it was high (0.62 ± 0.1) indicating that the sample was polydisperse and most likely prone to be unstable for this reason. Chan et al. (2008) found that increasing PLGA viscosity decreased the NP size. Chan et al. (2008) formulated PLGA–lecithin–PEG core–shell NPs as a controlled release drug delivery system using PLGA with a 50:50 monomer ratio, ester-terminated (Durect Corporation, Pelham, AL). By holding the lipid/polymer mass ratio constant they could vary the PLGA polymer inherent viscosity to study its effect on the NPs formed. PLGA with 0.82 dl/g inherent viscosity for the polymeric core was used in their optimal formulation. The viscosity of RG752H is lower than that for RG504H, which may be contributing to the final PLGA NPs size.

Mittal et al. (2007) used PLGA 50:50 of different molecular weights (14,500, 45,000, 85,000, 137,000 and 213,000 Da) and PLGA of different copolymer compositions: PLGA (50:50; MW-85,000 Da), PLGA (65:35; MW-97,000 Da), and PLGA (85:15; MW-87,000 Da) purchased from Birmingham Polymers, Inc (Birmingham, AL) to prepare estradiol loaded PLGA NPs using an emulsion–diffusion–evaporation method. They reported that as the Mw of the PLGA increased the NP size decreased with the PDI being highest at the lowest Mw, which was in contrast to the observation here where the lower Mw sample RG752H resulted in NPs with a larger particle size. This could indicate that the current studies used a method that was less suitable to PLGA RG752H possibly due to differences in the solubility of these polymers or their behaviour in the emulsification or solvent diffusions steps.

Table 3.1 Properties of PLGA nanoparticles prepared using different types of PLGA. Two types of PLGA were used RESOMER RG52H and RESOMER RG504H.

PLGA Type	Size (nm)	PDI	ZP (mV)
RESOMER RG752H	484 ± 24	0.62 ± 0.12	+ 9 ± 2
RESOMER RG504H	269 ± 33	0.08 ± 0.02	- 41 ± 1

Based on the observed results when comparing these PLGA types it was decided to choose PLGA RG504H in all subsequent studies as it gave more desirable size (nm), PDI and ZP (mV) values for the PLGA NPs.

3.2.1.2 Surfactant Concentration

The surfactant concentration influences the physical properties of PLGA NPs as it is a vital part of the emulsification step during NP production and influences the NP stability in their dispersion medium post formation. As Pluronic is a non-ionic surfactant it stabilises the system by sterically preventing the nanoparticles from interacting, this makes it a wise surfactant choice. It was decided to test another non-ionic surfactant to see if there would be interesting differences between them or an obvious benefit to preferentially choosing one. Tween 80 has also been a popular choice in previous studies producing NPs due to its biocompatibility. Zhang et al. (2006) reported that Tween 80 is widely used as an emulsifier/surfactant/stabiliser in the fabrication of polymeric nanoparticles, liposomes, or solid lipid nanoparticles. Koochehi et al. 2011 chose Tween 80 to form their PLGA RG 752S and RG752H based implant membranes as Tween 80 is an excipient that is used to stabilise aqueous formulations of medications for parenteral administration making it biocompatible and safe. Tween 80 has a molecular weight of 1310 g/mol (Zhang et al. 2006) while Pluronic F-68 has a molecular weight of 8400 g/mol (Santander-Ortega et al. 2006).

In the current studies it was observed that when Pluronic F-68 was used the size (nm) of the NPs decreased with increasing surfactant concentration and then increased again at the highest Pluronic F-68 concentration used (2.4% w/v) (**Table 3.2**). Santander-Ortega et al. (2006) showed that increasing the concentration of Pluronic F-68 in PLGA NPs coated with Pluronic F-68 helped to stabilise the NPs by forming a surrounding layer on the surface. Their work

suggested that when a critical concentration was reached, micelles formed on the nanoparticles surface increasing their size. This may have been the case for the current studies when the concentration of surfactant reached 2.5% w/v Pluronic F-68.

As shown in **Table 3.2**, the ZP reduced as the concentration of Pluronic F-68 in solution increased, most likely due to steric blocking of the surface charges, but it still remained in a stable range below -30 mV.

Tween 80 was used for comparison: it offered no formulation benefits on observation of the physical characteristics, as the PLGA NP size was larger than that with Pluronic F-68. The ZP was within the recommended range indicating good stability (± 30 mV) at -31 ± 1 mV and the PDI was low at 0.13 ± 0.02 but given the size differences it was decided that Pluronic F-68 would be more suitable for these experiments. Non-ionic surfactants are advantageous surfactants to use as they do not interact by charge with the other NPs in the dispersion or with the dispersion medium itself. If the particles are dispersed in a dispersion medium with a high ionic strength such as buffers or biological fluids it could be proposed that a non-ionic surfactant would be desirable. Amiji and Park (1992) prepared solutions of ten different types of Pluronics dissolved in phosphate buffered saline (PBS, pH 7.4) at bulk concentrations of Pluronic from 0.01 to 10 mg/ml. Fibrinogen adsorption and platelet adhesion on to dimethyldichlorosilane-treated glass and low-density polyethylene were examined. Fibrinogen adsorption on the Pluronic-coated surfaces was reduced by more than 95% compared to the adsorption on control surfaces. The ability of Pluronics to prevent platelet adhesion and activation was mainly dependent on the number of propylene oxide residues and was effective by repelling fibrinogen and platelets though steric repulsion.

Table 3.2 Influence of surfactant concentration on particle size (nm), PDI and ZP of PLGA NPs.

Concentration (% w/v)	Size (nm)	PDI	ZP (mV)
Pluronic F-68 0.8%	269 ± 3	0.08 ± 0.01	- 41 ± 1
Pluronic F-68 1.0%	200 ± 2	0.20 ± 0.04	- 28 ± 2
Pluronic F-68 2.4%	237 ± 9	0.32 ± 0.03	- 30 ± 1
Tween 80 1.0%	408 ± 11	0.13 ± 0.02	- 31 ± 1

Based on the results it was decided to use Pluronic F-68 at 1.0 % w/v concentration as it produced PLGA NPs with the smallest NP size, a low PDI value and a ZP value indicating good physical stability.

3.2.1.3 Number of Washing Steps

While the surfactant is important for the formation of the PLGA NPs, after preparation they are washed to remove excess surfactant, free drug and free polymer. It was thought in the current studies that removing these excess components would improve the safety profiles of the NPs and also to allow the NP behaviour to be predicted more accurately.

The washing steps appeared to be increasing the NP size and the sizes were found to be significantly different after each wash ($p<0.05$). This size increase is most likely due to the loss of the surfactant from the formulation, which would act to stabilise the NPs. It is desirable to reduce the surfactant content to the minimal amount necessary for stabilisation of the NPs in order to reduce side-effects due to the surfactant. Sahoo et al. (2002) prepared PLGA (50:50) NPs using PVA (0.5% w/v - 5% w/v) as the surfactant. This study proved that residual PVA resulted in the PLGA NPs having a relatively lower cellular uptake despite their smaller particle size.

Table 3.3 shows that the size increase between PLGA NPs washed once and twice was approximately 54 nm with the ZP decreasing from -23 ± 1 nm to -38 ± 1 mV. The ZP was more negative after each wash, which may be due to loss of Pluronic F-68 from the surface covering the charged $-\text{COOH}$ group on PLGA.

This is in accordance with Santander-Ortega et al. (2006) who reported that the presence of Pluronic F-68 on surface of PLGA nanoparticles neutralised the surface charge.

The differences observed between the second and third wash were not as large with a size increase of approx. 20 nm and an increase in ZP from -38 ± 0.3 to -32 ± 1.0 mV. For this reason it was decided to use two washes only for the following experiments to keep the preparation process efficient while at the same time allowing that excess polymer, drug and some of the surfactant was removed without destabilising the systems.

Table 3.3 Changes observed in the physical properties of PLGA NPs after each washing step to remove unnecessary components from the system.

Wash	Size (nm)	PDI	ZP (mV)
1	234 ± 1	0.08 ± 0.01	-23 ± 1
2	288 ± 4	0.12 ± 0.02	-38 ± 1
3	308 ± 5	0.11 ± 0.05	-32 ± 1

3.2.1.4 Centrifugation of PLGA NPs

In order to separate PLGA NPs during the washings steps they were centrifuged. Filtration was tried as a method to separate the PLGA NPs from the dispersion medium but this proved to be inefficient because it was only possible to recover a small quantity of PLGA NPs from the filter paper thus resulting in low PLGA NP yield post-washing.

The centrifugation speed was investigated to choose the speed that allowed efficient separation without damaging the PLGA NPs and at the same time gave an acceptable PLGA NP yield post-washing. Increasing the centrifugation speed did not have a major effect on the size of the PLGA NPs once redispersed after centrifugation at 8,000rpm, 10,000 rpm and 13,000 rpm. It did cause a decrease in the size of the empty PLGA NPs at 15,000 rpm which possibly indicated that the smaller PLGA NPs remained suspended for a longer period of time in the NP

dispersion (**Table 3.4**). High speeds may be necessary to completely separate these PLGA NPs from their dispersion medium and thus the average size would be lower. The higher PDI value observed at the fastest speed 15,000 rpm may be due to the separation of smaller NPs from the dispersion medium. Greater separation is important for increasing PLGA NP yield post-production. As with the size, the ZP did not show large differences between 8,000 rpm, 10,000 rpm and 13,000 rpm but at 15,000 rpm the ZP value increased possibly due to surfactant loss from the surface of the PLGA NPs at higher speeds.

Table 3.4. Effect of centrifugation speed on properties of NPs.

Centrifugation Speed (rpm)	Size (nm)	PDI	ZP (mV)
8,000	280 ± 3	0.13 ±0.05	-38 ± 2
10,000	255 ± 13	0.13±0.04	-37 ± 2
13,000	267 ± 3	0.08 ±0.02	-41 ± 1
15,000	218 ± 1	0.20 ±0.06	-28 ± 1

The size spanned from 280 ± 3 nm to 218 ± 1 nm when the centrifugation speed increased so the effect on size was significant. Centrifuging at higher speeds made reconstitution of the NPs difficult as the pellet was more compacted in the tube and it was harder for water to penetrate and redisperse the PLGA NPs. For this reason the centrifugation speed chosen for further experiments was the second highest speed 13,000 rpm.

Time did not have major effects on the size, PDI or ZP of the samples once it was over 10 minutes so 15 minutes was chosen as the centrifugation time. If the centrifugation time was over 30 minutes the pellet became very compacted and thus was too difficult to reconstitute fully.

3.2.1.5 Volume of 0.1% Pluronic Solution Used for Solvent-Diffusion

The PLGA NPs were prepared using the emulsification – solvent diffusion method. In this method once the PLGA and ethyl acetate had been emulsified with the Pluronic 0.1% w/v solution it was added to a further volume of Pluronic

0.1% w/v solution to allow the solvent (ethyl acetate) to diffusion out leaving the PLGA NPs. Previous work by Li et al. (2009) had used four times the original emulsion volume (15 ml added to 90 ml) to precipitate their PLGA NPs but these current experiments were conducted to test if a lower volume could be used to make the process more efficient. The volume of water added is a key factor in the emulsion solvent-diffusion method (Quintanar-Guerrero et al. 1996). A large volume of water results in rapid diffusion and thus reduces NP size. Larger volumes are more difficult to work with so the lowest volume that did not result in a considerable increase in the particle size was desired.

The particle size was smallest and the ZP was lowest when 45 ml of water was used (**Table 3.5**). There was a minor difference in the size of NPs for 90 ml and 45 ml (288±4 nm compared to 269±3 nm, respectively), but a large change in size was observed by a further reduction of the Pluronic solution 0.1% w/v volume to 25 ml (372±4 nm). The ZP also increased as the volume of Pluronic 0.1% w/v solution was reduced with increasing size becoming less stable when the volume of water decreased to 25 ml.

Table 3.5. Effect of volume of water used on PLGA NP size (nm), PDI and ZP (mV).

Volume of water added	Size (nm)	PDI	ZP (mV)
90 ml	288 ± 4	0.12 ± 0.03	-38 ± 1
45 ml	269 ± 3	0.08 ±0.02	-41 ± 1
25 ml	372 ± 4	0.21 ±0.08	-27 ± 1

3.2.1.6 Summary of optimal properties for PLGA NPs

The results of optimisation experiments were collected to consolidate a method for formation of the PLGA NPs to be used in the current studies. The chosen factors are listed below and were used going forward to form drug loaded PLGA NPs.

- **PLGA Type:** RG504H
- **Solvent:** Ethyl Acetate
- **Surfactant:** Pluronic F-68 0.1% w/v
- **Diffusion Volume:** 45 ml
- **Centrifugation speed and time:** 13,000 rpm, 15 mins
- **Number of washes:** 2

3.2.2 Characterisation of PLGA NPs

Once the formulation variables had been optimised the next step was characterising these PLGA NPs formed using the chosen formulation method.

3.2.2.1 PLGA NP Imaging

Once the NP preparative process had been optimised, scanning electron microscopy (SEM) was used as the technique to view the PLGA NPs. SEM was used instead of transmission electron microscopy (TEM) because the TEM beam would have melted the samples given the low T_g of PLGA ($42.5 \pm 0.5^{\circ}\text{C}$ - Passerini and Craig 2001). The PLGA NPs were present as even, spherical, non-porous, smooth spheres (**Figure 3.1**). Given the low T_g of PLGA some fusion of the NPs was seen probably caused by some softening while imaging the PLGA NPs. Similar features of PLGA NPs have been observed by many other authors (Song et al. 2005, Nafee et al. 2007 and Corrigan and Li 2009). All

PLGA NPs observed were found to be in the nano-size range. This imaging technique complemented the DLS results as the size observed was similar for both.

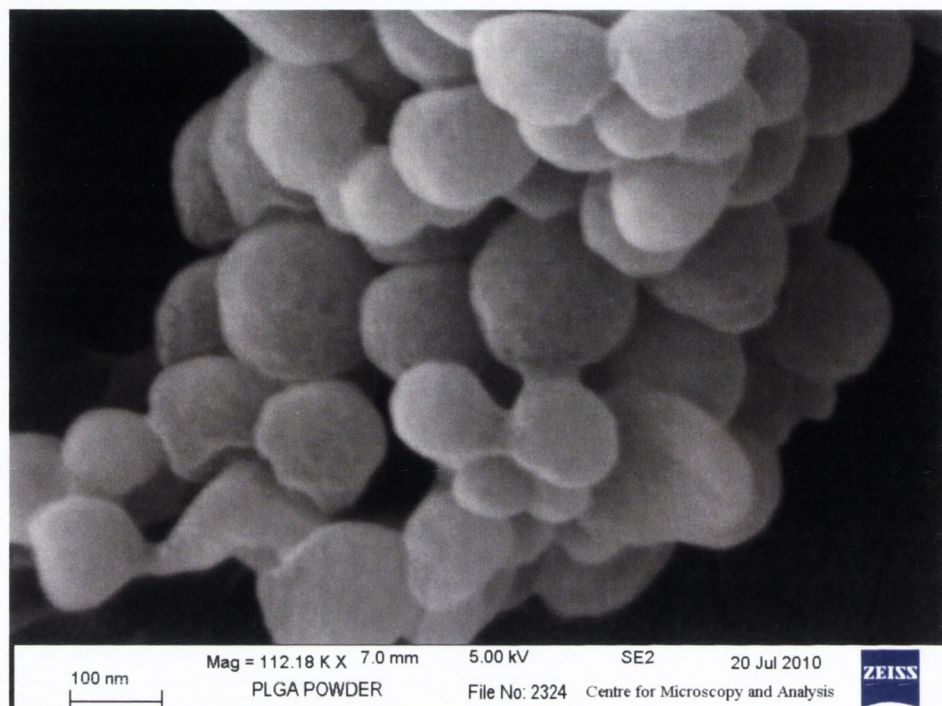


Figure 3.1 SEM image of empty PLGA NPs.

3.2.2.2 PLGA NP Particle Size, PDI and ZP Measurements using DLS

Unloaded PLGA NPs, prepared using the optimised manufacturing parameters as outlined in Section 3.2.1.6 were characterised using the Malvern Zetasizer Nano ZS. The properties of the optimised PLGA NPs (size (nm), ZP (mV), PDI) are listed in **Table 3.6**. The particle size was 267 ± 3 nm showing low standard deviation, the ZP – 41 ± 1 mV, indicating physical stability (outside of ± 30 mV) and the PDI was low 0.08 ± 0.02 . It was decided that it would not be necessary to optimise the PLGA NPs while loaded with KET and IND as these NPs are resilient to degradation due to their hydrophobic nature and so the drugs were not expected to affect the formulation stability as much as they would if the polymer was hydrophilic (Huh et al. 2003).

Table 3.6 Properties of PLGA NPs prepared using the optimised manufacturing method.

Polymer/Drug	Size (nm)	PDI	ZP (mV)
PLGA Empty	267 ± 3	0.08 ± 0.0	- 41 ± 1

3.3 Loading Ketoprofen (KET) into PLGA NPs

3.3.1 Drug Loading and Physical Characterisation

The formulation and process parameters established in **Section 3.2.1.6.** were used to prepare KET loaded PLGA NPs. To study DL, two concentrations of KET (0.1 mg/mg, 0.2 mg/mg) were tried to investigate how KET-loaded PLGA NPs would form with different amounts of drug added.

Empty PLGA NPs were smaller than those loaded with KET (**Table 3.7**). The size of KET loaded PLGA NPs increased from 267 ± 3 nm without the presence of KET to 344 ± 2 nm at KET 0.1 mg/mg loading concentration and 316 ± 3 nm at KET 0.2 mg/mg loading concentration. The addition of KET significantly increased the size ($p = 0.001$) of the loaded PLGA NPs compared to unloaded PLGA NPs.

The addition of KET increased the ZP of the PLGA NPs. The ZP increased from -41 ± 1 mV for empty PLGA NPs to -29 ± 1 mV at KET 0.1 mg/mg and -33 ± 1 mV at KET 0.2 mg/mg. The rise in ZP once KET had been added may be due to the partial blockage of surface charge by the KET. The difference between the ZP for KET 0.1 mg/mg and KET 0.2 mg/mg PLGA NPs was significantly different ($p = 0.003$) and the size difference between these was also significantly different ($p=0.0002$).

Although the surface charge of unloaded PLGA NPs is more negative than the surface charge of KET loaded PLGA NPs, the increase in surface charge with KET addition did not vary considerably with the quantity of KET loaded.

The PDI increased as KET was loaded into the PLGA NPs. This means that the addition of KET increased the variation between individual NPs within the KET loaded PLGA NP dispersion. It increased from 0.08 ± 0.02 for empty PLGA NPs to 0.22 ± 0.07 when loaded with KET 0.1 mg/mg and 0.30 ± 0.09 when loaded

with KET 0.2 mg/mg. As the PDI increases the PLGA NP samples may not exhibit constancy given that there is more variation within the samples.

The EE for PLGA KET loaded NPs at KET 0.1 mg/mg loading was 19 ± 4 % w/w and at KET 0.2 mg/mg DL was 41 ± 11 % w/w (**Table 3.7**). These encapsulation efficiencies corresponded to a DL value of 2 ± 1 % w/v for KET 0.1 mg/mg and 12 ± 3 % w/v for KET 0.2 mg/mg. This showed that addition of a higher concentration of KET 0.2 mg/mg actually resulted in higher KET loading. The higher DL at higher initial addition concentrations may be related to the concentration of drug being higher in the ethyl acetate solution during the formation process so each droplet emulsified had a higher concentration of KET dissolved in it. Corrigan and Li (2009) reported similar values for KET EE of 40.2 ± 1 % (initial loading drug concentration was 0.2 mg/mg)

Table 3.7 Physical properties and DL for PLGA NPs loaded with KET at two different concentrations.

Polymer/Drug	Size (nm)	ZP (mV)	PDI	EE%	DL % (w/w)
PLGA Empty	267 ± 3	-41 ± 1	0.08 ± 0.02	-	-
KET 0.1 mg/mg	344 ± 2	-29 ± 1	0.22 ± 0.07	19 ± 4	2 ± 1
KET 0.2 mg/mg	316 ± 3	-33 ± 1	0.31 ± 0.09	41 ± 11	12 ± 3

3.3.2 KET loaded PLGA NP Imaging

The KET loaded PLGA NPs at 0.2 mg/mg were chosen to view using SEM as this DL showed that at KET 0.2 mg/mg a higher concentration of drug had been incorporated and were thus a better formulation.

The KET 0.2 mg/mg loaded PLGA NPs were present as spherical, non-porous, smooth spheres similar to those observed for empty PLGA NPs (**Figure 3.2**). There was no evidence of KET obvious in these images and no loose or associated drug crystals were observed. All KET loaded PLGA NPs were found to be in the nano-size range and the SEM images complemented the dynamic light scattering (DLS) results. The average size was measured visually for the PLGA NPs by comparing to the scale bar. Given that for SEM the samples are dried it is expected that the average PLGA NPs size would be smaller due to less water being present in the PLGA NPs (Bootz et al. 2004).

Given that these KET loaded PLGA NPs have a similar appearance to the empty PLGA NPs the drug does not obviously affect the visible structure of the PLGA NPs.

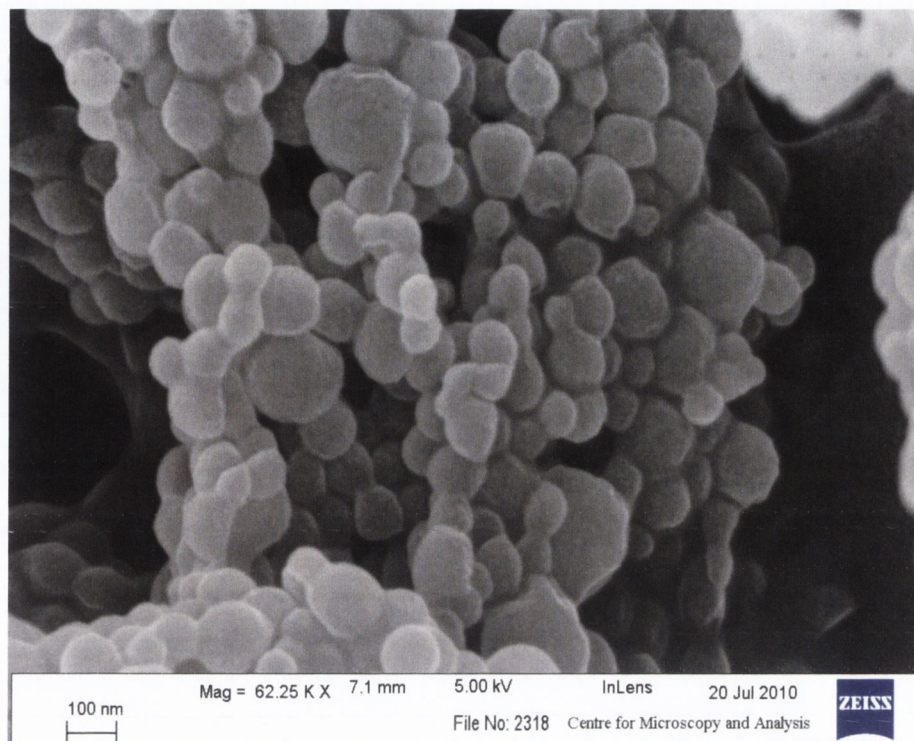


Figure 3.2 SEM image of PLGA NPs loaded with KET (0.2 mg/mg).

3.3.3 PXRD and DSC Analysis

Solid-state analysis was carried out to investigate in what physical form KET was present (crystalline or amorphous) within the PLGA NPs as this would have an effect on the behaviour of the NPs due to differences in KET's solubility. KET is classed as a BCS class II drug and its solubility depends greatly on the pH of the dissolution medium ([Sheng et al. 2006](#)). The aqueous solubility of crystalline KET is enhanced by conversion to the amorphous form. PLGA is amorphous by nature (Park 1995).

PXRD showed that the surface of all particles was amorphous. The peaks associated with crystalline KET were not visible (**Figure 3.3**). This showed that KET was amorphous on the surface of the PLGA NPs. It was considered possible that KET had precipitated in the amorphous form during the emulsification/ solvent-diffusion formation process.

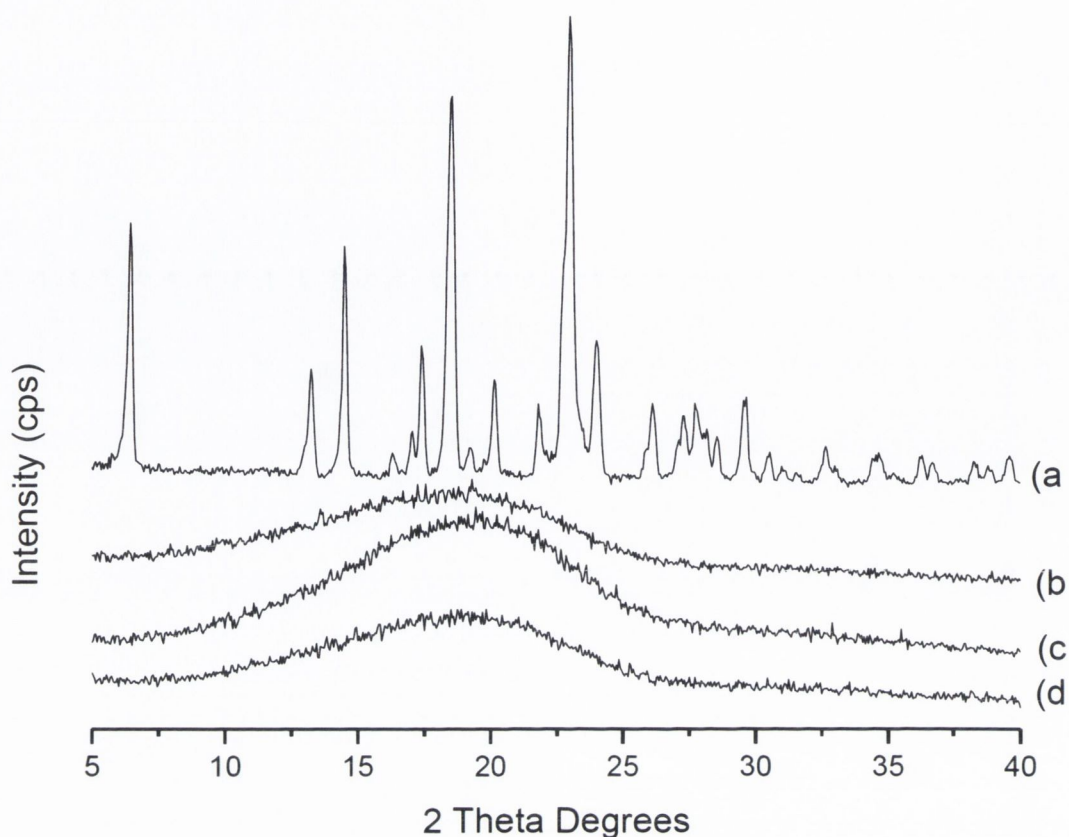


Figure 3.3 PXRD data for KET loaded PLGA NPs and empty PLGA NPs compared with free KET. a) = free KET, b) = KET 0.1 mg/mg, c) = KET 0.2 mg/mg ,d) PLGA NPs.

The DSC data showed that PLGA NPs contained KET in the amorphous form as there was no melting point observable for KET at 94.77 ± 0.26 °C (**Figure 3.4**). The KET-loaded PLGA NPs did not show any differences in their thermal behaviour for 0.1 mg/mg or 0.2 mg/mg of KET so the drug was amorphous in both. It was observable from the DSC traces that PLGA exhibited an endothermal relaxation process underlying the glass transition temperature (T_g) event as was observed previously by Corrigan and Li (2009). The T_g (°C) of PLGA was not considerably affected by the presence of KET (**Table 3.8**).

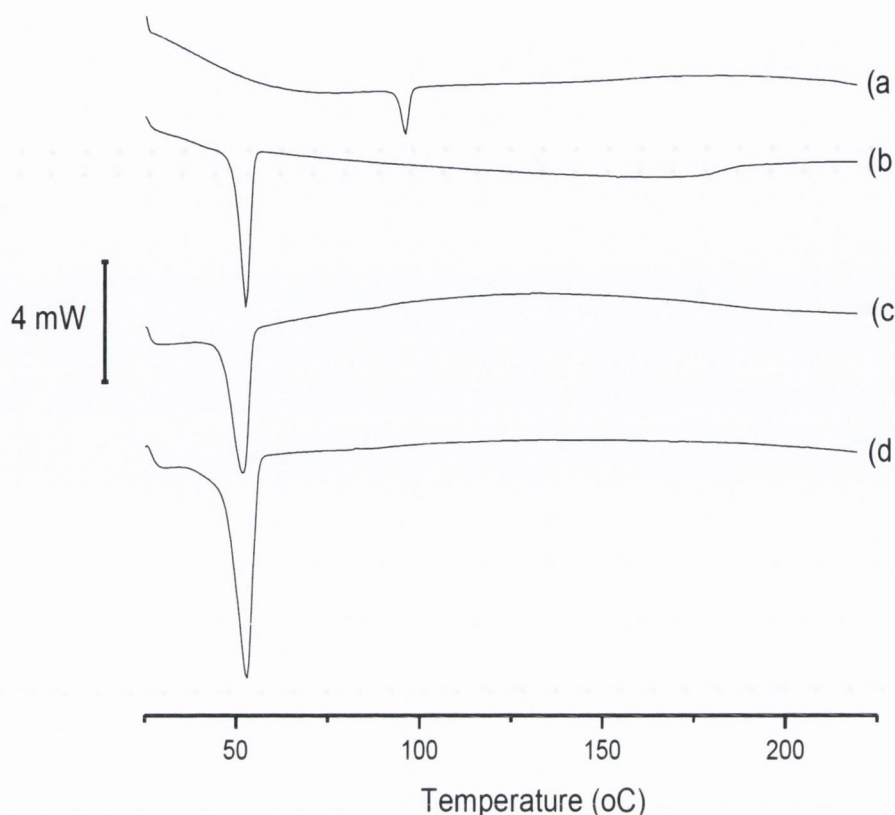


Figure 3.4 DSC data from KET loaded and empty PLGA NPs compared with free KET. a) = free KET, b) free PLGA, c) = KET 0.1 mg/mg, d) = KET 0.2 mg/mg

Table 3.8 Thermal properties of KET loaded PLGA NPs as determined by DSC. The table includes Tg values and relaxation process (J/g) for KET loaded PLGA NPs and empty PLGA NP.

Sample	PLGA Empty NPs	KET 0.1 mg/mg PLGA NPs	KET 0.2 mg/mg PLGA NPs
Tg (°C)	49.10 ± 1.10	46.15 ± 0.69	47.27 ± 1.05
Relaxation process (J/g)	86.33 ± 2.54	64.42 ± 1.34	71.06 ± 0.98

Previous work by Corrigan and Li (2009) has shown that empty PLGA NPs and those loaded with KET showed a Tg for PLGA only and no other events indicating the presence of KET within these NPs. In their work, the Tg of PLGA NPs varied depending on whether the NPs were KET loaded or not.

Their observed T_g for PLGA NPs loaded with KET was 48 °C and for their empty PLGA NPs it was 54°C. They concluded that both the manufacturing process and dispersed drug may affect conformation of the polymer molecular chains and result in a decrease in T_g.

3.3.4. Stability Studies

The stability of KET loaded PLGA NPs was studied over a 14 day period to look for changes in the physical stability of the sample over time. Post-preparation the NPs samples were stored as aqueous dispersions at room temperature (25 °C) and measured for size (nm), ZP (mV) and PDI using DLS (Malvern Zetasizer Nano ZS).

The results showed that over the 14 day period there were only small fluctuations in the size of the KET loaded PLGA NPs with this being reflected in the consistent PDI values. The size ranged from 94 – 102 % of the original size over the 14 days for KET 0.1 mg/mg NPs and for 0.2 mg/mg KET loaded PLGA NPs the range of variation over 14 days was 95 – 102 % of the original size. For empty PLGA NPs the % size change from the original size over 14 days ranged from 96 – 103 %, which was close to the range for KET loaded PLGA NPs. KET itself rapidly increased in particle size to 216% on day 9 compared to the starting particle size on day 1 (566nm). After day 9 KET precipitated out as larger sediments indicating that the PLGA NPs were playing a role in stabilising KET.

The PDI for the PLGA NPs did not vary significantly over the 14 day time period. For 0.1 mg/mg KET loaded PLGA NPs the PDI range spanned 0.03 – 0.08 and for 0.2 mg/mg the range was 0.05 – 0.09 over the 14 days. For empty PLGA NPs the range over 14 days was 0.08 – 0.14, which was no different to the KET loaded PLGA NPs.

For KET 0.1 mg/mg the ZP ranged from - 19 mV to - 21 mV and for KET 0.2 mg/mg the ZP ranged from -16 mV to - 21 mV from day 1 to day 14 again showing very little change. For empty PLGA NPs the range over 14 days for the ZP was - 21 mV to - 31 mV, which was actually a greater range of variation than was seen for the KET loaded PLGA NPs. The differences in ZP from those

originally observed (**Table 3.7**) may have been connected to changes in the behaviour of the surfactant present in the sample.

The drug content in the supernatant for KET 0.1 mg/mg on day 1 was 0.5 ± 0.1 % of the KET loaded (the actual KET loading was treated as 100%) and on day 14 it was 7.6 ± 1.2 %. For KET 0.2 mg/mg on day 1 there was 0.4 ± 0.2 % and on day 14 was 5.7 ± 1.8 % of the actual KET loaded present in the supernatant. This shows that there was some drug leakage over the 14 day period into the surrounding dispersion medium (**Figure 3.5**).

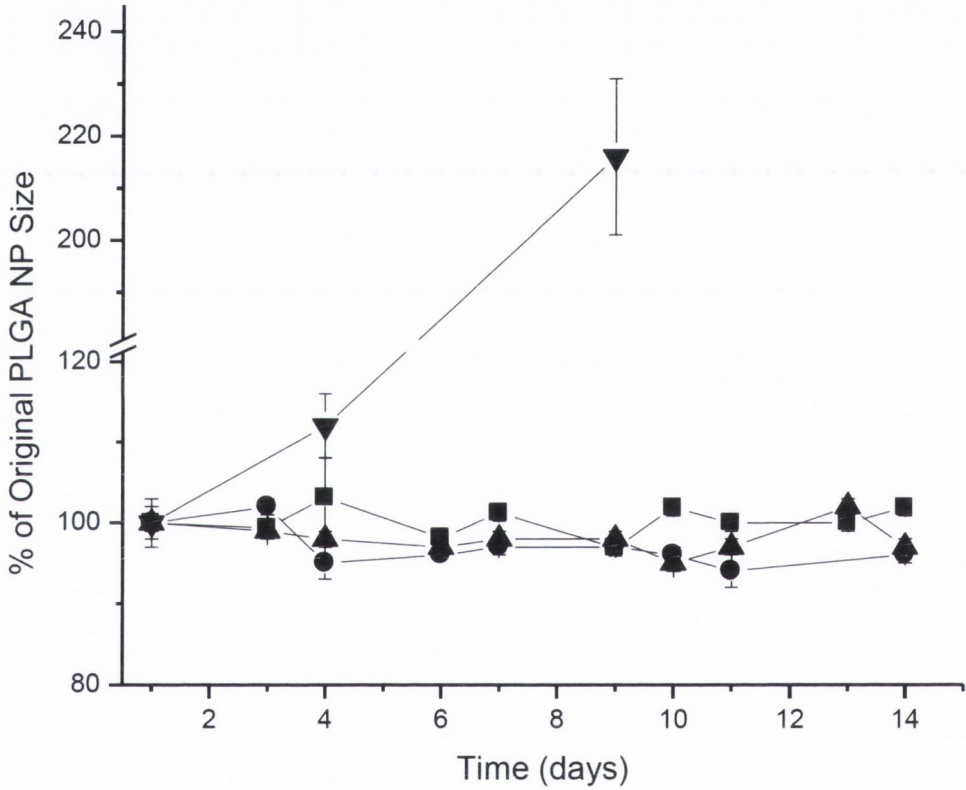


Figure 3.5 Particle size of KET loaded PLGA NPs over a 14 day period expressed in terms of the % change in the NP size over time. ▼ = Free KET ethanolic solution, ● = PLGA KET 0.1 mg/mg NPs, ▲ = PLGA KET 0.2 mg/mg, ■ = PLGA empty NPs.

3.3.5. Release Studies

Drug release from KET loaded PLGA NPs was studied over a 14 day period to look at the release profiles and duration for these KET loaded PLGA NPs. These studies were carried out using PBS buffer at pH 7.4 under sink conditions for KET. The drug release here was observed as having two distinct phases. There was a rapid burst release of KET followed by a lag phase and then very slow release of the remaining drug. The studies were conducted over a 14 day period as past this point the drug release appeared to decline probably due to drug degradation. Corrigan and Li (2009) studied drug release from PLGA NPs containing KET loaded at under 10 % DL and found that the t_{max} (time to peak concentration) for KET release was at 10.9 ± 0.5 days and had complete drug release at 20 days.

In the current studies the burst release of KET resulted in a release of 61.1 ± 3.2 % of the KET loaded for KET 0.1 mg/mg and 86.1 ± 1.4 % for KET 0.2 mg/mg within the first hour which rose to 73.2 ± 8.3 % for KET 0.1 mg/mg and 86.6 ± 2.2 % for KET 0.2 mg/mg within 24 hrs (**Figure 3.6**). The initial release phase for KET 0.1 mg/mg lasted for longer than KET 0.2 mg/mg and it took 50 hrs for the lag phase to begin at a release of 83.5 ± 4.1 %. Measurements over the next 23 days showed a lag phase until about day 10 at both KET 0.1 mg/mg and KET 0.2 mg/mg, when the KET release started to slowly rise again to 92.3 ± 4.2 % for KET 0.1 mg/mg and 96.0 ± 5.7 % for KET 0.2 mg/mg (**Figure 3.7**). The final amount of drug was not released and after day 14 a drop in drug release was seen probably due to KET degradation or else the KET was deeply trapped inside the PLGA NPs. The high burst release could be attributed to the solubility of KET. KET's aqueous solubility is approximately 0.005 mg/ml and in the amorphous form, as in the PLGA NPs, this increased to approximately 0.019 mg/ml (Hancock and Parkes 2000) combined with the possibility that much of the KET was located on the surface of the PLGA NPs. The KET present on the surface of the PLGA NPs would be expected to release first so it is likely that a high amount of the drug is present on the particle surface as the burst release was so rapid. Gabor et al. (1999) found the initial burst release to increase with the drug content and the increasing inherent viscosity of their KET loaded PLGA (RG502 50:50, Mw = 12,000 Da) microspheres with a KET loading of 3.9 % w/w. This could explain why the KET 0.2 mg/mg PLGA NPs had a higher initial burst release than KET 0.1 mg/mg loaded PLGA NPs. The KET burst release in these

current studies for KET 0.2 mg/mg was higher than for PLGA KET NPs prepared by Corrigan and Li (2009) who had a release of 42.2 % in the first 24hrs. This could be due to variations in the method of preparation and size of the PLGA NPs as Corrigan and Li used the surfactant 1% w/v PVA for emulsification while these current studies used Pluronic F-68 (0.1% w/v) and they also use a greater volume of surfactant solution for diffusion in the diffusion step of their emulsification-solvent diffusion method (90 ml of 0.03% w/v PVA solution) while these current studies used 45 ml of Pluronic F-68 1.0 % w/v solution. As it is likely that KET was amorphous, as observed using DSC and PXRD, this may have contributed to the fast release.

Following the lag phase the final amount of the KET loaded would have been expected to slowly escape the PLGA NPs by slow degradation of the PLGA polymer. Corrigan and Li (2009) reported that certain API regions in the polymer particle are encapsulated in polymer and require polymer degradation to initiate drug release, resulting in the second polymer degradation release phase. This phase is dependent on polymer composition and molecular weight, the higher the molecular weight and lactide content of the polymer the longer the lag time as well the influence of the drug itself which may interact with the polymer to lower the polymer Tg and degradation rate (Blasi et al 2007), or facilitate autocatalysis by altering the pH within the polymer.

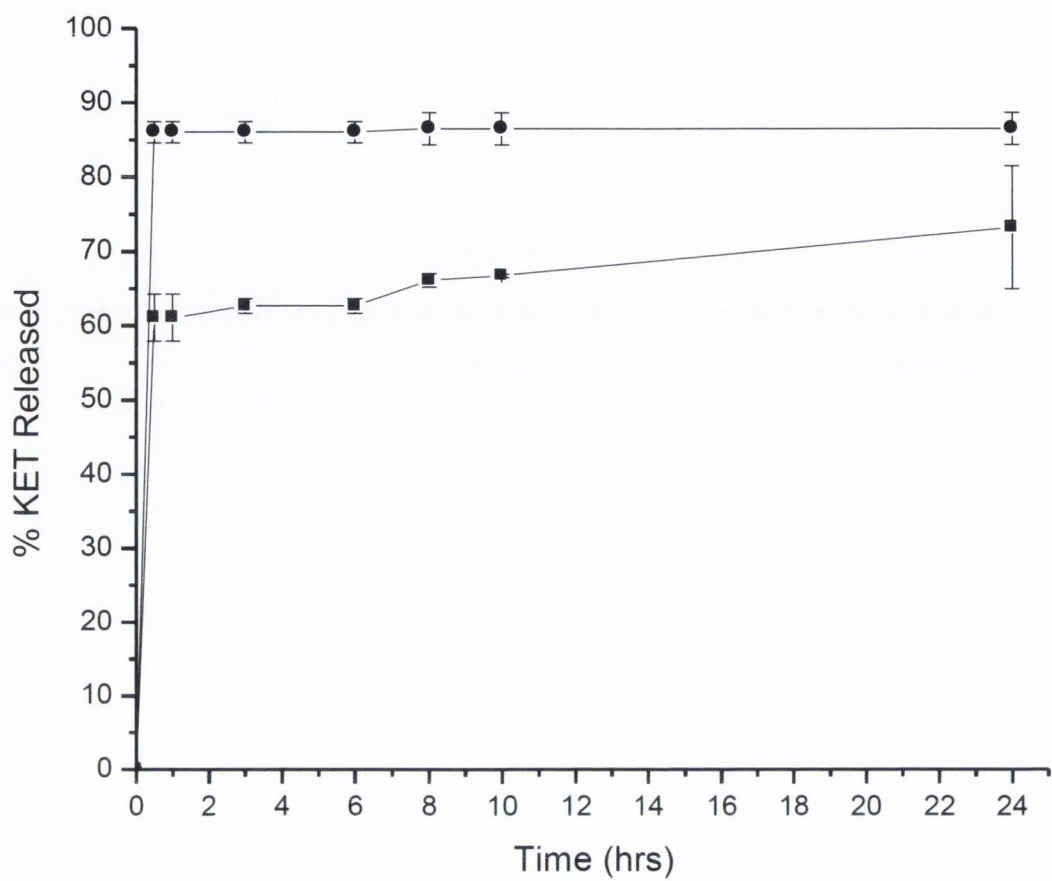


Figure 3.6 Release studies for PLGA NPs loaded with KET over a period of 24 hrs. ■ = KET 0.1 mg/mg PLGA NPs, ● = KET 0.2 mg/mg PLGA NPs.

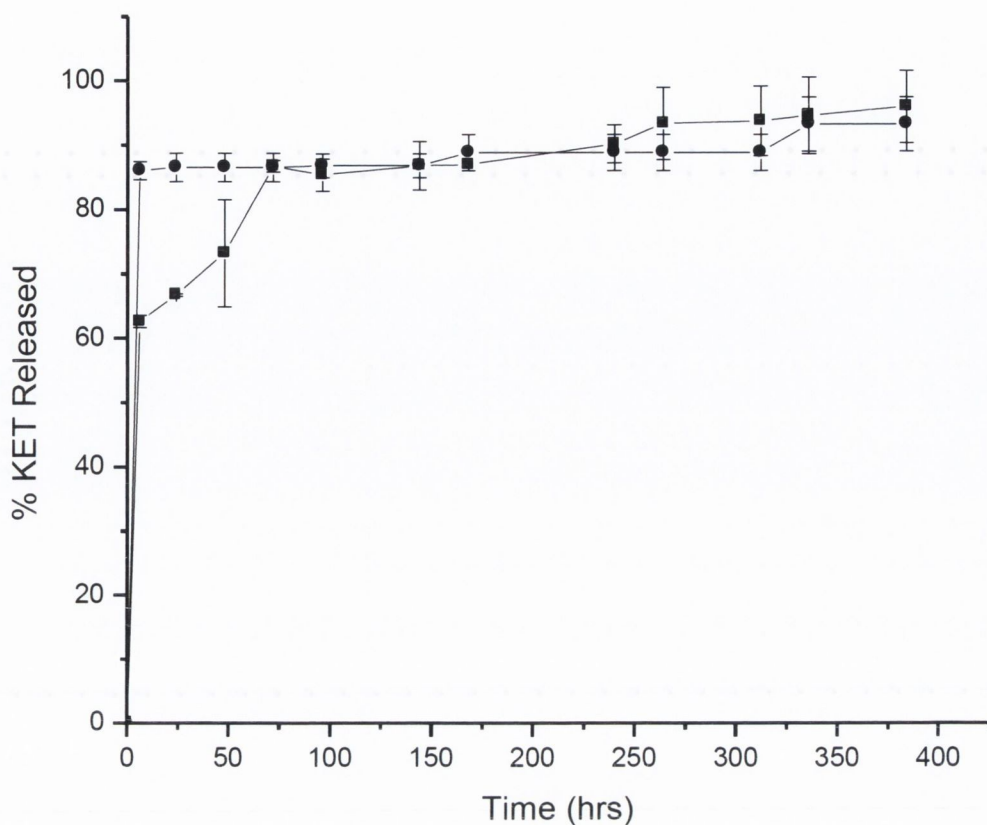


Figure 3.7 Release studies for PLGA NPs loaded with KET over a period of 24 days. ■ = KET 0.1 mg/mg PLGA NPs, ● = KET 0.2 mg/mg PLGA NPs.

Complete drug release was not seen over the duration of the study. This KET may be deep inside the PLGA NP structure and thus not easily released due to poor penetration of the dissolution medium. Since KET was amorphous, as observed using DSC and PXRD, this may have contributed to the fast release.

3.4 Loading Indometacin (IND) into PLGA NPs

3.4.1 Drug Loading and Physical Characterisation

IND was the second NSAID chosen to be loaded into the optimised PLGA NPs. IND has lower aqueous solubility than KET. The difference in solubility was expected to have an impact on the PLGA NPs formed as the preparation method involved firstly emulsification, which is influenced by the solubility of the components and then precipitation of the NPs as a result of solvent diffusion which may vary in rate and total precipitation depending on the component solubility.

The manufacturing parameters as established in **Section 3.2.1.6**, were used to prepare IND loaded PLGA NPs. For DL two concentrations of IND (0.1 mg/mg and 0.2 mg/mg) were tried to see how the PLGA NPs would form with different concentrations of IND added.

IND loaded PLGA NPs were larger than empty PLGA NPs (**Table 3.9**). The size of IND loaded PLGA NPs increased from 267 ± 3 nm without the presence of IND to 291 ± 3 nm at IND 0.1 mg/mg loading concentration and 323 ± 8 nm at IND 0.2 mg/mg loading concentration. The addition of IND significantly increased the size of the PLGA NPs when compared to the unloaded PLGA NPs ($p=0.0001$). There was also a significant difference between the size of IND 0.1 mg/mg PLGA NPs and IND 0.2 mg/mg PLGA NPs ($p = 0.003$). The size of the PLGA NPs increased as the concentration of IND increased.

The addition of IND also increased the ZP of the PLGA NPs. The ZP increased from -41 ± 1 mV for empty PLGA NPs to -32 ± 1 mV at IND 0.1 mg/mg and -35 ± 2 mV at IND 0.2 mg/mg. There was no significant difference between the ZP for IND 0.1 mg/mg loaded PLGA NPs and for IND 0.2 mg/mg loaded PLGA NPs ($p = 0.081$). The rise in ZP once IND had been added may be due to the partial blockage of surface charge of the PLGA by the IND. Despite the increase in drug content the ZP for IND 0.2 mg/mg had only a slightly more negative surface charge than IND 0.1 mg/mg. The ZP values measured for IND loaded PLGA NPs were still optimal values for NP physical stability (± 30 mV).

PDI increased as IND was loaded into the PLGA NPs. This suggests that the addition of IND increased the variation within the IND loaded PLGA NP dispersions. It increased from 0.08 ± 0.02 for empty PLGA NPs to 0.16 ± 0.14 when loaded with IND 0.1 mg/mg and 0.20 ± 0.10 when loaded with IND 0.2 mg/mg. There was a significant increase between empty PLGA NPs and IND loaded PLGA NPs ($p = 0.002$) but there was no significant difference seen between the IND loaded PLGA NPs at 0.1 mg/mg and 0.2 mg/mg ($p = 0.708$).

The EE for PLGA IND loaded NPs was 91 ± 4 % w/v for IND 0.1 mg/mg loading and it was 93 ± 2 % w/w at IND 0.2 mg/mg loading. This was equal to a DL of 9 ± 0.8 % w/v for IND 0.1 mg/mg and 19 ± 0.4 % w/v for IND 0.2 mg/mg. These encapsulation efficiencies were very high for IND 0.1 mg/mg and IND 0.2 mg/mg as the majority of the IND loaded was encapsulated. Corrigan and Li (2009) achieved an EE of 65 ± 1 % w/w and a DL of 7 ± 0.1 % w/w when they loaded 0.2 mg/mg of KET into the PLGA NPs. The differences could be due to differences in the preparation method or operator technique differences. This showed that IND was very compatible with this formulation and readily incorporated successfully by using this method.

Table 3.9 Physical properties and DL for PLGA NPs loaded with IND at two different concentrations (0.1 mg/mg and 0.2 mg/mg)

Polymer/Drug	Size (nm)	ZP (mV)	PDI	EE % (w/w)	DL % (w/w)
PLGA Empty	267 ± 3	-41 ± 1	0.08 ± 0.02	-	-
IND 0.1 mg/mg	291 ± 3	-32 ± 1	0.16 ± 0.14	91 ± 4	9 ± 1
IND 0.2 mg/mg	323 ± 8	-35 ± 1	0.20 ± 0.10	93 ± 2	19 ± 1

3.4.2 IND loaded PLGA NP Imaging

The IND loaded PLGA NPs at 0.2 mg/mg were chosen for SEM as these PLGA NPs had a higher concentration of IND incorporated than IND 0.1 PLGA NPs and so it would be easier to see any IND present in the PLGA NPs.

The IND 0.2 mg/mg loaded PLGA NPs were present as spherical, non-porous, smooth spheres similar to those observed for empty PLGA NPs (**Figure 3.8**). There was neither evidence of free IND crystals observable in these images nor

IND crystals associated with the PLGA NPs. All IND loaded PLGA NPs were found to be in the nano-size range as calculated visually by comparison to the scale bar. The SEM images complemented the dynamic light scattering (DLS) results as the size observed was similar for both with a decrease in size for the SEM samples due to loss of water (Bootz et al. 2004).

The IND did not change the structure of PLGA NPs from that observed for empty PLGA NPs by a noticeable amount.

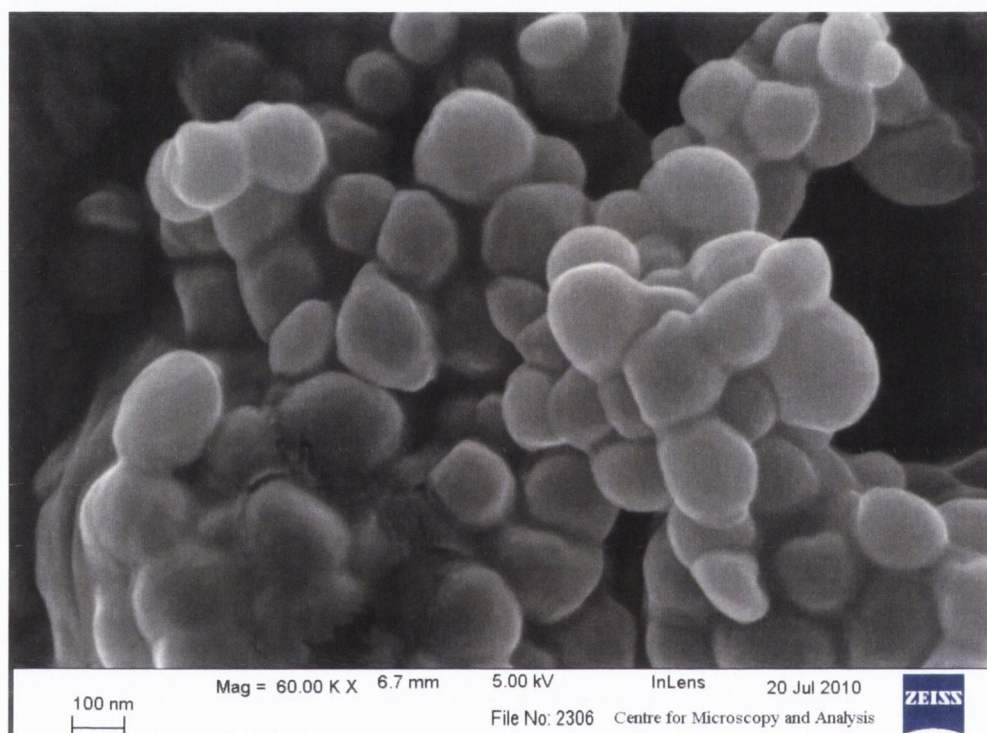


Figure 3.8 SEM Image of PLGA NP loaded with IND.

3.4.3 PXRD and DSC Analysis

Solid-state analysis was carried out to investigate the physical form of IND present associated with the PLGA NPs. Like KET, IND is classed as a BCS class II drug but it is less soluble than KET. The aqueous solubility of crystalline IND may be enhanced by conversion to one of its five polymorphic crystalline forms or by conversion to the amorphous form (Surwase et al. 2013).

PXRD analysis showed that the surface of all IND loaded PLGA NPs was amorphous. The Braggs peaks visible for free IND were not visible for IND loaded PLGA NPs (**Figure 3.9**). This showed that IND present in the PLGA NPs

was converted to the amorphous form during the emulsification and precipitation process.

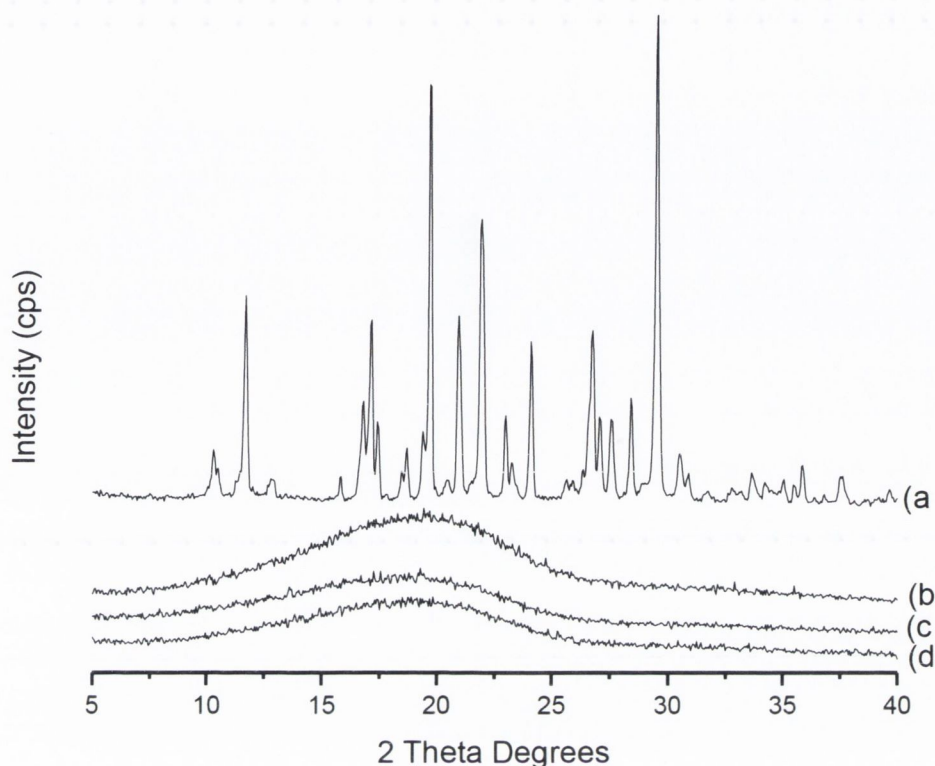


Figure 3.9 PXRD data from IND loaded PLGA NPs and empty PLGA NPs compared with free IND. a) free IND, b) IND 0.1 mg/mg, c) IND 0.2 mg/mg, d) PLGA NPs.

DSC results for IND loaded PLGA NPs showed that IND was amorphous (**Figure 3.10**) as there was no melting point observable for IND at 159.56 ± 0.05 °C. No differences in the DSC traces were seen between PLGA IND loaded NPs at 0.1 mg/mg or 0.2 mg/mg loading. The Tg of PLGA shifted in the presence of IND but the shift was not considerable (**Table 3.10**). It was observable from the DSC traces, as was the case with KET loaded PLGA NPs, that PLGA showed a relaxation process.

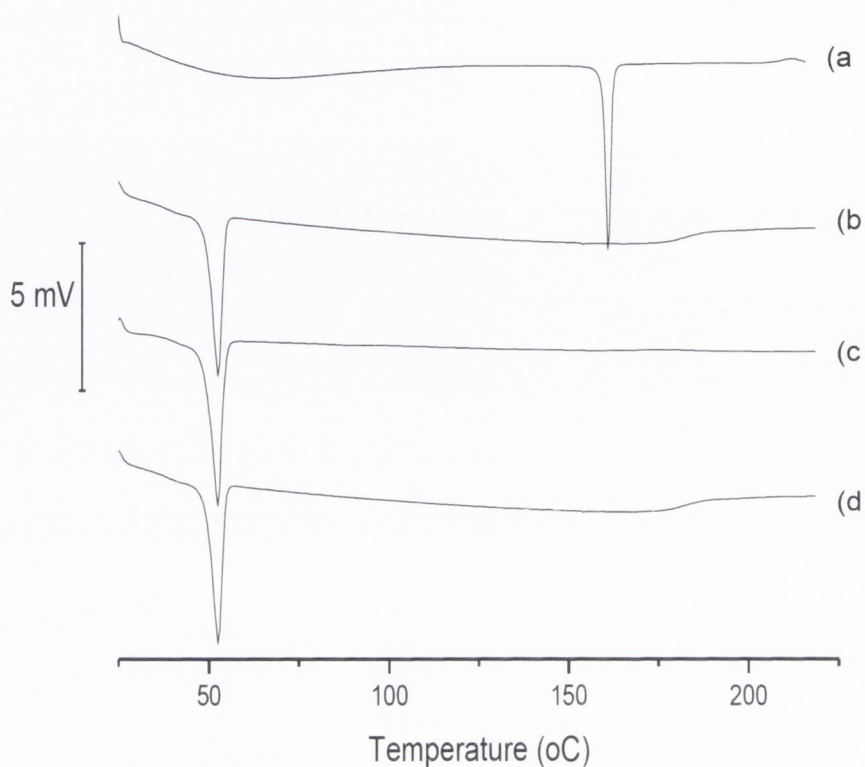


Figure 3.10 DSC data from IND loaded and empty PLGA NPs compared with free IND. a) = free IND, b) = IND 0.1 mg/mg, c) = IND 0.2 mg/mg, d) = empty PLGA NPs.

These observations were very similar to those by Corrigan and Li (2009) who found that IND in their IND 0.2 mg/mg loaded PLGA NPs was amorphous and there was a minimal effect on the T_g of PLGA when IND was added.

Table 3.10. Tg and Relaxation Process (J/g) values for IND loaded PLGA NPs.

Sample	PLGA	IND	IND 0.2 mg/mg
	Empty NPs	0.1mg/mg PLGA NPs	PLGA NPs
Tg (°C)	49.10 ± 1.10	44.74 ± 0.80	48.90 ± 0.97
Relaxation Process (J/g)	86.33 ± 2.54	75.24 ± 1.68	74.13 ± 1.65

3.4.4. Stability Studies

The stability of IND loaded PLGA NPs was studied over a 14 day period to look for changes in the physical stability of the sample over time. Post-preparation the NPs samples were stored as aqueous dispersions at room temperature (25 °C) and measured for size (nm), ZP (mV) and PDI using DLS (Malvern Zetasizer Nano ZS).

The results showed that over the 14 day period there were only small fluctuations in the size of the IND loaded PLGA NPs with this being reflected in the low PDI values (**Figure 3.11**). The size of the NPs, expressed as % of original size, on day 14 ranged from 95 -101 % for IND 0.1 mg/mg NPs and from 96 - 113 % for 0.2 mg/mg IND loaded PLGA NPs. The size variation was greater than that seen for empty PLGA NPs (96 – 103%). Free IND increased in particle size to 1020 % of the starting particle size (100 nm) on day 6. After day 6 IND precipitated out as larger sediments indicating that the PLGA NPs were playing a role in stabilising IND.

The PDI for the PLGA NPs did not vary significantly over the 14 day time period. For 0.1 mg/mg IND loaded PLGA NPs the PDI ranged from 0.07 - 0.11 over 14 days, for 0.2 mg/mg the PDI ranged from 0.07 – 0.38 over 14 days. This showed that for IND 0.2 mg/mg loaded NPs there were some shifts in the particle structure over time as the PDI increased. The PDI range was greater for these IND loaded NPs than the empty PLGA NPs (0.08 – 0.14)

For IND 0.1 mg/mg the ZP jumped from - 21.8 to - 31.6 mV for 14 days. For IND 0.2 mg/mg the ZP ranged from - 22.4 to - 34.1 mV over 14 days. Since the

empty PLGA NPs had more negative surface charge than the IND loaded PLGA NPs it is possible that drug loss may have caused the increase in ZP. The decrease in ZP may also have been related to the increasing PDI within the sample. Visually all samples did not appear to change over the course of the 14 day study. Except for PLGA IND 0.2 mg/mg where some precipitation was visible after day 11. The range for ZP was close to that observed for free PLGA NPs (-21.2 to -31.2 mV). The differences in ZP from the originally observed values may have been connected to the behaviour of the surfactant present in the sample.

The drug content in the supernatant for IND 0.1 mg/mg on day 1 was 0.4 ± 0.1 % w/w of actual drug loaded and on day 14 was 3.2 ± 1.1 % w/w. For IND 0.2 mg/mg loaded PLGA NPs the IND loaded on day 1 was 1.2 ± 0.4 % w/w and on day 14 this had risen to 5.2 ± 0.8 % w/w. The increased PDI for IND 0.2 mg/mg loaded PLGA NPs could have been due to leakage of IND from the PLGA NPs.

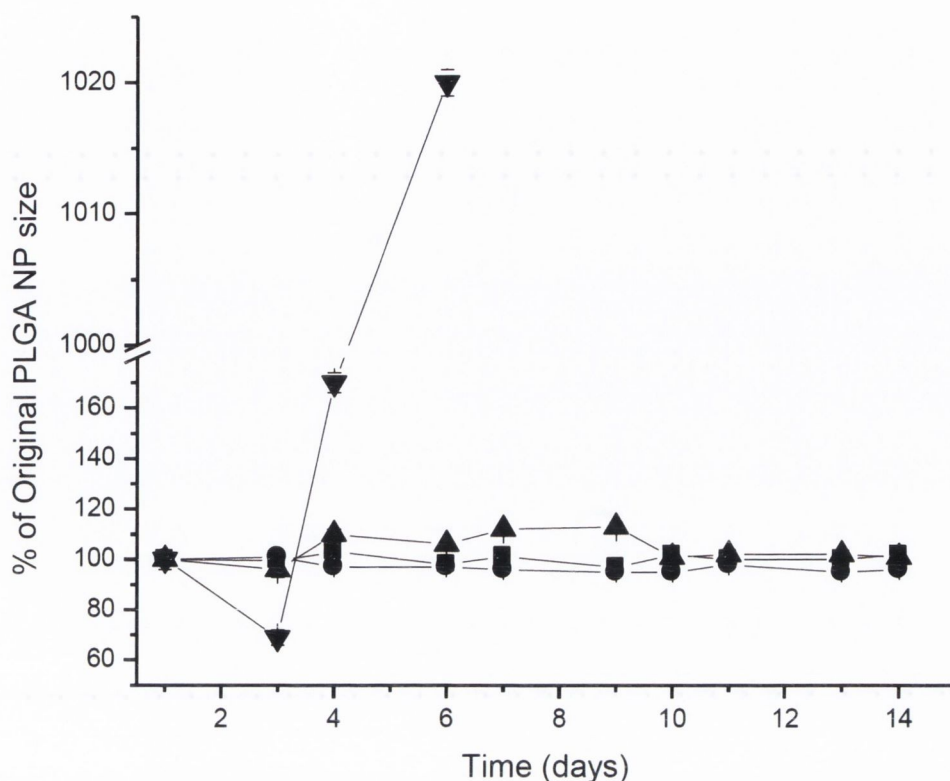


Figure 3.11 Stability studies of IND loaded PLGA NPs over a 14 day period expressed as a % of the original particle size. ▼ = Free IND ethanolic solution, ● = PLGA IND 0.1 mg/mg NPs, ▲ = PLGA IND 0.2 mg/mg, ■ = PLGA NPs.

3.4.5. Release Studies

Drug release from IND loaded PLGA NPs was studied over a 24 day period to look at the release profile and duration for these IND loaded PLGA NPs. The release studies were carried out at 37 °C in PBS buffer (pH 7.4) under sink conditions. The drug release here was observed as having two distinct phases. There was a rapid burst release of IND followed by a very slow release of the remaining IND.

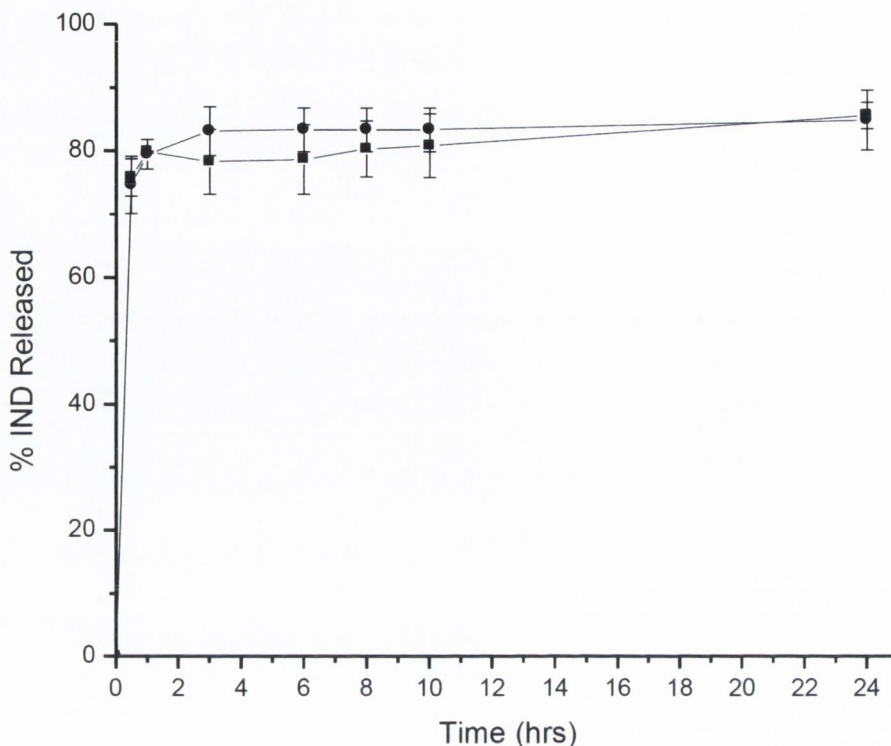


Figure 3.12 Release studies for PLGA NPs loaded with IND over a period 24 hours. ■ = IND 0.1 mg/mg PLGA NPs, ● = IND 0.2 mg/mg PLGA NPs.

The burst release of IND resulted in a release of $75.8 \pm 2.9\%$ for IND 0.1 mg/mg and $74.6 \pm 4.5\%$ for IND 0.2 mg/mg loading which rose to $85.6 \pm 2.1\%$ for IND 0.1 mg/mg and $83.4 \pm 3.5\%$ for IND 0.2 mg/mg within 24 hrs (**Figure 3.12**). Corrigan and Li (2009) had a 26.3% burst release for IND PLGA NPs when 0.2 mg/mg was loaded which was much lower than the current studies. This indicates that the current work prepared IND loaded PLGA NPs with a high surface content of IND. Both concentrations of IND (0.1 and 0.2 mg/mg) showed very similar release profiles regardless of the fact that the IND 0.2 mg/mg formulation had almost double the concentration of IND loaded. Additional IND on the surface of the PLGA NPs for IND 0.2 mg/mg than IND 0.1 mg/mg could explain the larger size observed for IND 0.2 mg/mg.

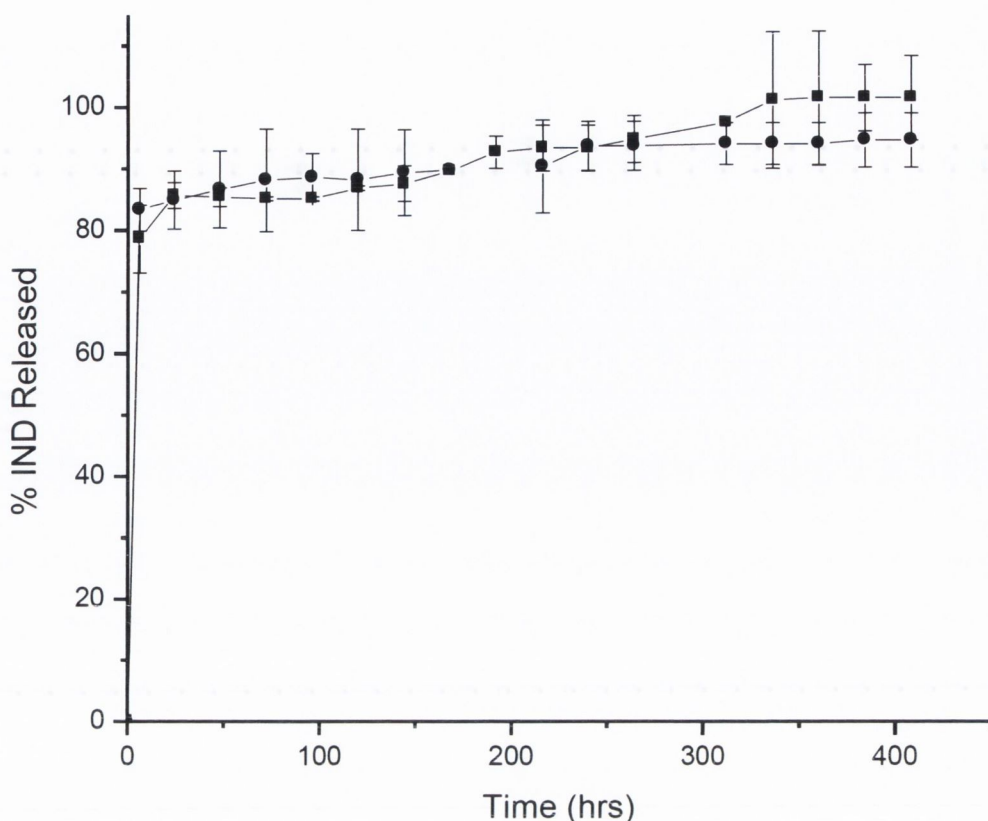


Figure 3.13 Release studies for PLGA NPs loaded with IND over a period 14 days. ■ = IND 0.1 mg/mg PLGA NPs, ● = IND 0.2 mg/mg PLGA NPs.

Measurements over the next 23 days showed a slow release of the final amount of IND from the PLGA NPs with IND 0.1 mg/mg release rising to $96.4 \pm 5.4 \%$ and for IND 0.2 mg/mg it rose to $95.3 \pm 3.9 \%$ on day 24 (**Figure 3.13**). This meant that in the last 13 days 20% w/v of the total IND DL was released for IND 0.1 mg/mg and 10% w/v of the total DL for IND 0.2 mg/mg. This portion of the loaded IND was most likely deep inside the IND 0.2 mg/mg loaded PLGA NPs and so could not be released as quickly. The lag phase observed here was not as pronounced as that seen for KET with a steady rise being seen in the drug release throughout the 28 days period. It is possible that the higher IND loading could be the cause of this or the IND location in these PLGA NPs is closer to the surface. Corrigan and Li (2009) reported that IND released from PLGA NPs had a t_{max} (time to peak concentration) of 12.5 ± 0.4 days. Like with KET PLGA NPs he found that the entire IND content was completely released by 20 days.

Complete drug release was not seen over the duration of the study for IND 0.2 mg/mg but it was for IND 0.1 mg/mg possibly due to IND 0.1 mg/mg having a lower DL.

3.4 Conclusions

PLGA NPs were successfully optimised to have desirable physicochemical properties for further DL studies. These optimisation steps allowed the influence of each variable to be tested, which gave valuable information to allow predictability for the formulation and to quickly solve problems or understand variations if they arise.

PLGA NPs could be successfully formed loaded with the hydrophobic, NSAIDs IND and KET. These PLGA NPs showed good physical stability once prepared making them possible to keep as dispersion over time without immediate concern that the PLGA NPs will degrade.

The addition of either of KET or IND increased the size of the PLGA NPs but the SEM images confirmed that they did not affect the structure of the PLGA NPs. The PLGA polymer was initially amorphous and remained amorphous post-formation of the IND and KET loaded PLGA NPs. IND and KET both were incorporated into the PLGA NPs in the amorphous form as observed using PXRD or DSC analysis. The degree of shift in the T_g of PLGA observed was not substantial for either drug.

KET is more hydrophilic than IND and this was the most likely reason that KET was not loaded into the PLGA NPs with as high EE as was seen for IND. Loading KET and IND increased the NP size from that for the empty PLGA NPs. The increase seen was greater for KET loaded PLGA NPs than for IND loaded PLGA NPs despite KET loading being lower. It is possible that the high IND loading indicates that IND incorporates well into the PLGA NPs that the particles form in a consistent manner while given KET's lower DL and lower solubility there may be more variation in KET's association with the PLGA NPs.

The ZP of loaded PLGA NPs became more positive with the addition of drug for both IND and KET. It could be assumed that the presence of drug was blocking the PLGA groups giving rise to the negative surface charge of the NPs. The blocking is most likely by steric means as the drugs themselves should have negative charges as they are acidic and thus the drugs would not be expected to neutralise the negative PLGA charge.

The DL for IND was significantly higher than for KET as it is more hydrophobic than KET and thus would have been easier to dissolve in the PLGA-ethyl acetate solution and would have been less likely to partition into the aqueous phase than KET, while KET may have stayed in the aqueous phase or migrated into the aqueous phase during the formation of these PLGA NPs. The poorer encapsulation of KET could be linked to its higher aqueous solubility, IND loaded PLGA NPs show a lower amount of IND leakage to the external aqueous medium, during preparation of the PLGA NPs (Barichello et al. 1999).

Although PLGA NPs are described as slow release formulations (Danhier et al. 2012) that was not the case here as the burst releases of IND and KET resulted in a large amount of the drug release occur within the first 24 hrs. This could reflect the location of the drug as IND or KET located on the surface will be exposed to the dispersion medium. The final amount of loaded drug did release slowly with time but the majority of the DL was released during the rapid burst release. These PLGA NPs showed most of their drug release within the first few hours of release so they would be suited to release formulations designed to release drug over a few hours. It could be expected that the presence of KET and IND in the PLGA NPs may be accelerating the PLGA erosion as they are acidic drugs, which could facilitate acidic hydrolysis of PLGA thus rendering the release faster (Corrigan and Li 2009). This could be the reason why IND at higher loading showed a higher burst release and shorter lag time during the IND release. The higher IND loading could also be the reason why the burst release seen in the current studies was higher than that observed by Corrigan and Li (2009).

SEM photomicrographs indicated spherical NPs, with smooth surfaces for both drugs. Good physical stability was observed for all KET and IND loaded PLGA NPs over a 14 day period while the particle size of the free drugs rose quickly over the first few days of the stability study. This indicates that KET and IND are being stabilised by PLGA NPs.

The Optimisation and Characterisation of Empty and Ketoprofen Loaded Hyaluronic Acid:Chitosan Polyelectrolyte Nanoparticles

Chapter 4

4.1 Introduction

HA:CS PECNs were chosen as NCs to use in these studies as they are fast release systems and NCs from the same PECN class have been proven to form physically stable particles capable of being loaded with drugs (Umerska et al. 2012, Lu et al. 2011, de la Fuente et al. 2008). The polymers individually are suitable for use in PECNs. CS has been shown to be both bioactive and biodegradable (Muzzarelli et al. 1988) as well as being useful in wound healing due to its mucoadhesive properties (Madhumathi et al. 2010). HA is found naturally in the body in connective tissue and is also under investigation for its uses in wound healing (Almond 2007). Their biological properties make them an interesting combination to combine with anti-inflammatory drugs such as KET and IND.

HA:CS PECNs were previously prepared using crosslinkers such as TPP to stabilise them (de la Fuente et al. 2008, Oyarzun-Ampuero et al. 2009). Attempts were made to prepare these PECNs without stabilisers with limited success due to aggregation of the PECNs (Boddohi et al. 2009). Umerska et al. (2012) improved the stability by introducing a sonication step into the preparation method so HA solution (0.1% w/v) was sonicated for between 0.5 and 6.0 hrs to reduce its molecular weight allowing the easy and instantaneous formation of HA:CS PECNs. It was decided primarily to recreate the HA:CS PECNs formulated previously by Umerska et al. (2012) and characterise them prior to the addition of drugs.

In the second part of this chapter the focus is on the preparation of these HA:CS PECNs loaded with KET, a hydrophobic (BCS class II) NSAID. Cheow et al. (2012) have investigated loading PECNs, which are hydrophilic systems, with hydrophobic drugs using one polymer and salt to form the complexes. Specifically they focused on the drugs ciprofloxacin, curcumin and ibuprofen with poly(allylamine hydrochloride) and dextran sulphate as the single polymers used. In their studies their systems were mainly consisting of drug nanocrystals with polymer and salt balance being crucial to maintain their stability. This current work firstly focused on using a second polymer to stabilise the PECN systems instead of using a salt and secondly it concentrated on the solid state properties of the NC systems. Many research groups are developing NCs and focusing in

their publications on the DL of the NP systems but are not further investigating the drug solid state characteristics in the system.

4.2 The Development and Characterisation of Unloaded HA:CS PECNs

4.2.1 Introduction

To choose the optimal HA:CS PECNs the parameters involved in the formation of these systems were explored in this chapter. Previous work by Umerska et al. (2012) had explored the optimisation of HA:CS PECNs to study the formulation and understand how the parameters affect the end PECNs. In the work of Umerska et.al. (2012) polymer ratio, the counter-ion of the CS salt and the total polymer concentration were studied. Umerska et al. (2012) concluded that the CS chloride salt (CL113) was more suitable to the formulation than the glutamate salt (GL113) as it only had one negative charge while the glutamate salt had two which made it more complex. Also, Umerska and coworkers had previously tested HA:CS PECNs, consisting of the CS chloride salt (CL113) and HA, for cytotoxicity using an MTS assay on Caco-2 cells. Cell viability was found to be not significantly different to the control. The total polymer concentration was also chosen as 0.1% w/v as Umerska et al. (2012) reported that the samples became more polydisperse if the concentration was raised to 0.2% w/v. Importantly, the 0.1% w/v HA solution was ultrasonicated and it was found that 2 hrs is the optimal sonication time as it depolymerised the HA solution to a chain length ($mW = 257 \pm 8.43$ kDa) that balanced a suitable dynamic viscosity (2.14 ± 0.162 mPa/s) for HA:CS PECN formation with an efficient duration of time for sonication. Moving forward from the work by Umerska et al. (2012), the current work built on characterising these HA:CS PECNs to design them for the purpose of loading the hydrophobic, NSAIDs KET and IND.

4.2.2 Optimisation of the HA:CS PECN Formulation Variables

4.2.2.1 Varying HA:CS Polymer Ratio

The polymer ratio was the most important variable according to Umerska et al. (2012) influencing the formation of these PECNs as the PECNs could only form with desirable physical properties at certain HA:CS weight ratios. This parameter was varied to find the optimal conditions for formation. Particle size (nm), ZP (mV) and the PDI were used as measures to determine which particles were the most desirable. The weight ratio of HA:CS was varied from 10:4 to 5:9 as outside this range HA:CS PECNs with desirable physical properties were not formed and flocculation was observed either immediately upon mixing the HA and CS polymer solutions or else it was observed within a period of 20 mins of mixing. Physically stable HA:CS PECNs formed over a range of weight ratios, HA:CS PECNs with a positive charge formed at ratios of HA:CS 7:7, 7.5:6.5, and 8:6 and HA:CS PECNs with a negative charge were acceptable at a weight ratio of HA:CS 10:4 only. Optimal weight ratios for the HA:CS PECNs were taken to be those that produced HA:CS PECNs that had a particle size less than 200 nm with a zeta potential outside ± 30 mV indicating physical stability and a PDI below 0.2 showing that the sample has a narrow distribution of particle sizes (Dautzenberg and Kriz 2003). If the weight ratios of HA:CS moved outside of these ideal weight ratios the resulting samples either did not produce HA:CS PECNs in a concentration large enough to be detectable by dynamic light scattering or the HA:CS PECNs were large in particle size and highly polydisperse. The 9:5 weight ratio gave the least desirable results with particles outside the nano-size range being formed and aggregation was quickly observed. This aggregation would be expected as the ZP here was almost neutral at 5.3 ± 0.5 mV (**Table 4.1**).

The charge ratios for HA:CS were calculated to reveal the stable ratios for combining the polymers. The most stable values were 1.83 at a weight ratio of 10:4 HA:CS and 0.69 at a weight ratio of 7.6:6.5 HA:CS. This indicates that for positively and negatively charged HA:CS PECNs the polymer contributing most to the overall charge varies. This is in contrast to the fact that HA solution has always the greater weight in these 10:4 and 7.5:6.5 HA:CS systems. The charge ratio for positively charged HA:CS PECNs shows that the positive charge from CS is in excess and for negatively charged HA:CS PECNs the negative charge

from HA is in excess. This is reflected in the charge of the ZP for these PECNs. The closer to 1 the charge ratio was, the more unstable the PECNs became with a HA:CS weight ratio of 9:5 being the most unstable with a charge ratio of 1.12. Chen et al (2007) combined dextran sulphate (DS) and CS at varying charge ratios to form PECNs and found that close to the charge ratio of 1 the PECNs increased in size and for their system containing the polymers at a charge ratio (negative charge: positive charge) of 1.12 the particle size was 1894 ± 187 nm and a PDI of 0.64 ± 0.19 . Other studies have reported PECNs with undesirable properties such as large size, a ZP close to zero or high PDI at a charge ratio close to 1 (Boddohi et al. 2009, Rodriguesa et al. 2012, Umerska et al. 2012). In the current work when the ratio of excess polymer to the oppositely charged polymer was at approximately 2:1 or 1:2 the system was stable.

The values obtained in these studies were similar to those obtained previously by Umerska et al. (2012) at the HA:CS 10:4 weight ratio.

Table 4.1. Properties of empty HA:CS NPs at varying polymer ratios.

HA:CS volume (mls)	pH	Size (nm)	PDI	ZP (mV)	Mass Mixing Ratio	Charge Mixing Ratio
10:4	5.8	160 ± 5	0.116 ± 0.019	-29 ± 1	2.50	1.83
9:5	4.9	$>1\mu\text{m}$	0.761 ± 0.253	$+5 \pm 1$	1.80	1.12
8:6	4.4	168 ± 2	0.132 ± 0.019	$+32 \pm 3$	1.33	0.80
7.5:6.5	4.4	173 ± 2	0.102 ± 0.017	$+33 \pm 1$	1.15	0.69
7:7	4.4	189 ± 3	0.182 ± 0.009	$+36 \pm 3$	1.00	0.60
6:8	4.3	325 ± 25	0.306 ± 0.032	$+36 \pm 2$	0.81	0.48
5:9	4.2	989 ± 173	0.609 ± 0.097	$+31 \pm 5$	0.56	0.33

4.2.2.2 Varying HA Solution pH to form HA:CS PECNs

Experiments were carried out to manipulate the % ionisation of HA. Therefore 0.1% w/v HA solutions with different pH values (2, 4, 5.7 (native pH), 8 and 10) were prepared to discover how the HA:CS PECN properties could be influenced by the initial charge of the HA solution involved in formation. The HA:CS weight ratio of 7.5:6.5 was used for all experiments to test how pH changes could have

an impact on the formation of these HA:CS PECN systems. It was decided to vary the pH of HA solution as opposed to CS solution because both the positively charged and negatively charged HA:CS PECNs contained HA solution in greater volume than CS solution. Another reason why HA solution was varied for this study was that all components of the formulation are added to the 0.1% w/v HA solution during the formation of both positively (HA:CS 7.5:6.5) and negatively (HA:CS 10:4) charged PECNs so it is the starting solution. At the pH values of the 0.1% w/v HA solution investigated, the polymer was 100% ionised at pH 5.7, pH 8 and pH 10, 91% ionised at pH 4 and 9% ionised at pH 2. After addition of the CS solution, the pH of the final dispersion varied based on the starting pH of the HA solution as the quantities of both polymers were constant. While the PECNs produced from each starting HA solution had similar sizes there were differences in the ZP and PDI that would impact upon the physical stability of the system. As the pH of HA solution moved further away from the native pH, pH 5.7, the PDI moved away from 0.120 ± 0.071 increasing to values above 0.2 for all samples. The 0.1% w/v CS solution was 100% ionised post mixing with the 0.1% w/v HA solutions at pH 2, 4, and 5.7 and 99% ionised at pH 8 and 10 (**Table 4.2**), so ionisation of the CS solution did not show large changes when different pH values were tried for the HA solution. The largest variation in the pH of the HA:CS PECN dispersion was observed when the pH of HA was changed from pH 2 to pH 4 resulting in the final HA:CS PECN dispersion changing pH from 2.5 to 3.8. The change between pH 2 and pH 4 may be due to the fact that the pKa for HA was at pH 3 so above this value HA is more ionised. Only small changes in the final HA:CS PECN dispersion parameters (size, ZP, PDI) were seen as the pH of HA solution was altered from pH 5.7 to pH 10. The resulting pH of the formed PECN dispersions ranged from pH 2.5 – 4.5. Based on these results it was decided that altering the pH of the HA solution did not benefit the PECNs which was reflected in the higher PDI values and it was concluded that the HA solution should be used in its natural state without pH adjustment.

Table 4.2. Properties of (7.5:6.5) HA:CS PECNs prepared using 0.1% w/v HA solutions with different starting pH values adjusted using 0.01M HCl and 0.01M NaOH. The non-adjusted pH is also included for comparison (pH=5.7). The % ionised was calculated based on the Henderson-Hasselbalch equation (Eq. 4.1).

HA sol pH	% Ionised HA	pH of NP disp	pKa CS	% ionised CS	pKa HA	% ionised HA	Size (nm)	PDI	ZP (mV)
2	9	2.5	6.5	100	3	24	190 ± 3	0.252 ± 0.014	+ 23 ± 2
4	91	3.8	6.5	100	3	86	244 ± 2	0.273 ± 0.021	+ 33 ± 1
5.7	100	4.2	6.5	100	3	94	196 ± 1	0.120 ± 0.071	+ 38 ± 1
8	100	4.3	6.5	99	3	95	203 ± 2	0.211 ± 0.061	+ 37 ± 1
10	100	4.5	6.5	99	3	97	235 ± 6	0.284 ± 0.050	+ 38 ± 1

$$pH = pKa + \log_{10} \frac{(A-)}{(HA)} \text{ Eq 4.1}$$

Equation 4.1. Henderson-Hasselbalch equation used to predict the drug solubility in the aqueous polymer solutions. pKa is the acid dissociation constant for the drug, (A-) is the concentration of ionised drug present, (HA) is the concentration of unionised drug present.

4.2.2.3 Optimal Formulation for HA:CS PECNs

The optimal PECN HA:CS ratios in **Table 4.3** were chosen based on the desirable size, ZP, PDI and physical stability to represent a negatively and a positively charged HA:CS PECN system. The positively charged HA:CS PECN has a higher particle size than the negatively charged HA:CS PECN (173 ± 2 nm for the positively charged 7.5:6.5 HA:CS and 160 ± 5 nm for negatively charged 10:4 HA:CS). The PDI of the negatively charged particles was not significantly different to that of the positively charged particles (**Table 4.3**). The PECN dispersions were turbid with no evidence of aggregation seen for 21 days post formation. Outside of the optimal weight ratio the solutions were clear indicating no PECNs had been formed or else visible aggregates could be seen quickly after formation showing that the PECNs were physically unstable.

Table 4.3 Composition and properties of optimal unloaded HA:CS PECN systems as used for subsequent drug loading experiments.

HA:CS weight ratio	Size (nm)	ZP (mV)	PDI
7.5:6.5	173 ± 2	+ 33 ± 1	0.102 ± 0.017
10:4	160 ± 5	- 29 ± 1	0.116 ± 0.019

The empty carriers prepared in these studies were compared to those prepared previously by Umerska et al. (2012) as they had been prepared using the same method and materials (**Table 4.4**). It was observed that the carriers in this study had a smaller size than those prepared by Umerska et al. Looking firstly at the 10:4 HA:CS ratio the particle size difference was not significantly different for the 10:4 ratio ($p = 0.056$), where the size observed in these studies was 160 ± 5 nm and those prepared by Umerska et al. had a mean size of 172 ± 6 nm. Although the size difference at 10:4 was not significantly different the ZP value at this weight ratio measured by Umerska (-38 ± 2 mV) and the ZP value measured in this current work (-29 ± 1 mV) were significantly different ($p = 0.001$). The current ZP value for negatively charged HA:CS PECNs was in a stable range (Hunter 1988) as was the ZP value measured by Umerska et al. The PDI for both experiments was low, Umerska and coworkers: 0.112 ± 0.027 and the current studies: 0.116 ± 0.019 and they were not significantly different ($p = 0.844$).

Comparing the 7.5:6.5 HA:CS weight ratio was less direct as Umerska et al. (2012) did not test this exact ratio with 7.5:6.0 being their closest weight ratio so t-tests were not used for comparison. Comparing these it was observed that again her HA:CS PECNs had a larger size 222 ± 12 nm compared to 173 ± 2 nm in the current studies, her ZP value was higher but both were still in stable range $+38 \pm 4$ mV for Umerska et al. and $+33 \pm 1$ mV for the current work and their PDI was low 0.131 ± 0.030 as was that for the current study 0.102 ± 0.017 .

Table 4.4 Properties of unloaded HA:CS PECNs systems prepared by Umerska (2012) by the same method. The closest weight ratio used by Umerska to the HA:CS 7.5:6.5 used in current studies is HA:CS 7.5:6.0 so that formulation was used for comparison here.

HA:CS weight ratio	Size (nm)	ZP (mV)	PDI
7.5:6	222 ± 12	+ 38 ± 4	0.131 ± 0.030
10:4	172 ± 6	- 38 ± 2	0.112 ± 0.027

4.2.3 Characterisation of the HA:CS PECNs

4.2.3.1 Isoelectric Point Determination

The isoelectric point of PECNs is a key parameter when characterising the physical stability as at this pH the ZP of the PECNs is zero and the PECNs lose their physical stability allowing the particles to interact freely by Brownian motion as their high surface charge allowed the PECNs to repel each other. The ZP according to DLVO theory allows the PECNs to repel each other and stay in solution so as the ZP reaches neutral the repelling forces are lessened enough for particles to collide. Determining the isoelectric point of the PECNs could help predict the conditions under which the PECNs would be physically stable and to assign them to a particular method of drug delivery that would suit their physical stability.

The empty carriers were titrated as a positive carrier (HA:CS 7.5:6.5 weight ratio) and a negative carrier (HA:CS 10:4 weight ratio). The pH titration studies revealed that the positively charged carrier had an isoelectric point at pH 7 (**Figure 4.1**) while the empty negatively charge carriers displayed an isoelectric point at pH 3.2 (**Figure 4.2**). This highlighted the fact that these carriers have very different surface charges and thus will have different behaviour to suit different purposes. This indicates that, at the measured conditions, the positively charged HA:CS PECNs may be most stable in the stomach at pH 2 but they may lose their charge and be prone to collapse in the intestines at pH 7-9. The opposite is true for the negatively charged HA:CS PECNs which would be stable

at the intestinal pH 7-9 and in blood pH but not in the stomach at pH 2. The negatively charged HA:CS PECNs appear to be a more stable choice for medicinal applications as they are stable at pH 7.4. It is worth taking into account that the concentration of HA:CS PECNs localised at the site of delivery will be much lower than in the native dispersions, thus it is possible that the HA:CS PECNs may not collide *in vivo* despite the neutral ZP and thus many remain physically stable.

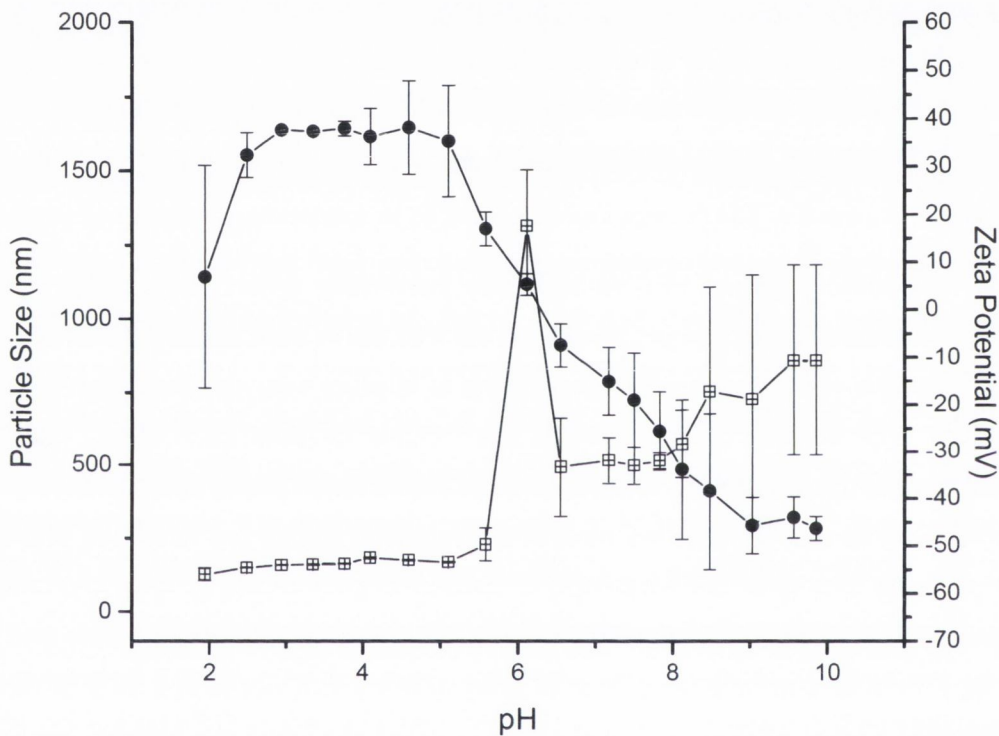


Figure 4.1 Titration profile for positively charged HA:CS 7.5:6.5 (weight ratio) PECNs. • = ZP (mV), □ = size (nm)

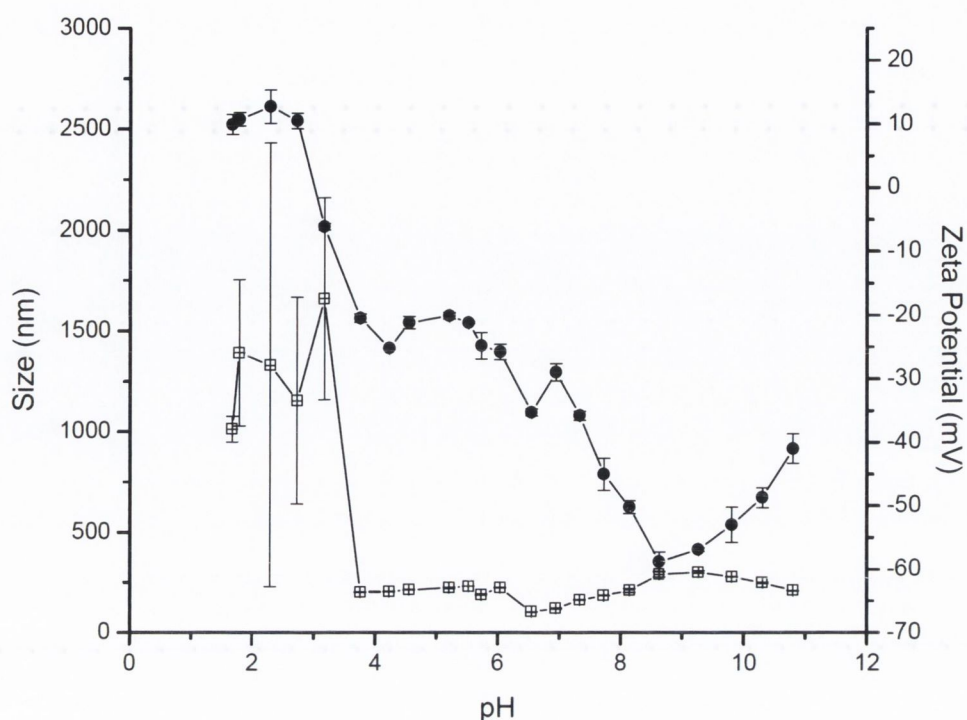


Figure 4.2 Titration profile for negatively charged HA:CS 10:4 (weight ratio) PECNs. . • = ZP (mV), □ = size (nm)

4.2.3.1 Stability Studies

The physical stability of the empty carriers was tested over a 14 day period to learn more about the resilience of the carriers and their ability to resist aggregation or degradation (**Figure 4.3**).

The positively charged HA:CS 7.5:6.5 carriers showed a gradual decrease in the particle size over the 14 day period but the PDI remained low showing that there was not extensive aggregation taking place. The particle size on day 14 was 75% of the original size. The ZP for these HA:CS positively charged HA:CS PECNs spanned 22 mV – 32 mV over 14 days.

For negatively charged HA:CS PECNs the decrease observed in size was not as large with the particle size being 99% of the original size on day 14. The ZP for

the empty HA:CS PECNs had a span of - 24.7 to - 34.6 mV over the 14 day period. This showed that the particle size of negatively charged HA:CS PECNs was more stable over time and so these structures are more resilient to change.

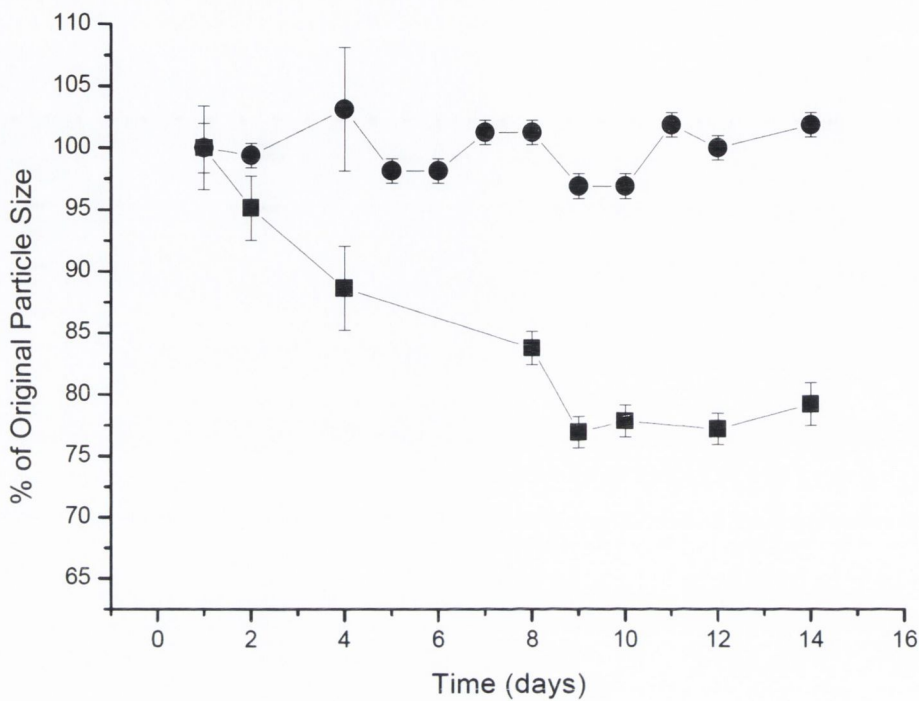


Figure 4.3 Stability profile for empty HA:CS PECNs. ● = negatively charged HA:CS 10:4 PECNs. ■ = positively charged HA:CS 7.5:6.5 PECNs

4.2.3.2 Electron Microscopy

Microscopy showed the morphology of the HA:CS PECNs and confirmed that the particle size as visualised by microscopy was consistent with that measured by dynamic light scattering. Positively charged HA:CS PECNs were observed as having an irregular shape(**Figure 4.4**) which was also the case for negatively charged HA:CS PECNs (**Figure 4.5**). Looking at the HA:CS PECNs it was difficult to distinguish between them based on charge.

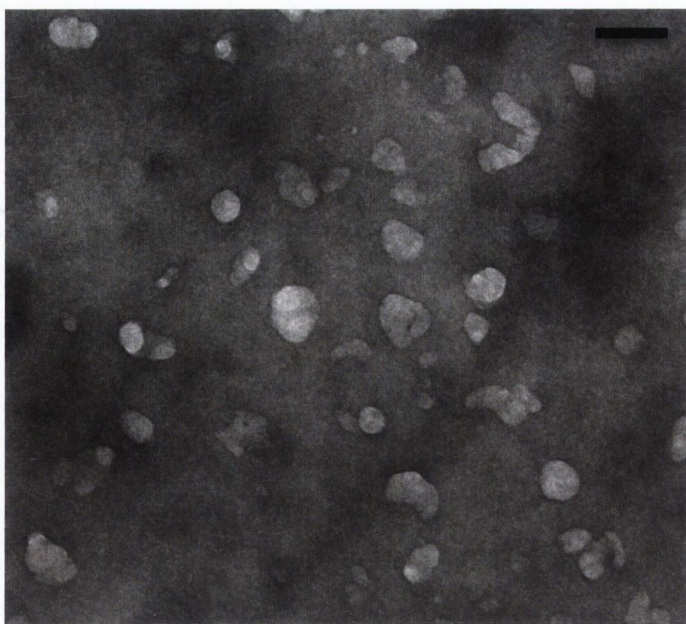


Figure 4.4 TEM image of positively charged unloaded HA:CS PECNs. Scale bar = 100 nm

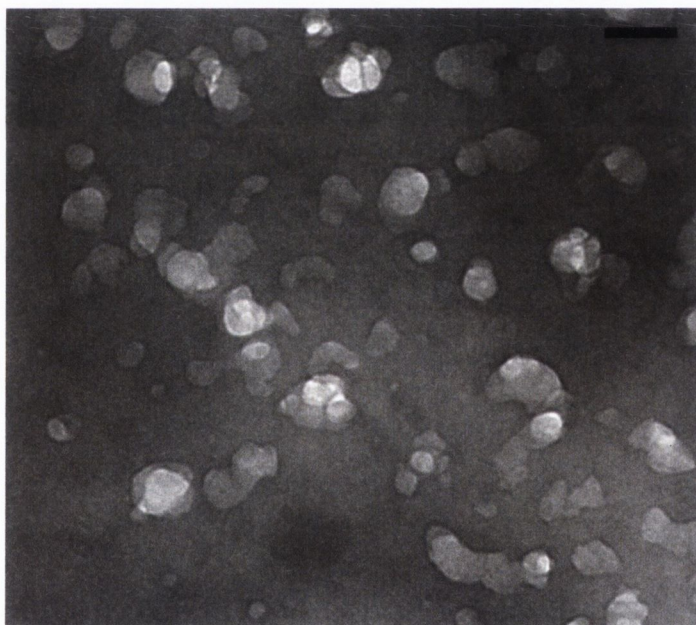


Figure 4.5 TEM image of negatively charged unloaded HA:CS PECNs. Scale bar = 100 nm.

4.3 The Development and Characterisation of KET loaded HA:CS PECNs

4.3.1 Optimisation of the KET loaded HA:CS PECN Formulation Variables

4.3.1.1 Solubility of KET in Aqueous Solutions

Experiments were carried out in the current work to investigate the solubility of KET in the components of the HA:CS PECNs. The solubility of KET in the various solvents/solutions can be ranked, in order of increasing solubility, as: 0.1% w/v CS < 0.1% w/v NPs < water < 0.1% w/v HA < ethanol (**Table 4.3**). With the exception of ethanol the solubility increased as the pH of the dissolution medium increased, this is to be expected due to the acidic nature of the KET (KET pKa = 4.23). The solubility of KET in the polymer solutions was compared with the solubility of KET in water at the corresponding pH values pre and post KET addition as predicted using the Henderson-Hasselbalch equation (**Table 4.5, Figure 4.6, Eq. 4.1**). The solubility of KET was predicted both at the pH both pre and post KET addition because the actual solubility in the 0.1% HA and 0.1% CS solutions for KET loaded HA:CS PECNs will be in a range between the pre and post KET addition solubility prediction. This is because the experimental solubility value is based on a saturated KET solution but in the case of KET loaded HA:CS PECNs the concentration of drug added may not reach the saturation point and thus the pH will differ from that at the final equilibrium pH value.

The predicted solubility of KET in water at the corresponding pH of the component solutions showed differences from the actual measured solubility in the polymer solutions. This indicated that pH might not be the only factor influencing the drug solubility. Yalkowsky and He (2003) reported the aqueous solubility of KET in water to be 0.14 mg/ml, which is consistent with the value measured experimentally in this work, 0.12 ± 0.04 mg/ml. The predicted solubility at pH 4.1 of 0.13 mg/ml, was lower than the actual solubility measured in 0.1% w/v HA solution (0.17 ± 0.01 mg/ml). For 0.1 % w/v CS solution the predicted solubility (0.08 mg/ml) in water at pH 4.0 was slightly lower than the actual

solubility (0.10 ± 0.01 mg/ml). The increase in solubility of KET in the polymer solutions may be due to interactions between the polymers and the KET molecules that are not present between KET and water molecules.

KET in aqueous dispersions (0.1% w/v) of the positively charged HA:CS 7.5:6.5 and the negatively charged HA:CS 10:4 PECNs showed higher solubility compared to the predicted values at the equivalent pH values. This may be due to KET interaction or incorporation into the PECNs.

The polymer charge may have an influence on the solubility of KET as the negative charge of HA may reduce the solubility of KET while the positive charge of CS may enhance the solubility of KET. Cheow and Hadinoto (2012) prepared nanoplexes from ciprofloxacin and dextran and found that as the drug content increased the hydrophobic interactions increased stabilising the particles. It is likely that inter-drug (ciprofloxacin-ciprofloxacin) hydrophobic interactions played a role in stabilising this system. Inter-drug interactions are likely to have stabilised the PECNs formed in these experiments too due to the hydrophobic nature of KET. The solubility of KET in solution containing the positively charged HA:CS PECNs (0.13 ± 0.01 mg/ml measured solubility) was higher than expected in water at the same pH (0.09 mg/ml calculated solubility) as was the case for KET solubility in a solution containing negatively charged HA:CS PECNs (0.14 ± 0.01 mg/ml measured solubility) compared to its calculated solubility at the equivalent pH (0.11 mg/ml). This rise in solubility could be related to the possible salt formation with CS.

Table 4.5 Solubility of KET in the constituents of the PECN formulations. pH is given both pre-addition of KET and post-dissolution of KET. The predicted solubility of KET at the pre-dissolution pH calculated based on the Henderson-Hasselbalch equation is also given (**Eq. 4.1**).

Solvent	pH of pre-solubility studies	Measured Solubility (mg/ml)	pH of post-solubility studies	Predicted Solubility in Aqueous Solution at pre-solubility study pH (mg/ml)	Predicted Solubility in Aqueous Solution at post-solubility study pH (mg/ml)
Ethanol		368.69 ± 40.40			
Water	5.9	0.12 ± 0.04	4.0	3.61	0.12
0.1% HA solution	6.2	0.17 ± 0.01	4.1	7.12	0.13
0.1% CS solution	4.0	0.10 ± 0.01	3.2	0.12	0.08
0.1% Pos HA:CS PECNs	4.5	0.13 ± 0.01	3.6	0.22	0.09
0.1% Neg HA:CS PECNs	5.7	0.14 ± 0.01	3.8	2.30	0.11

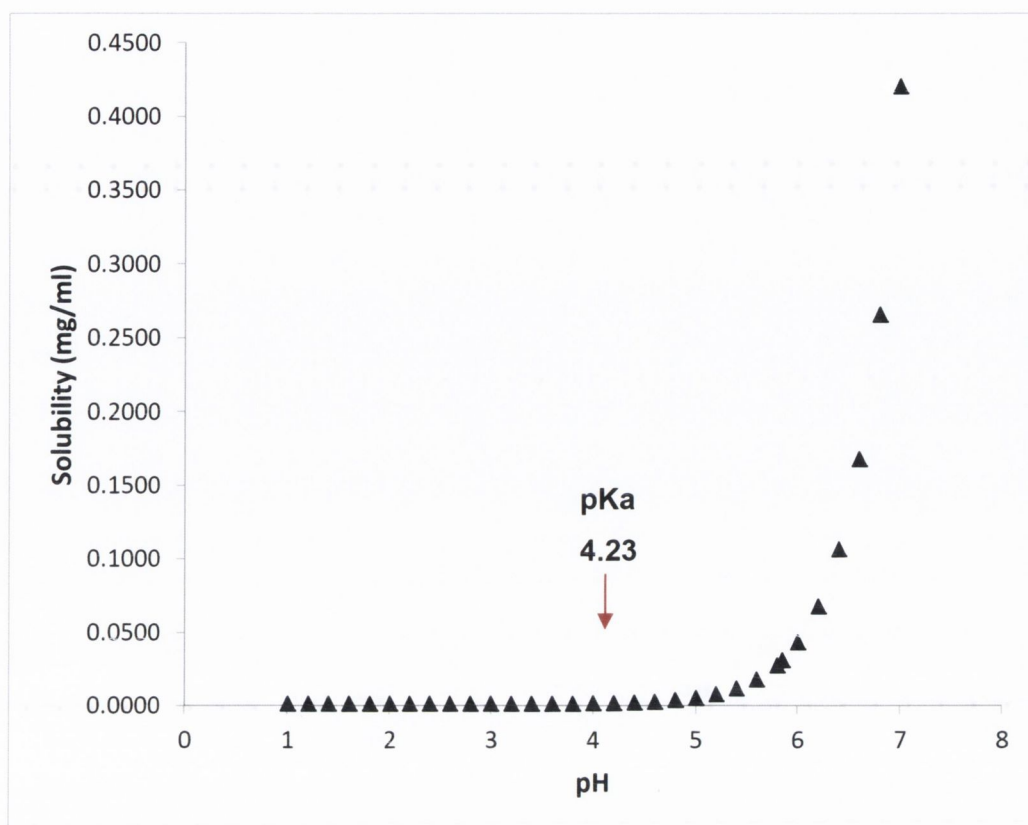


Figure 4.6 The solubility of KET versus pH as calculated using the Henderson-Hasselbalch equation (Eq. 4.1). Once the pH = pKa of 4.23 for KET is reached the solubility rises rapidly.

An ionisation profile was constructed to give an overview of the percentage ionisation of the components of the system: KET, HA and CS (**Figure 4.7**). This showed the value at which each of the components were ionised to an optimal degree. Since KET loaded HA:CS PECNs are formed primarily based on electrostatic interactions, the initial ionisation of the components is important to take into account when preparing the PECNs. KET reached 100% ionisation at pH 7.6 but it was 99% ionised from pH 6.4 and the pH rose from that point onwards while CS was 100% ionised up to pH 3 and remained 99% ionised until pH 4.5 at which point the ionisation started to decline. HA solution was not predicted to be 100% ionised until pH 6.4 but it was 99% ionised from pH 4.9. Therefore, if the pH of the system remained close to pH 5 all components would have a high degree of ionisation present.

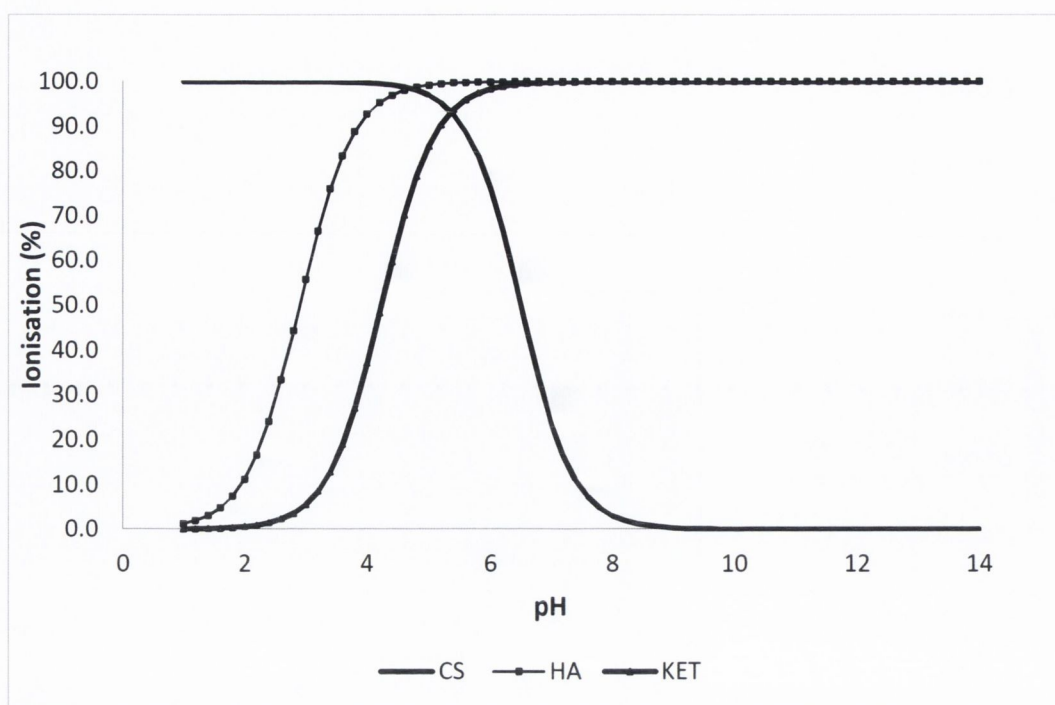


Figure 4.7 Ionisation % profile versus pH for the components of KET HA:CS PECNs.

4.3.1.2 Varying Polymer Ratio

The HA:CS polymer weight ratio was varied to test the optimal conditions for the formation of KET loaded HA:CS PECNs.

Particle size, ZP, PDI and physical stability were the parameters used to determine which HA:CS ratio produced the most suitable PECNs once loaded with KET. The weight ratio of HA:CS was varied from 10:4 to 5:9 as for empty HA:CS PECNs. Physically stable KET loaded HA:CS PECNs with a positive charge formed at HA:CS 7:7, 7.5:6.5, and 8:6 and PECNs with a negative charge were possible at HA:CS 10:4. As was the case for unloaded HA:CS PECNs, the 9:5 ratio gave the least desirable results with particles outside the nano-range being formed, this would be expected as the ZP was almost neutral at 7 ± 2 mV so the charge had been neutralised. This is expected as the charge ratio for HA:CS in the HA:CS 9:5 PECNs is 1.01, a value close to 1 indicating charge neutralisation (**Table 4.6**). When the components in PECNs have a charge ratio of 1:1, charge neutralisation results either in the formation of large particles outside the nano-range or else polyelectrolyte complexes do not

successfully form at all (Boddohi et al. 2009, Rolland et.al. 2012, Schatz et al. 2004).

The charge ratios were calculated to reveal the stable ratios for combining the polymers. The optimal charge ratios for HA:CS were 1.53 at a HA:CS weight ratio of 10:4 and 0.62 for 7.6:6.5. This confirms that for positively and negatively charged PECNs the polymer, which has the same charge as the PECNs is in excess; CS charge is in excess for positively charged PECNs and HA is in excess for negatively charged PECNs. Although CS positive charge is in excess for the positively charged systems, HA is still in excess in the mass ratio in the positively charged KET loaded HA:CS PECNs. The closer to 1 the charge ratio was the more likely the KET loaded HA:CS PECNs were to aggregate. A HA:CS weight ratio of 9:5, equivalent to a charge ratio of 1.01, was the most unstable.

Table 4.6 Properties of HA:CS PECNs at varying polymer weight ratios.

HA:CS weight ratio	pH	Size (nm)	PDI	ZP (mV)	Mass Mixing Ratio	Charge Mixing Ratio
10:4	4.2	197±5	0.1	-28± 1	2.50	1.53
9:5	3.8	>1um	0.305	7± 2	1.80	1.01
8:6	3.7	184±8	0.122	33± 2	1.33	0.73
7.5:6.5	3.7	187±2	0.103	34± 0.4	1.15	0.63
7:7	3.8	191±15	0.115	34± 3	1.00	0.57
6:8	3.9	233±9	0.154	37± 3	0.81	0.47
5:9	3.9	359±108	0.342	38± 3	0.56	0.33

The HA:CS PECN charge ratio increased as the volume of HA increased. This increase in charge ratio was greater as the pH increased due to differences in the pH of the final HA:CS PECN dispersion. As KET was more soluble above its pKa at 4.23, the drug addition to formulations with higher initial pH resulted in a

larger decrease in pH. HA:CS 10:4 had a pH for empty NCs at pH 5.8 and once KET was added the pH dropped to 4.2 which was reflected in a difference in the charge ratio from 1.83 to 1.57 post KET addition. At the 7.5:6.5 HA:CS ratio the pH changed from pH 4.4 without KET to 3.7 with KET and the difference in charge ratio was less (0.69 without KET and 0.63 with KET). The optimal weight ratios for KET loaded HA:CS PECNs were the same as those chosen as most suitable for the HA:CS PECNs without KET: 7.5:6.5 for the positively charged system and 10:4 for the negatively charged system.

4.3.1.3 Varying the pH of HA Solution

As for the unloaded HA:CS PECNs (**Section 4.2.2.2**) the pH of HA solution was varied to investigate if it could be manipulated to influence HA:CS PECN formation when KET is loaded (**Table 4.7**). The HA:CS 7.5:6.5 combination yielding positively charged KET loaded PECNs was chosen to investigate this parameter. The KET concentration chosen was 0.2 mg/mg as it represented the median DL concentration. At pH 5.7 native, unadjusted pH of HA aqueous solution) KET is 97.4% ionised. Addition of drops of 0.1M NaOH saw the ionisation increase for KET to 100%, which is an increase of 2.6 %. The increase in pH above 5.7 did reduce ionisation of CS in the solution by 2% and thus reduced the amount of CS available for interaction via electrostatic bonds. Altering the pH of 0.1% w/v HA solution did not show any advantages in the physical characteristics of the PECNs formed and the measured size, ZP and PDI did not change considerably. KET loaded HA:CS 7.5:6.5 PECNs produced using a solution of HA with native pH had a small size, low polydispersity and a stable ZP and thus there was no reason to alter the pH. The percentage ionisation was calculated based on the Henderson–Hasselbalch equation (**Eq. 4.1**).

Table 4.7 The pH of HA solution was varied to assess its effects on the properties of the KET loaded PECNs.

HA pH	% Ionised HA	% Ionised KET	pH of NP Suspension	pKa CS	% ionised CS	pKa HA	% ionised HA	Size (nm)	PDI	ZP (mV)
2	9	0.6	2.8	6.5	100	3	39	204 ± 4	0.12 ± 0.01	33 ± 1
4	91	37.1	3.8	6.5	100	3	86	159 ± 4	0.10 ± 0.02	33 ± 1
5.7	100	97.4	4.2	6.5	100	3	94	186 ± 1	0.18 ± 0.10	34 ± 1
8	100	100.0	4.4	6.5	99	3	96	184 ± 2	0.11 ± 0.02	34 ± 1
10	100	100.0	4.9	6.5	98	3	99	182 ± 3	0.11 ± 0.02	33 ± 1

4.3.1.4 Varying Solvent for KET

Solubility studies carried out on KET in water and in aqueous PECN dispersions showed that KET solubility in the components was lower than the intended DL for 0.4 and 0.2 mg/mg KET addition and just above for 0.1 mg/mg KET addition (**Table 4.5**). In order to increase the drug solubility to increase the chance of KET mixing with the polymers to form PECNs it was decided to use a cosolvent for KET. Once dissolved the KET could bind to the CS polymer chains by electrostatic interactions, the main interaction type, which allows the PECNs to form (Thünemann et al. 2004). The ionised KET in the solution may have competed with HA for CS charged functional groups. Lankalapalli and Kolapalli (2009) described how an increase in the ionic strength of a solution of polyelectrolytes could influence the PECN formation as the small ions compete for binding sites on the polymers chains. It is expected that binding of KET would occur by competition with HA charged units, as KET is a low molecular weight ionic molecule. It was decided to test a range of solvents to investigate if they offered any advantages to the physical properties of the resulting HA:CS PECN system. The experiments were carried out using a HA:CS combination yielding positively charged HA:CS PECNs with KET loading of 0.2 mg/mg to study differences between the solvents.

Positively charged HA:CS 7.5:6.5 KET loaded PECNs formed using different solvents did not show large variations in the physical properties of the PECNs (**Table 4.8**). The size of the PECNs did not show any significant differences ($p=0.914$). The PECNs produced from ethanol measured at 194 ± 2 nm, from ethyl acetate at 186 ± 54 nm, from acetone at 194 ± 8 and from dimethylsulfoxide (DMSO) at 202 ± 6 nm. The amount of solvent used was very low at a ratio of 1:57 for solvent : polymer solutions and it did not appear to influence the formation of the KET loaded HA:CS PECNs by itself. The largest change for size data was seen in the standard deviations of the KET loaded HA:CS PECNs, with the values increasing for ethanol < DMSO < acetone < ethyl acetate. This could be due to the miscibility of the solvents with water as the standard deviation values increase as miscibility with water decreased e.g. ethyl acetate has very limited miscibility with water (Godfrey 1972). The ZP values for the KET loaded HA:CS PECNs were significantly different ($p=0.001$) but the range was narrow spanning only 4.6 mV so this was not expected to have a

large influence on the behaviour of the formed PECNs. The ZP values observed were $+ 34 \pm 1$ mV for ethanol, $+ 35 \pm 1$ mV for ethyl acetate, $+ 37 \pm 1$ mV for DMSO and $+ 32 \pm 4$ mV for acetone PECN samples (**Table 4.8**).

No significant differences were observed for the PDI of the KET loaded HA:CS PECNs ($p = 0.434$) and all PDI values were low. This shows that the solvent did not greatly affect the formation process of the HA:CS PECNs as the size variations within the samples remained low. The ethyl acetate sample showed the highest PDI value at 0.190 ± 0.081 , which is due to the large standard deviation value seen for size 186 ± 54 nm.

Ethanol and DMSO samples presented the lowest standard deviation and PDI and these two solvents had lower vapour pressures of 44.6 mmHg and 0.42 mmHg, respectively, than acetone 184 mmHg and ethyl acetate 77 mmHg. Slower evaporation of the solvent could allow the drug more time to dissociate and interact with the polymers. Rapid solvent evaporation may precipitate the drug prior to association. Rapid solvent evaporation was found to be advantageous in the emulsion solvent-evaporation/diffusion methods for PLGA NPs preparation to reduce NP size (Quintanar-Guerrero et al. 1996) as the drug is already emulsified in the organic phase so evaporation of solvent does not affect the initial NP formation but in the case of these KET loaded HA:CS PECNs the drug is not emulsified and thus slower evaporation may be advantageous.

Ethanol was chosen as the solvent of choice over DMSO as it is miscible with water, cheap, non-toxic and also it can be removed more easily than e.g. DMSO as DMSO has a high boiling point of $189\text{ }^{\circ}\text{C}$ compared to ethanol at $78\text{ }^{\circ}\text{C}$ (Riddick 2008).

Table 4.8 The properties of KET loaded HA:CS 7.5:6.5 PECNs formed using different solvents to dissolve the drug. KET was dissolved in the solvent at a concentration of 1 mg/89.3 μ l.

Solvent	Size (nm)	PDI	ZP (mV)
Ethanol	194 $\pm$ 2	0.16 $\pm$ 0.011	+ 34 $\pm$ 1
Ethyl acetate	186 $\pm$ 54	0.19 $\pm$ 0.081	+ 35 $\pm$ 1
Acetone	194 $\pm$ 8	0.15 $\pm$ 0.022	+ 37 $\pm$ 1
DMSO	202 $\pm$ 6	0.13 $\pm$ 0.014	+ 32 $\pm$ 4

4.3.1.5 Varying Drug Concentration and Drug Loading

According to the B.P. KET is classified as freely soluble in ethanol (B.P.,2013). The experimental solubility value measured for KET in ethanol was 368.69 $\pm$ 40.40 mg/ml (**Table 4.5**). It was decided to use three different concentrations of KET to prepare the PECNs to investigate the capacity of the PECNs to load KET. The low concentration was 0.1 mg KET/mg polymers which was the equivalent of 10% loading, the medium concentration was 0.2 mg KET/mg polymers equivalent to 20% DL and the high concentration was 0.4 mg KET/mg polymers equivalent to 40% DL. Above 0.4 mg KET/mg polymers aggregation was seen. This was due to the increasing volume of ethanol as addition of 0.6 mg KET/mg polymers at a lower concentration of ethanol did not result in precipitation. The ratio of KET:ethanol was kept constant at 1 mg KET in 89.3 μ l of ethanol (1.12 % w/v) and thus as the quantity of KET increased the volume of ethanol also increased proportionally: 0.1 mg KET / mg polymers = 1.4 mg KET/ 125 μ l ethanol, 0.2 mg KET / mg polymers = 2.8 mg KET/ 250 μ l ethanol and 0.4 mg KET /mg polymers = 5.6 mg KET / 500 μ l ethanol. The fixed volume of polymer solutions used was 14 mls in total. Ethanol was seen to affect the physical stability of the PECN system when its volume was raised above 700 μ l in the system, which had a total volume of 14 mls. The KET:ethanol concentration remained fixed so the behaviour of KET in formation of the KET loaded HA:CS PECNs would not be due to variations in the ethanol concentration.

The correlation between the amount of KET added and the actual KET content gave $R^2 = 0.811$, for KET loaded 7.5:6.5 HA:CS (positively charged) PECNs (**Figure 4.8**). In contrast, the correlation between the amount of KET added and the correlation between the actual KET content post-wash in 10:4 HA:CS (negatively charged) PECNs had $R^2 = 0.995$ and the DL increased with an increasing amount of KET added. This was further investigated by studying the drug solubility in the polymer solutions and the charge ratios of the optimal formulations.

Table 4.9 Properties of KET loaded positively charged HA:CS 7.5:6.5 PECNs. EE = encapsulation efficiency, DL = drug loading.

Polymer/Drug	Size (nm)	ZP (mV)	PDI	EE (%w/w)	DL (%w/w)	Drug Content (mg/mg)
HA-CS Empty	173 ± 2	+ 33 ± 1.	0.10 ± 0.02	-	-	-
KET 0.1 mg/mg	182 ± 1	+ 37 ± 1	0.11 ± 0.03	38.4 ± 7.3	3.7 ± 0.1	0.038
KET 0.2 mg/mg	187 ± 2	+ 34 ± 1	0.10 ± 0.05	28.0 ± 4.5	5.3 ± 0.8	0.053
KET 0.4 mg/mg	282 ± 3	+ 41 ± 1	0.10 ± 0.04	14.9 ± 1.7	5.9 ± 0.4	0.059

The properties of negatively charged KET loaded HA:CS PECNs differed from those of positively charged HA:CS PECNs. The negatively charged KET loaded HA:CS PECNs were larger than the positively charged KET loaded HA:CS PECNs. HA has a higher molecular weight (257 kDa) than CS (110 kDa, Umerska et al. 2012) so given that negatively charged NPs have a higher HA content it is may be expected that these NPs would be larger. Huang et al. (2004) reported that decreasing the molecular weight of the CS polymer used to prepare their fluorescein-5-isothiocyanate labelled CS NPs showed a corresponding decrease in the final NP size as measured by DLS. For KET loaded positively charged HA:CS PECNs the addition of KET increased the size of the NCs (**Table 4.9**) and for KET loaded negatively charged HA:CS PECNs the NCs size decreased on addition of KET (**Table 4.10**).

Table 4.10 Properties of KET loaded negatively charged HA:CS 10:4 PECNs.
 EE = encapsulation efficiency, DL = drug loading

Polymer/Drug	Size (nm)	ZP (mV)	PDI	EE (%) w/v	DL(%) w/w	Drug Content (mg / mg)
HA-CS Empty	192 ± 1	- 32 ± 5	0.12 ± 0.02	-	-	-
KET 0.1 mg/mg	187 ± 1	- 30 ± 1	0.15 ± 0.03	94.0 ± 0.9	8.6 ± 0.1	0.086
KET 0.2 mg/mg	173 ± 1	- 28 ± 1	0.10 ± 0.03	63.1 ± 5.6	11.4 ± 1.1	0.114
KET 0.4 mg/mg	170 ± 2	- 27 ± 1	0.09 ± 0.01	51.0 ± 3.6	18.7 ± 1.0	0.187

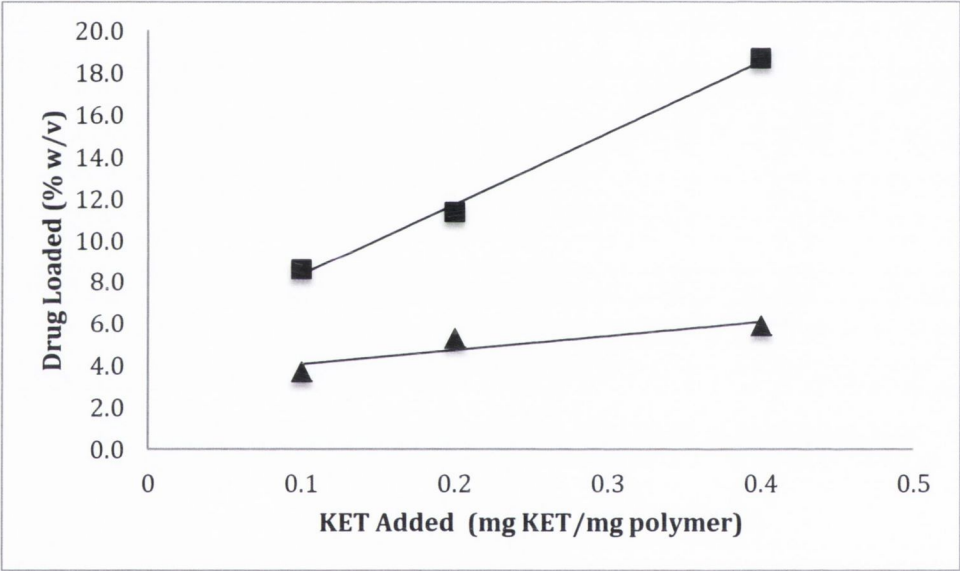


Figure 4.8 Relationship between the actual amount of drug loaded and initial KET added for positively (▲) and negatively (■) charged HA:CS PECNs.

4.3.1.6 Precipitation of KET in Aqueous Environment

KET was prepared as an ethanolic solution at a concentration of 1.12% w/v. This KET ethanolic solution was added to water in the same volumes as was added to form KET loaded HA:CS PECNs (0.1 mg/mg = 125 µl, 0.2 mg/mg = 250 µl and

0.4 mg/mg = 500 μ l) to give three final concentrations of KET ethanolic solution at 0.89 % w/v, 1.79 % w/v and 3.57 % w/v in the total volume of water. These KET ethanolic solution concentrations corresponded to a final concentration of KET of 0.1 mg/ml, 0.2 mg/ml and 0.4 mg/ml. The aim of this experiment was to investigate whether stable KET NPs would form without the use of PECNs to stabilise KET. Adding KET as ethanolic solution to water without the presence of polymers, in the same volume as that used to form KET loaded HA:CS PECNs did not result in physically stable KET NPs with monomodal particle size distribution. KET did appear to form a nanosized colloidal dispersion but the particle size was not stable over time and the PDI was high.

The size measurements carried out using DLS showed that the KET addition as an ethanolic solution without simultaneous addition of the polymers HA and CS resulted in a polydisperse ($PDI = 0.426 \pm 0.013$) system. Some NPs were present with a mean size of 847 ± 223 nm as well as larger particles, which could not be accurately measured. Some of the KET added dissolved in water as its solubility was found experimentally to be 0.12 ± 0.04 mg/ml (**Table 4.5**), which is 60% of the total concentration of KET added (0.2 mg/mg). If some KET dissolved it would reduce the concentration of NPs present in the suspension and thus the concentration of NPs may have been too low for accurate measurement. If a higher amount of KET solution is added with KET content equivalent to that added to form 0.4 mg/mg KET loaded HA:CS PECNs the PDI remained high at 0.551 ± 0.037 , the main size peak was seen at 651 ± 98 nm. KET added in the ethanolic solution to water without the presence of HA and CS polymers formed particles with a positive ZP (KET: $+ 7 \pm 1$ mV). This may be the reason why the ZP of HA:CS PECNs increased once KET was loaded. The size and ZP distributions measured using DLS for free KET were polymodal and unstable, while the KET loaded HA-CS PECN samples showed a monomodal size and ZP distribution (**Figures 4.9, 4.10, 4.11 and 4.12**). This indicates that the drug is being stabilised by association with the HA:CS PECNs as sedimentation and variation in size and ZP was not visible for some time (see **Stability Studies, Section 4.2.2.3**).

If the concentration of KET was tested at the higher DL value of 0.4 mg/mg there was still no precipitation seen. The peaks observed during size measurements using DLS were erratic and many populations were observed so an accurate estimate of size was not possible to define.

It was attempted to precipitate KET from 0.1% w/v HA and 0.1% w/v CS solutions at a concentration of KET 0.1, 0.2 and 0.4 mg/mg. No precipitation was seen at these concentrations for KET, all solutions remained clear. The size measurements using DLS revealed many peaks suggesting that the KET NPs had precipitated out of the solutions but many have been too polydisperse in size to gather an accurate reading of the average PECN size.

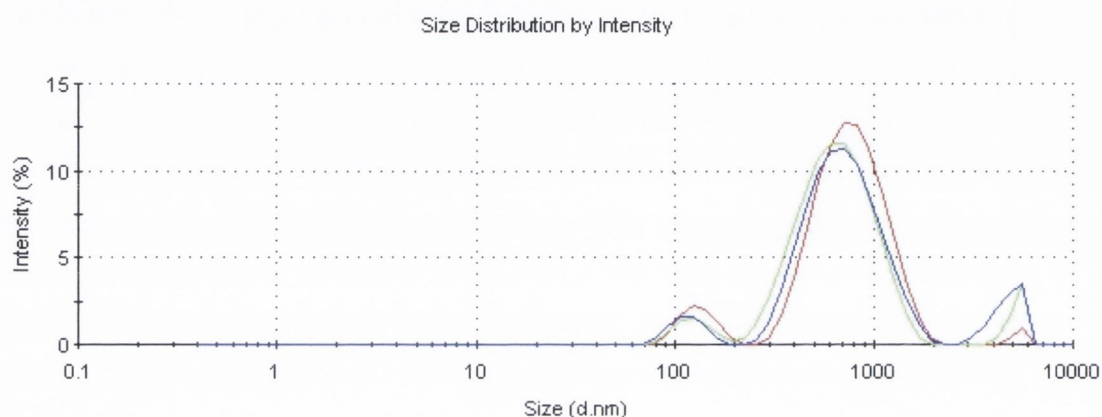


Figure 4.9 Particle size distribution of free KET precipitate, no PECNs

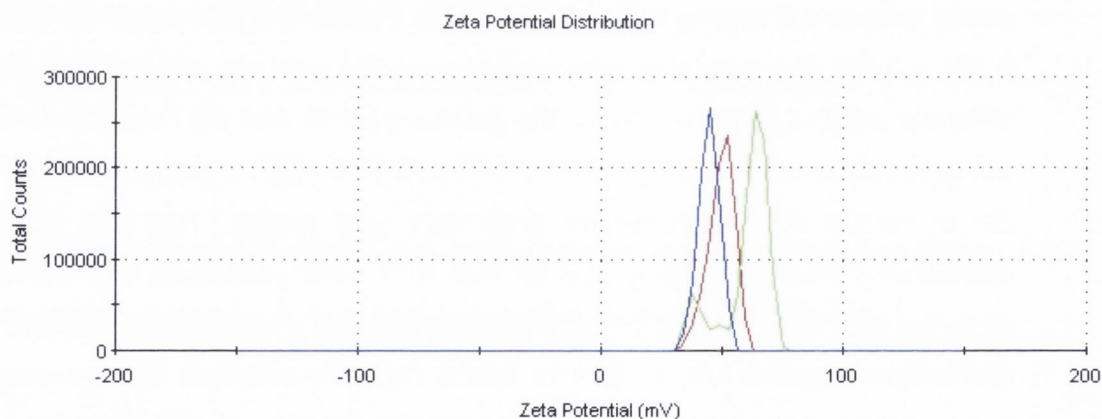


Figure 4.10 Zeta potential distribution of free KET precipitate, no PECNs.

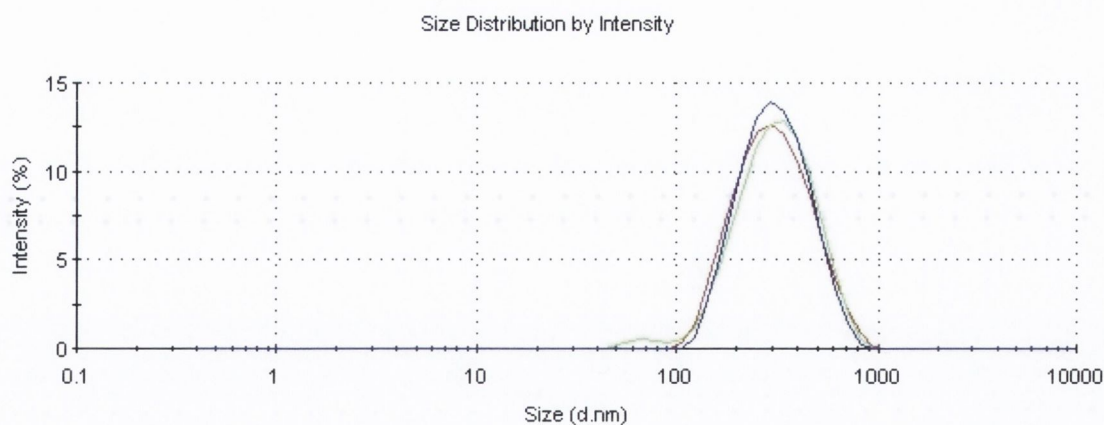


Figure 4.11 Particle size distribution of HA:CS PECNs, positively charged, with 0.2 mg/mg KET loading.

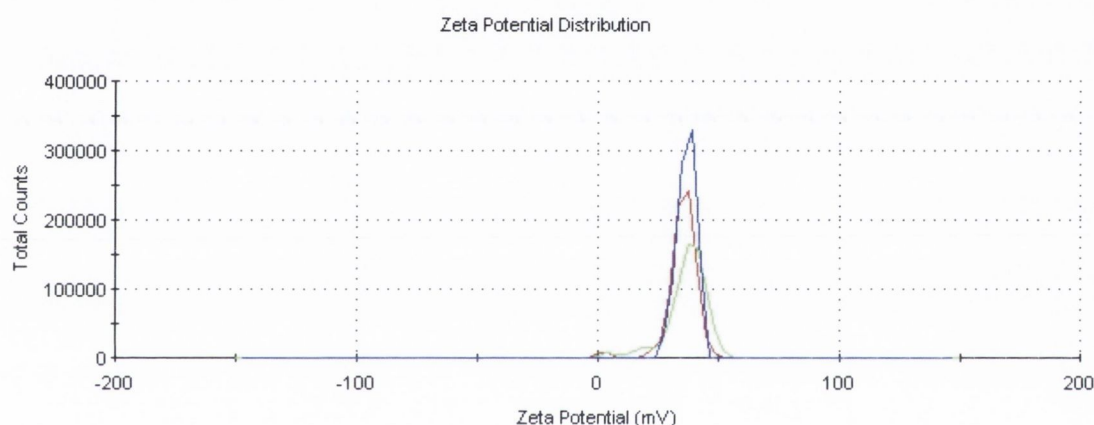


Figure 4.12. Zeta potential distribution of HA:CS PECNs, positively charged, with 0.2 mg/mg KET loading.

4.3.1.7 Summary of KET loaded HA:CS PECNs composition

Once KET was loaded the physical characteristics of the formed HA:CS PECNs were investigated sequentially. The particle size of positively charged KET loaded HA:CS 7.5:6.5 PECNs increased following addition of drug. Without the addition of KET, unloaded HA:CS 7.5:6.5 had an average size of 173 ± 2 nm which increased as drug content increased from 182 ± 1 nm at KET 0.1 mg/mg, 187 ± 2 nm at KET 0.2 mg/mg and 282 ± 3 nm at KET 0.4 mg/mg. Negatively charged KET loaded HA:CS 10:4 PECNs decreased in size with increased DL from 160 ± 5 nm for empty PECNs, 187 ± 1 nm at KET 0.1 mg/mg, and 173 ± 1 nm at KET 0.2 mg/mg and 170 ± 2 nm at KET 0.4 mg/mg (**Table 4.9**). The

difference in direction for the size change for positive and negative charge may be due to physical stability or the nature and strength of drug binding. Higher DL was observed for negatively charged KET loaded HA:CS 10:4 PECNs than for KET loaded positively charged HA:CS 7.5:6.5 PECNs (**Table 4.9, 4.10**). The increase in the KET content for the negatively charged HA:CS PECNs may be due to compaction of the structure of the PECNs because the KET molecule has lower molecular weight than HA and it is possible that if the drug replaces a HA molecule in competition for the positive binding sites on the CS molecules the size decreases due to loss of larger HA molecules. In the case of the KET loaded positively charged HA:CS PECNs the size increased when the drug content increased so it is possible that the drug was less tightly bound and so particle size increases as the drug content increases.

Also for negatively charged KET loaded HA:CS 10:4 PECNs the HA content is higher and thus drug solubility in the polymers is higher as KET has a solubility value in HA of 0.017 ± 0.01 mg/ml compared to 0.012 ± 0.04 mg/ml in water and 0.010 ± 0.003 mg/ml in CS solution which could result in drug dissolution in the polymers and thus the PECNs may decrease in size.

4.2.2 Characterisation of the KET Loaded HA:CS PECNs

4.3.2.1 Final Chosen Formulations

The optimal empty PECN formulations HA:CS 7.5:6.5 and 10:4 were chosen as the most stable formulations when loaded with KET. This was verified by the optimisation studies. **Table 4.11** summarises the constitution of these optimal KET loaded HA:CS PECNs used and **Table 4.12** shows their charge related properties. The specific weight ratio and charge ratio of HA:CS is crucial for physically stable HA:CS PECN formation. Positively charged KET loaded HA:CS 7.5:6.5 PECNs were prepared and negatively charged KET loaded HA:CS 10:4 by varying the polymer weight ratio. The optimal formulations had a charge ratio with one polymer in excess close to the charge ratio of 2:1 or 1:2 number of negative charges: number of positive charges as is summarised in **Table 4.12**. HA:CS PECNs appeared stable at all drug KET DL with no precipitation seen over a one week period. This visual evidence was complemented by the fact that all charge ratios are close in value for each DL concentration: positively charged KET 0.1 mg/mg 0.63, KET 0.2 mg/mg 0.65 and KET 0.4 mg/mg 0.63, negatively charged KET 0.1 mg/mg 1.76, KET 0.2 mg/mg 1.32 and KET 0.4 mg/mg 1.35.

Table 4.11 Summary of the constitution of all HA-CS NPs prepared. Positive and negatively charged are shown. + = positive charge, - = negative charge. The table also outlines the ratios of polymer to drug, ethanol to polymer and ethanol to drug.

Sample	0.1% w/v HA (ml)	0.1% w/v CS (ml)	EtOH Solution (ml)	Added Drug Conc (mg/mg polymer)	HA:CS ratio (w/w)	EtOH: Polymer Ratio (v/v)	Drug: EtOH Ratio (w/v)
KET 0.1 +	7.5	6.5	0.125	0.1	1:0.87	1:114	1:89.3
KET 0.2 +	7.5	6.5	0.250	0.2	1:0.87	1:57	1:89.3
KET 0.4 +	7.5	6.5	0.500	0.4	1:0.87	1:28.5	1:89.3
KET 0.1 -	10	4	0.125	0.1	1:0.4	1:114	1:89.3
KET 0.2 -	10	4	0.250	0.2	1:0.4	1:57	1:89.3
KET 0.4 -	10	4	0.500	0.4	1:0.4	1:28.5	1:89.3

Table 4.12 Summary of the ratios of formation of the KET loaded PECNs including the mass ratio and the charge ratio calculated as number of negative charges (HA+ KET) divided by the number of positive charges. n^- = number of negative charges, n^+ = number of positive charges, , Neg – negative charge, Pos – positive charge, CMR – charge mixing ratio.

Sample HA:CS	HA (ml)	CS (ml)	HA (mg/ml)	CS (mg/ml)	Drug (mg/mg)	HA/CS/drug MR	pH Sample	n- HA	n+ CS	n- Drug	CMR HA:CS:Drug	CMR (Neg/Pos)
KET 0.1 +	7.5	6.5	0.54	0.46	0.10	5.4 :4.6 :1	3.7	0.012	0.019	0.00009	134.40 : 217.78 : 1.00	0.625
KET 0.2 +	7.5	6.5	0.54	0.46	0.20	2.7 :2.3 :1	3.7	0.006	0.010	0.00019	31.69 : 50.5: 1.00	0.647
KET 0.4 +	7.5	6.5	0.54	0.46	0.40	1.4 :1.2 :1.0	3.5	0.003	0.005	0.00025	10.85 : 8.83 : 1.00	0.629
KET 0.1 -	10	4	0.71	0.29	0.10	7.1: 2.3: 1.0	4.0	0.0017	0.0010	0.00014	116.54 :66.47 : 1.00	1.768
KET 0.2 -	10	4	0.71	0.29	0.20	3.6 :1.5 :1.0	3.7	0.008	0.006	0.00018	44.18 : 34.17 : 1.00	1.322
KET 0.4 -	10	4	0.71	0.29	0.40	1.8 :0.7 :1.0	3.61	0.004	0.003	0.00031	12.33 : 9.88 : 1.00	1.349

4.3.2.2. Isoelectric Point Determination

The ZP of the positively charged 0.2 mg/mg KET loaded HA:CS 7.5:6.5 PECNs was neutralised at approximately pH 6.8 (**Figures 4.13**). A sharp increase in the HA:CS PECN size was observed around the isoelectric point, which verified the necessity for the stabilisation of the surface charge in order to maintain physically stable HA:CS PECNs. Following the increase in size, the HA:CS PECNs never returned to their original size

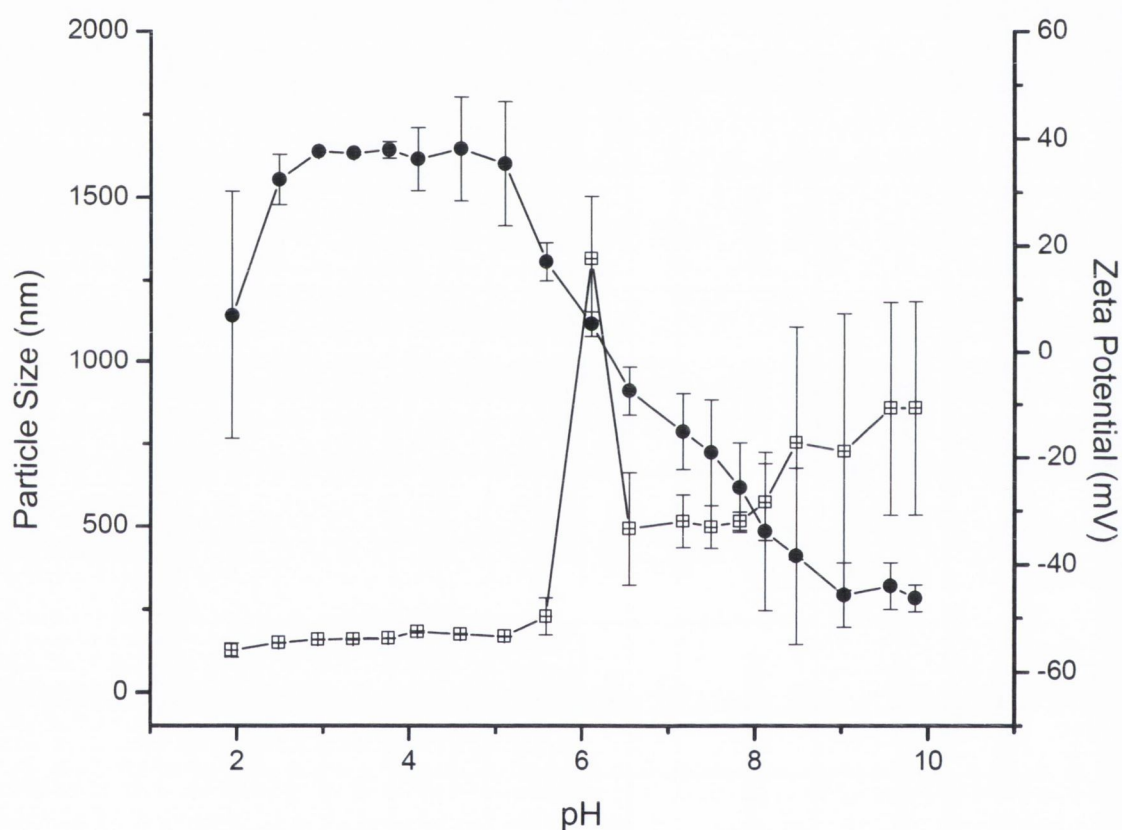


Figure 4.13 Titration profile for KET-loaded HA:CS positively PECNs. Chart shows the effect of pH on the ZP and size of the PECNs. ● = ZP (mV), ■ = size (nm).

For negatively charged HA:CS 10:4 PECNs the isoelectric point was at a lower pH value than for positively charged KET loaded HA:CS PECNs. For empty

PECNs the isoelectric point was approximately 3.8 and for KET loaded negatively charged PECNs it was approximately 4.5 (**Figure 4.14**).

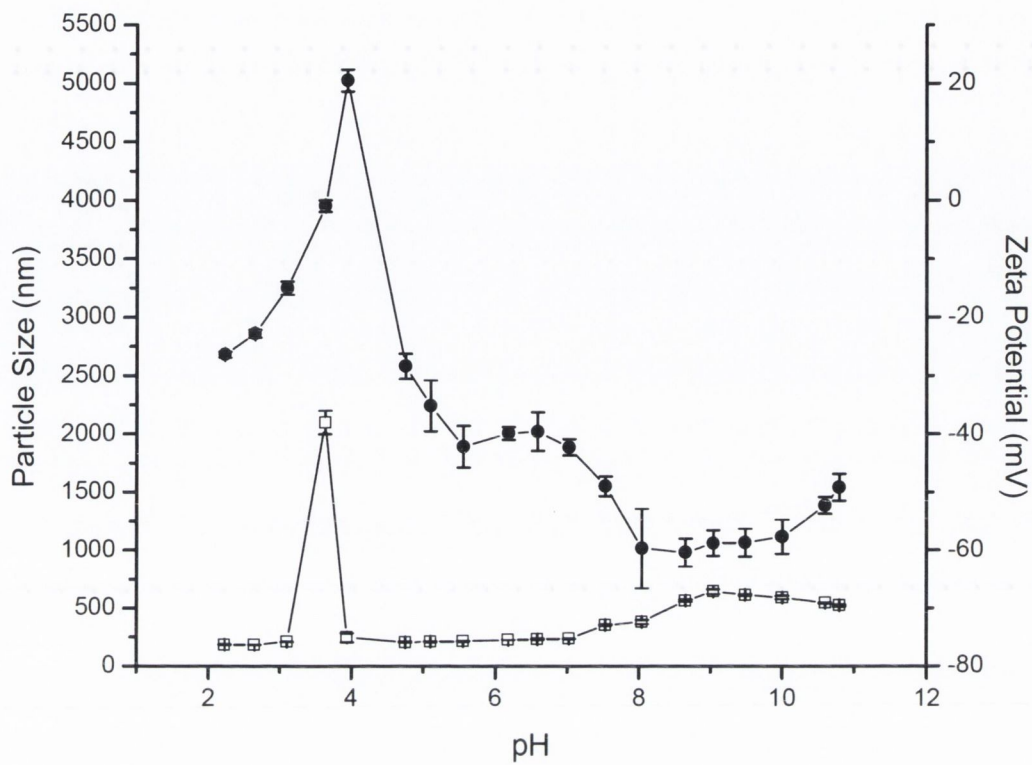


Figure 4.14 Titration profile for KET-loaded HA:CS negatively charged PECNs. Chart shows the effect of pH on the ZP and size of the PECNs. ● = ZP (mV), ■ = size (nm).

4.3.2.3 Stability studies

The physical stability of the KET loaded HA:CS PECNs with a positive or a negative charge was tested over a period of 14 days. All KET loaded HA:CS PECN formulations remained in the nano-range with low PDI over a 14 day period. Both charges of KET loaded HA:CS PECNs decreased in size with time; this may indicate drug loss or disassembly of the particles themselves. No precipitation was observed during the 14 days (**Figure 4.15 and 4.16**). The particle size of positively charged HA:CS PECNs changed the most with KET 0.1 mg/mg loaded dropping to 81% of the original particles by day 14 and at 0.2 mg/mg KET dropping to 72% of the original starting size.

Negatively charged KET loaded HA:CS PECNs displayed only minimal reductions in the particle size. KET 0.1 mg/mg HA:CS PECNs were 88% of their original size on day 14 and at 0.2 mg/mg they were 97% of the original size on day 14. The fluctuations in size observed over 14 days may have been due to particles equilibrating with their environment if there were changes in the HA:CS PECNs constitution. The positively charged HA:CS PECNs had a greater drop in size than the negatively charged HA:CS PECNs. As DL was also higher for negatively charged KET loaded HA:CS PECNs than positively charged HA:CS PECNs the drug content was not expected to be the reason unless the addition of drug was holding the PECN structures more stable. The HA in excess may have provided a protective halo around the PECNs stabilising the size and shape of the HA:CS PECN. Umerska et al. (2012) noted a halo effect around their HA:CS PECNs using transmission electron microscopy.

Free KET (KET added as ethanolic solution to water, as described in **Section 4.3.1.6** appears to have partly dissolved as the free drug suspension gave results similar to water after a few hours and there was no precipitation of the drug apparent within 14 days. The ZP of free drug was erratic and varied widely indicating a lack of stability if the drug was suspended. KET may have precipitated and sedimented or KET may have been in a solution and so viable NPs were not formed without the polymers.

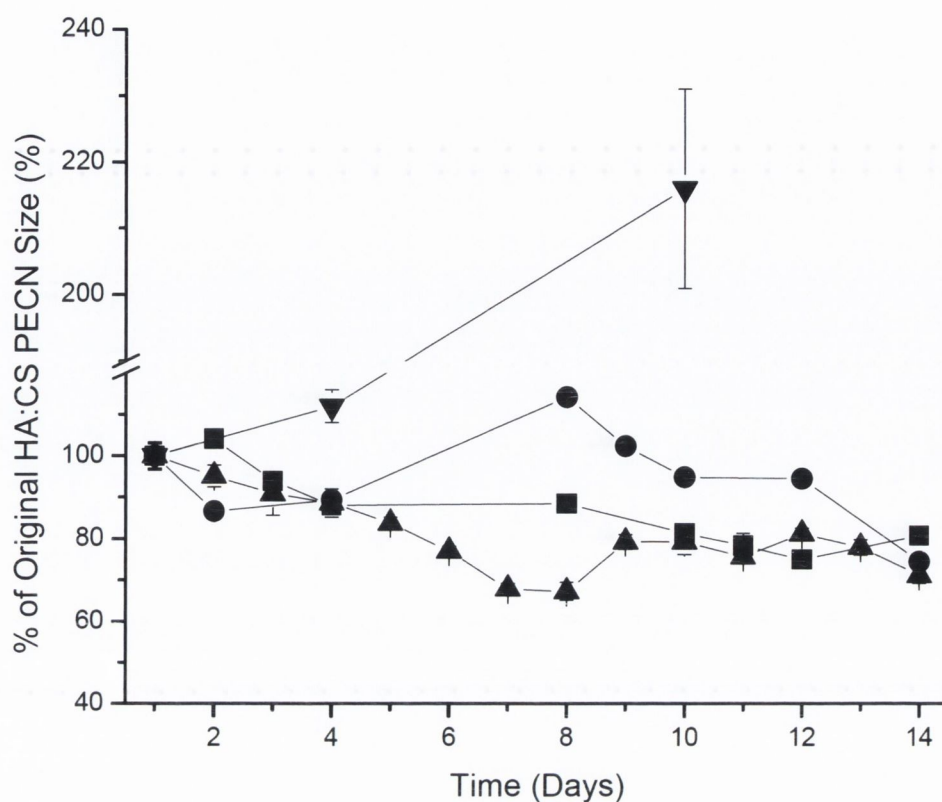


Figure 4.15 Positively charged HA:CS PECNs physical stability over 14 days reported in size (nm). ▼ = Free KET ethanolic solution, ■ = HA:CS KET 0.1 mg/mg PECNs, ● = HA:CS KET 0.2 mg/mg PECNs, ▲ = Empty HA:CS PECNs.

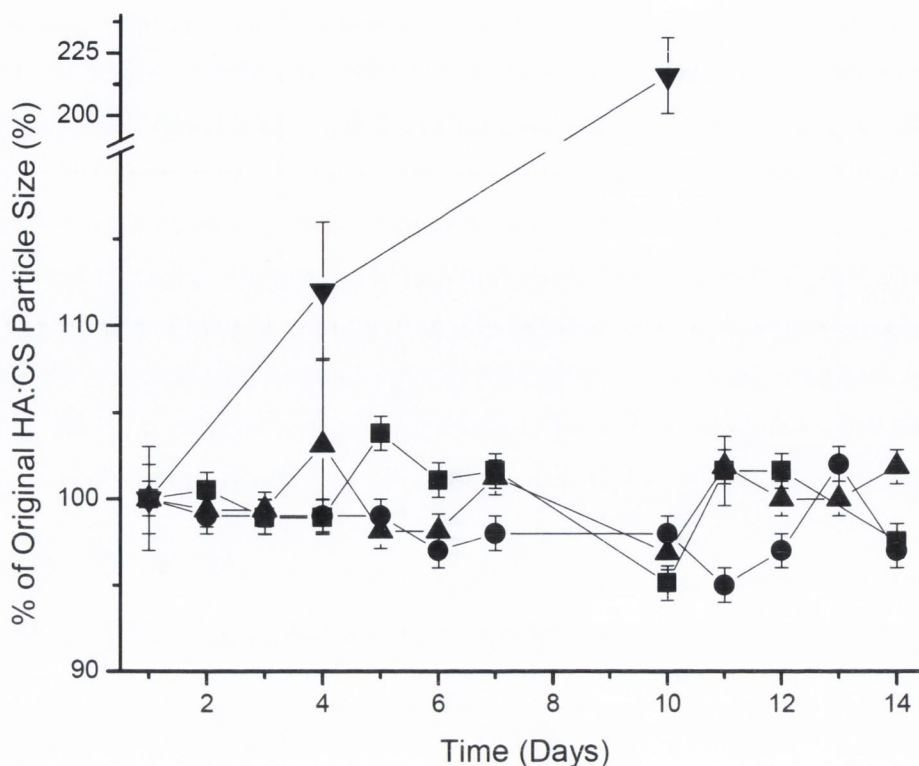


Figure 4.16 Negatively charged HA:CS PECNs physical stability over 14 days reported in size (nm). ▼ = Free KET ethanolic solution, ● = HA:CS KET 0.1 mg/mg PECNs, ■ = HA:CS KET 0.2 mg/mg PECNs, ▲ = Empty HA:CS PECNs.

The ZP values showed only very minor decreases over the 14 day period and so the HA:CS PECNs retained their ability to repel each other in the dispersion over the duration of the study. For positively charged KET loaded HA:CS PECNs at 0.1 mg/mg the range for ZP was + 35 mV to + 48 mV between day 1 to day 14, and for 0.2 mg/mg the range was + 32 mV to + 42 mV. For negatively charged KET loaded HA:CS PECNs at 0.1 mg/mg the range for ZP was - 25 to - 30 mV over the 14 days and for 0.2 mg/mg it was - 23 mV to - 33 mV. Since the drug itself, formulated as polymer-free NPs, possess a positive charge, drug loss from the particles could have reduced the charge as the lowest ZP values were recorded for all formulations on day 14. The ranges observed with KET loaded PECNs were not considerably different to the empty HA:CS PECNs, positively charged + 27 to + 38 mV and negatively charged - 25 to - 35 mV.

The drug content in the supernatant for positively charged KET 0.2 mg/mg HA:CS PECNs on day 1 was 1.5 ± 0.4 % of the KET loaded (the actual KET

loading was treated as 100%) and on day 14 it was 9.2 ± 1.3 %. For negatively charged KET 0.2 mg/mg HA:CS PECNs on day 1 there was 0.7 ± 0.3 % and on day 14 was 6.2 ± 1.3 % of the actual KET loaded present in the supernatant. This shows that there was some drug leakage over the 14 day period into the surrounding dispersion medium with higher leakage for positively charged HA:CS PECNs than negatively charged HA:CS PECNs. The leakage was also higher than for PLGA NPs.

4.3.2.4. Electron Microscopy of KET 0.2 mg/mg loaded HA:CS PECNs

The TEM images for HA:CS PECNs loaded with KET at 0.2 mg/mg did not show large differences in the appearance of the HA:CS PECNs from what was observed for empty HA:CS PECNs. The shape was not spherical and the size was close to that measured by DLS. It was also difficult to distinguish between positively and negatively charged HA:CS PECNs suggesting that the structure of this type of HA:CS PECNs are not very variable (**Figure 4.17 and 4.18**) in size and that all the differences in KET association noticed are not due to larger variations in the HA:CS PECNs shape.

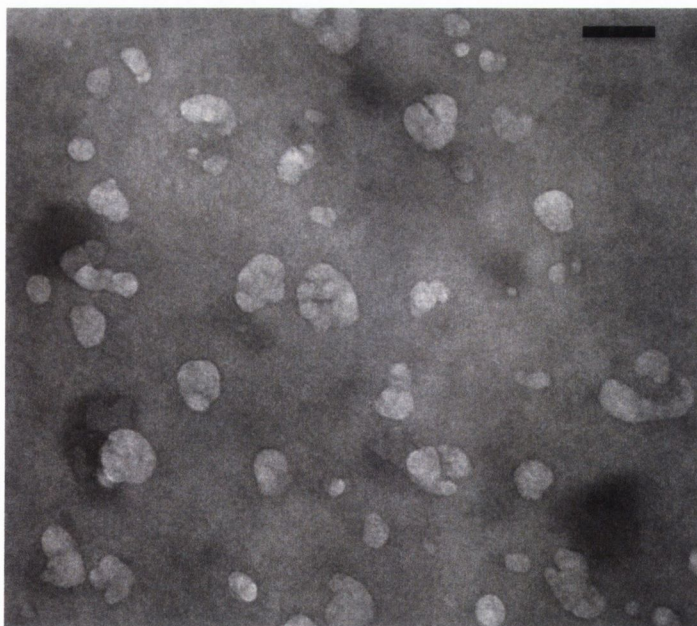


Figure 4.17 TEM image of positively charged KET 0.2 mg/mg HA:CS PECNs. Scale bar = 100nm.

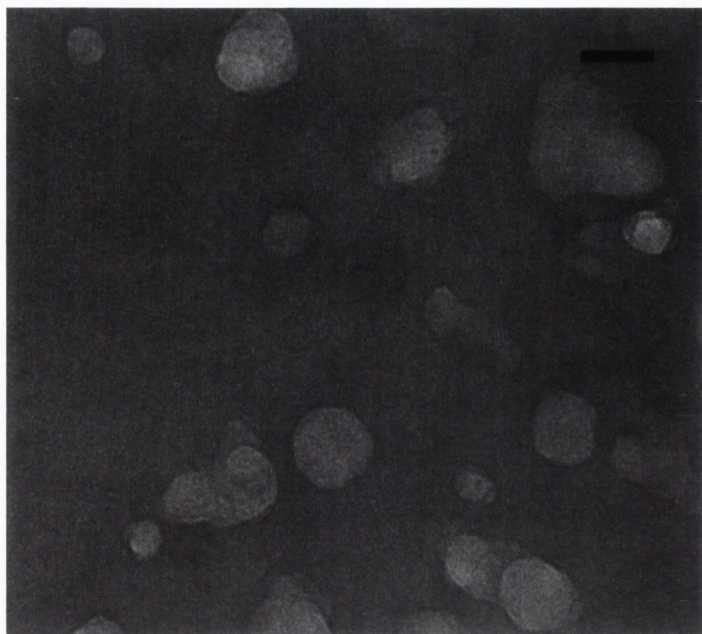


Figure 4.18 TEM image of negatively charged KET 0.2 mg/mg HA:CS PECNs. Scale bar = 100nm.

4.3.2.5 Release studies

Release studies were conducted using three different release media. The first was PBS buffer at pH 7.4 (**Figure 4.19**), the second release medium was 0.01 M HCL at pH 2, which was used to investigate at the effect of pH on the release from HA:CS PECNs (**Figure 4.20**), and the third was deionised water at pH 5.9, to observe if the ionic strength (in comparison to PBS) was affecting KET release (**Figure 4.21**). As the isoelectric point of positive KET loaded HA:CS PECNs was pH 6.8 (**Figure 4.13**) and the isoelectric point of negative KET loaded PECNs was pH 4.5 (**Figure 4.14**) it was expected that the HA:CS PECNs would behave differently depending on the pH and ionic strength of the release medium. PECNs have been reported to be very sensitive to the ionic strength of the medium in which they are dispersed (Schatz et al. 2004).

Addition of KET loaded HA:CS PECNs to PBS proved that these KET loaded HA:CS PECN systems are fast release systems with all samples reaching 100% release between 30-60 mins (**Figure 4.19**). The negatively charged particles displayed the same release pattern as the positively charged PECNs despite having a higher drug content. PBS has a higher ionic strength than water and was at pH 7.4. Positively charged KET loaded HA:CS PECNs were expected to

be more unstable in PBS than negatively charged based on their pI values. Evidence of this was not seen with both negatively and positively KET loaded HA:CS PECNs releasing their drug content rapidly. There were no important differences seen in the release profile for the negatively or positively charged PECNs in the different media showing that the media did not have a strong influence on these negatively and positively charged KET loaded HA:CS PECNs despite the difference in pH and ionic strength. The low concentration of particles present in the total volume of release medium (0.25 mg particles/ml of release medium) meant that the samples were less concentrated than the natively prepared dispersions and thus it is possible that the KET loaded HA:CS PECNs were not affected by medium as they were not present in high enough quantities to aggregate.

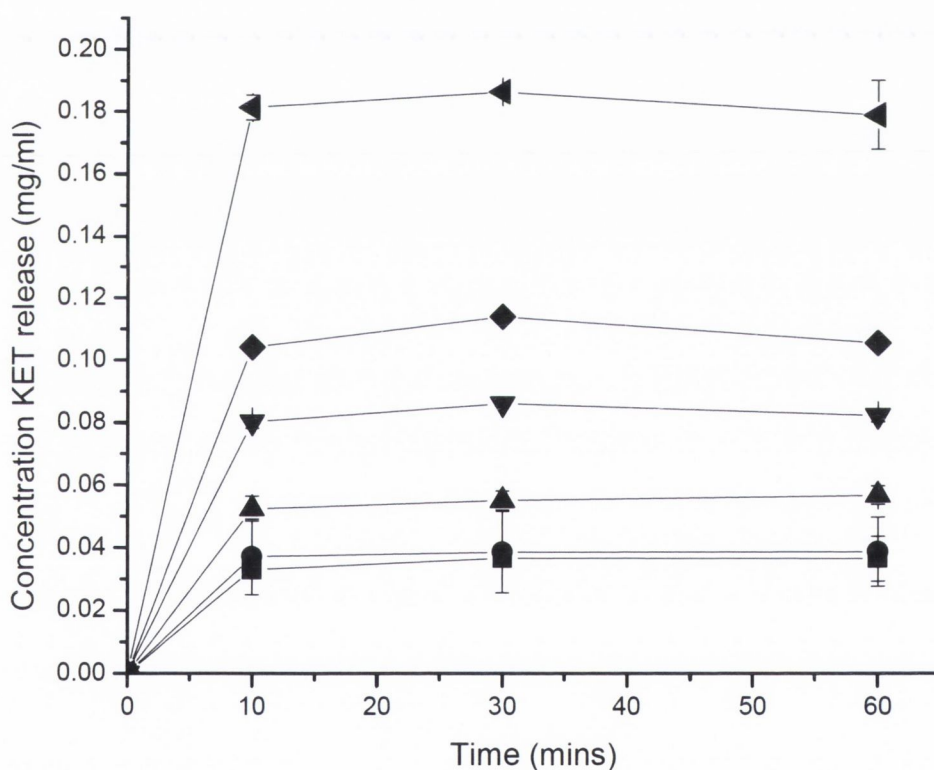


Figure 4.19 Release studies for KET loaded HA:CS PECNs in PBS. ■ = KET 0.1 mg/ml POS (positive) charge, ● = KET 0.2 mg/ml POS, ▲ = KET 0.4 mg/ml POS, ▼ = KET 0.1 mg/ml NEG (negative), ◆ = KET 0.2 mg/ml NEG, sideways triangle = KET 0.4 mg/ml NEG

The main difference observed for the release of KET from HA:CS PECNs in 0.01 M HCL was for negatively charged HA:CS PECNs at KET 0.4 mg/mg (**Figure 4.20**). While the release profiles for the positively charged HA:CS PECNs were not considerably different to those observed when release was in PBS, the release from negatively charged HA:CS PECNs at KET 0.4 mg/mg is much lower. This may be due to the pH of 0.01 M HCl being below the pKa of KET and thus when the DL was highest at KET 0.4 mg/mg the medium was saturated. The excess drug was not released as no precipitated KET crystals were observed in the release vessel.

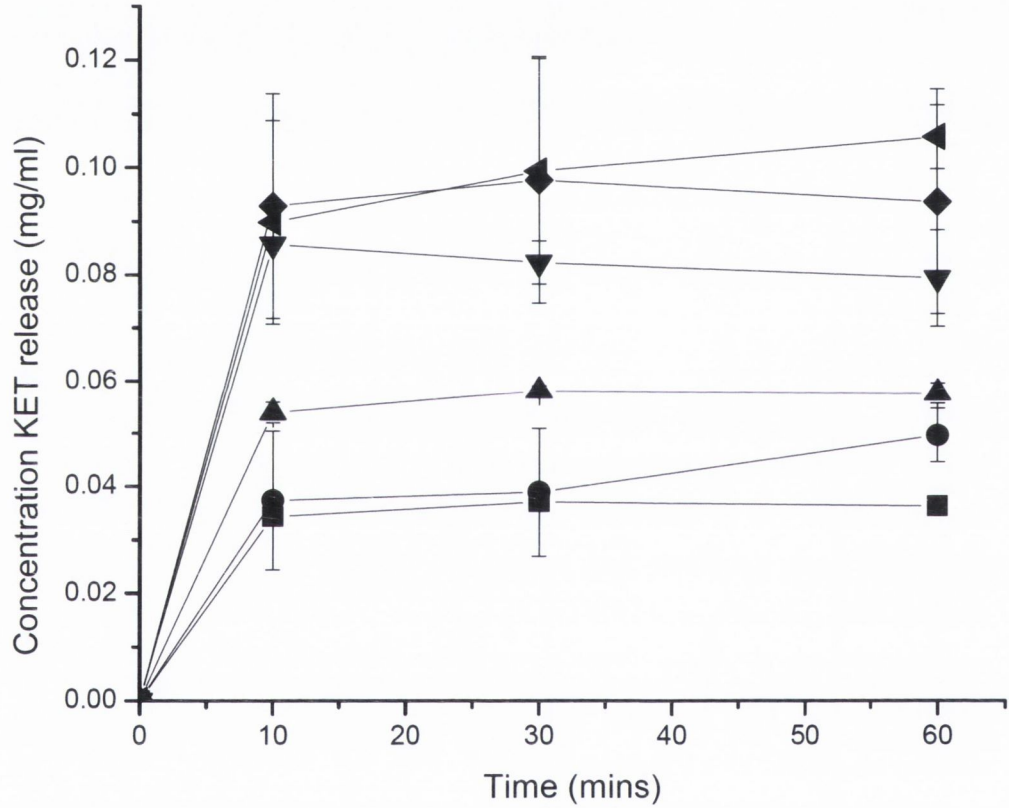


Figure 4.20 Release studies for KET loaded HA:CS PECNs in 0.01 M HCl. ■ = KET 0.1 mg/ml POS (positive) charge, ● = KET 0.2 mg/mg POS,▲ = KET 0.4 mg/ml POS, ▼ = KET 0.1 mg/ml NEG (negative), ◆ = KET 0.2 mg/ml NEG, sideways triangle = KET 0.4 mg/ml NEG.

Release in deionised water was almost identical to that observed in PBS buffer at pH 7.4 (**Figure 4.21**). Any differences can most likely be explained by

changes in pH. There may be influence due to the ionic strength allowing slightly greater KET release in water. If the ionic strength of PBS was playing a large role in the release then this would have caused noticeable differences in the release profiles and most likely changes between the positive and negatively charged PECNs when compared to water. Ions in the release medium would have preferred one charge of PECN and thus release for those charged particles would have been expected to be different to the release observed in deionised water.

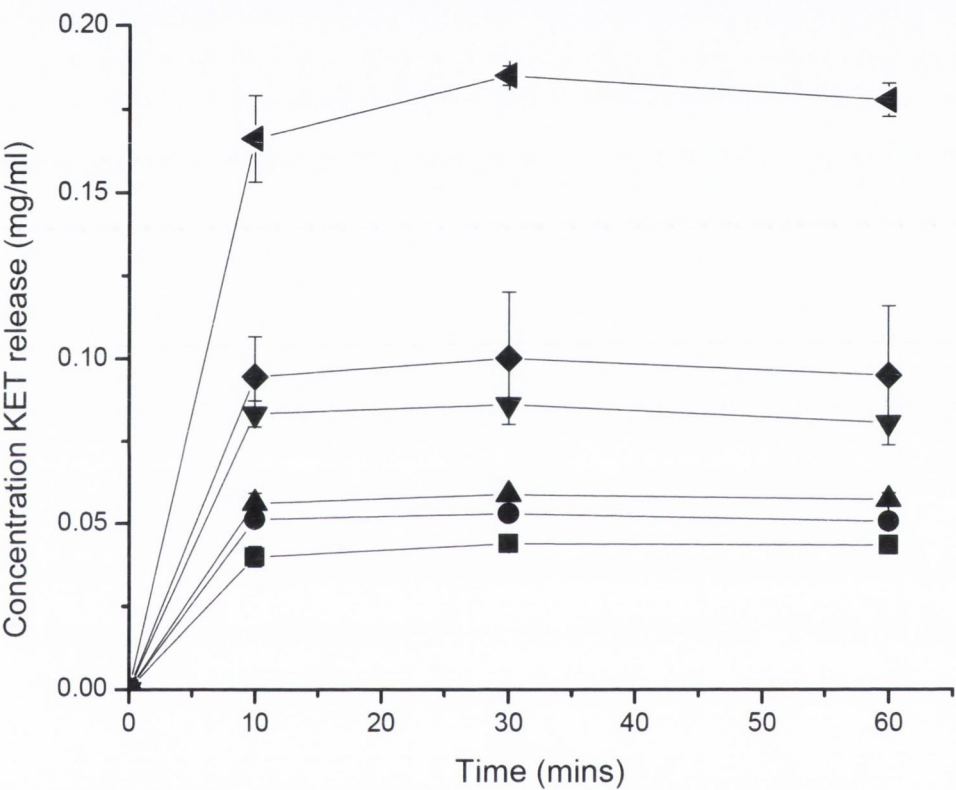


Figure 4.21 Release studies for KET loaded HA:CS PECNs in deionised Water.
■ = KET 0.1 mg/ml POS (positive) charge, ● = KET 0.2 mg/mg POS, ▲ = KET 0.4 mg/ml POS, ▼ = KET 0.1 mg/ml NEG (negative), ◆ = KET 0.2 mg/ml NEG, sideways triangle = KET 0.4 mg/ml NEG

4.3.3 Investigations into the solid state of the KET loaded HA:CS PECN

4.3.3.1 Introduction

KET may be present in different forms within HA:CS PECNs. It may form a complex with the CS polymer due to the electrostatic interaction, it may have dissolved and mixed with the polymers before precipitating in an amorphous state or it may have precipitated as nanocrystals stabilised by the polymers. Nanocrystals may be associated with the polymers or exist independently in the sample. KET may also be present in a combination of these forms within the same sample. The KET loaded HA:CS PECNs may also be freeze-dried for storage prior to redispersion in water for use and thus it is important to know the solid state of the drug and to question the association between KET and the polymers as the solid state may influence its behaviour on redispersion and storage. The solid state properties of the drug in a formulation may influence the drug stability and solubility and thus bioavailability in the body (Newman and Byrne 2003). The aim of these experiments was to investigate the solid state of KET in the PECNs.

4.3.3.2 PXRD and DSC Analysis

PXRD and DSC analysis were used as techniques to observe the behaviour of KET in HA:CS PECNs. Both, pre-wash and post-wash samples were investigated as pre-wash KET loaded HA:CS PECN samples had the all of the KET added still present in the sample with the HA and CS polymers while the post-wash KET loaded HA:CS PECNs had only tightly bound KET remaining as both free KET and loosely bound KET had been removed during the washing step.

In the pre-wash positively charged KET loaded HA:CS PECNs visible Bragg peaks of crystalline KET were absent in the PXRD analysis (**Figure 4.22**). It is possible that the absence of visible Bragg peaks in these pre-wash positively charged KET loaded HA:CS PECNs is due to the drug being amorphous or it

may be in a nanocrystalline state with the drug crystals too small to be detected by the PXRD analysis. PXRD analysis showed that the surfaces of positively charged post-wash KET loaded HA:CS PECNs were also amorphous or in a nanocrystalline state. The Bragg diffraction peaks associated with crystalline KET were not visible for this sample. No Bragg peaks were observed for any of the different concentrations of KET initially added during formation of the KET loaded HA:CS PECNs (0.1 mg/mg, 0.2 mg/mg or 0.4 mg/mg).

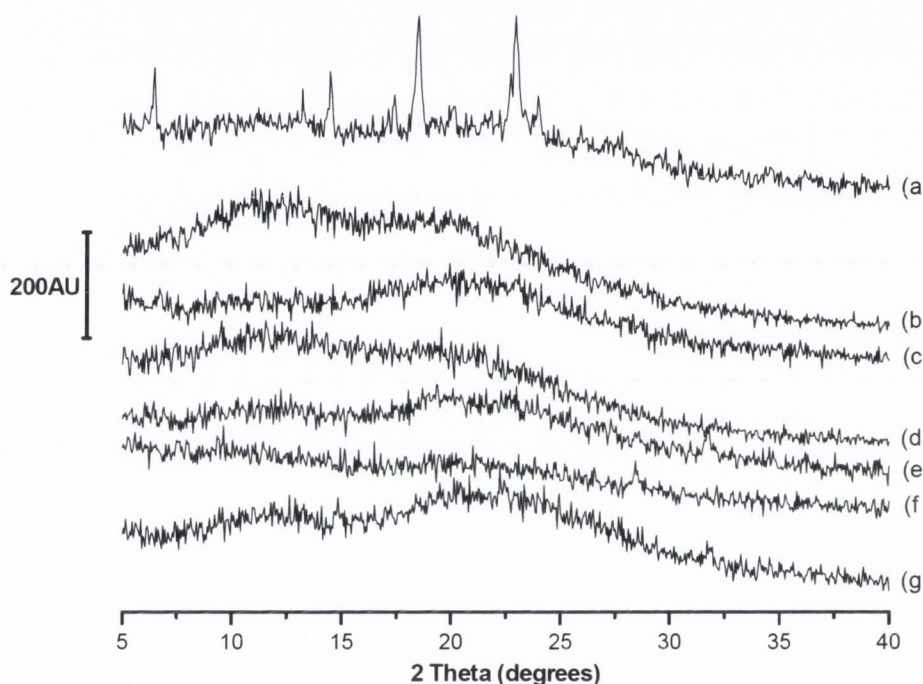


Figure 4.22 PXRD of HA:CS **KET** loaded PECNs **positively** charged and a polymer/drug physical mix. a) KET 0.2 mg/mg physical mix with HA and CS polymers, b) KET 0.4 mg/mg PECNs pre-wash c) KET 0.4 mg/mg PECNs post-wash, d) KET 0.2 mg/mg PECNs pre-wash, e) KET 0.2 mg/mg PECNs post-wash, f) KET 0.1 mg/mg PECNs pre-wash, g) KET 0.1 mg/mg PECNs post-wash.

Pre-wash negatively charged KET loaded HA:CS PECNs clearly showed peaks of crystalline KET for the sample at a concentration of 0.4 mg/mg with evidence of peaks in the result for 0.2 mg/mg KET loading (**Figure 4.23**). This showed that at 0.1 mg/mg KET was amorphous or nanocrystalline while KET had precipitated out in the form of larger crystals when it was loaded at higher concentrations.

This crystallised drug may have been stabilised by the polymers while the rest of the drug present would be expected to be in an amorphous state or complexed with the polymers. Any excess drug that is not tightly bound may have precipitated out as crystals post addition of the drug. This was different from the results obtained with the positively charged KET loaded HA:CS PECNs where no Bragg peaks were seen at any concentration. The difference between the KET loaded HA:CS PECNs was the weight ratio of HA:CS moving from 7.5:6.5 for positively charged to 10:4 for negatively charged PECNs. This showed that the polymer ratio was influencing the form of association between KET and the HA and CS polymers involved in KET loaded HA:CS PECN formation.

PXRD analysis showed that the surfaces of negatively charged post-wash KET loaded HA:CS PECNs were amorphous or in a nanocrystalline state as the Bragg peaks associated with crystalline KET were not visible for negatively charged post-wash KET loaded HA:CS PECNs as was the case for the positively charged post-wash KET loaded HA:CS PECNs.

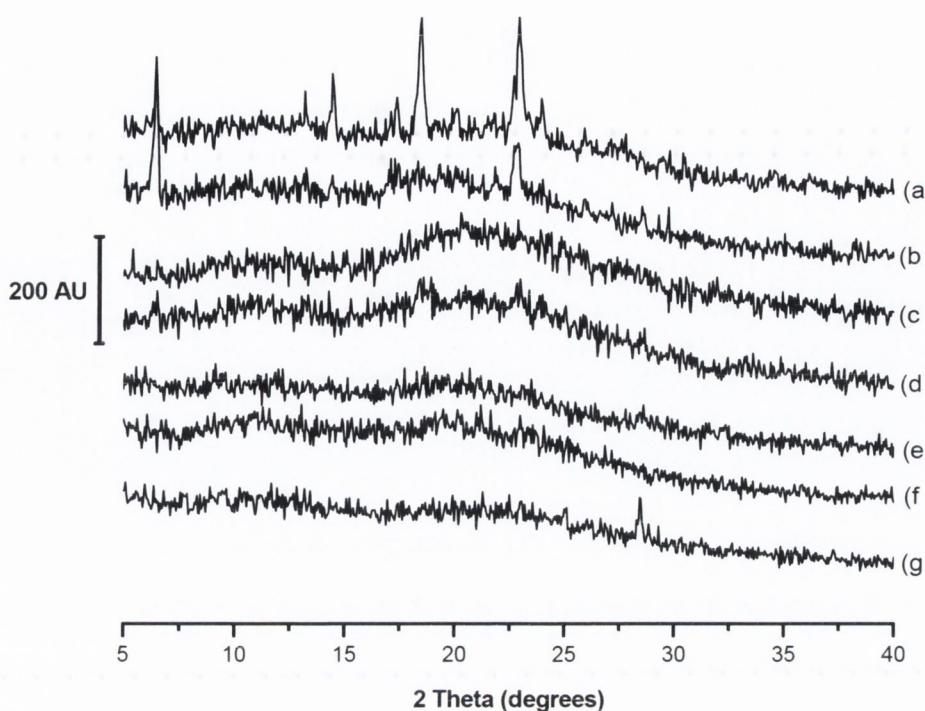


Figure 4.23 PXRD of HA:CS KET loaded PECNs **negatively** charged and a polymer/drug physical mix. a) KET 0.2 mg/mg physical mix with HA and CS polymers, b) KET 0.4 mg/mg PECNs pre-wash c) KET 0.4 mg/mg PECNs post-wash, d) KET 0.2 mg/mg PECNs pre-wash, e) KET 0.2 mg/mg PECNs post-wash, f) KET 0.1 mg/mg PECNs pre-wash, g) KET 0.1 mg/mg PECNs post-wash.

DSC was used to build upon the data from PXRD looking at the solid state of KET in the PECNs. Tests were again carried out both on samples pre-wash and post-wash for positive and negatively charged PECNs loaded with KET at all concentrations 0.1 mg/mg, 0.2 mg/mg, 0.4 mg/mg. KET has a melting point in the range 94°C – 97°C according to the BP. The melting point of KET starting material was experimentally determined to be 94.77 ± 0.26 °C with an enthalpy of fusion 33.43 ± 4.62 J/g. The melting point of KET in the physical mixes with the polymers HA and CS was at 93.39 ± 0.05 °C with an enthalpy of fusion at 33.94 ± 3.88 J/g (**Figure 4.24**).

In pre-wash positively charged KET loaded HA:CS PECNs there was a melting peak present at 90.19 ± 1.11 °C with an enthalpy of fusion 1.34 J/g for the KET concentration of 0.4 mg/mg only and at the KET loaded concentration of 0.2

mg/mg pre-wash the melting point was 90.65 ± 1.75 °C and the enthalpy of fusion was 0.06 J/g. Samples with the lower drug concentration of 0.1 mg/mg did not show melting peaks (**Figure 4.24**). This indicates that at the higher concentrations (0.4 mg/mg and 0.2 mg/mg) KET had precipitated out in crystal form but since these crystals were not detectable by PXRD the drug crystals may have been in a nanocrystalline state or have crystallised during the heating of the sample by DSC. The crystallisation process may not have been visible due to some other, overlapping thermal events.

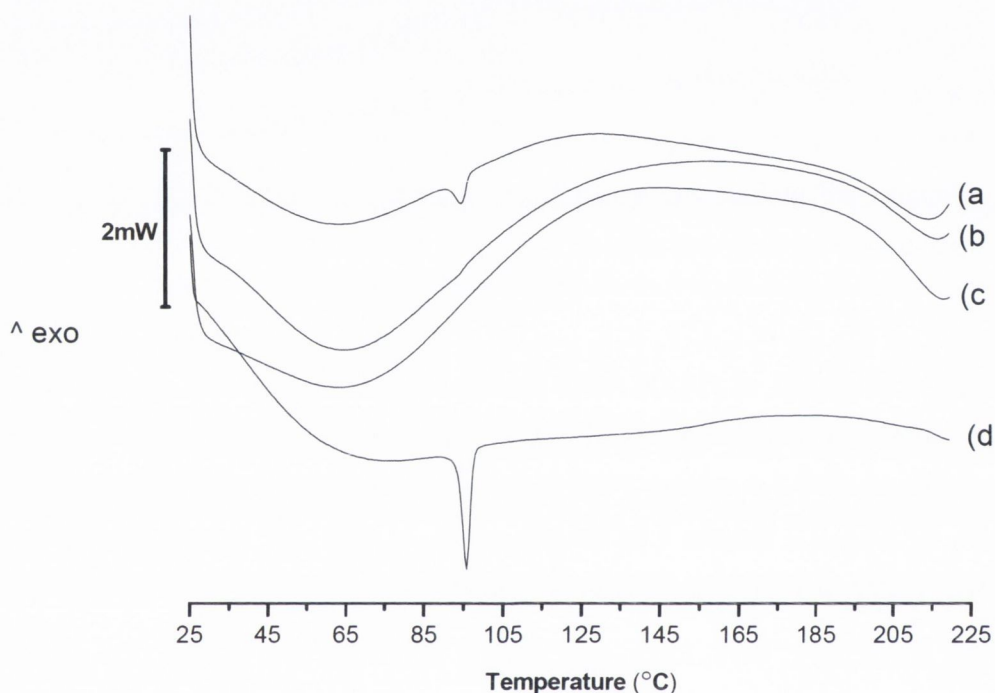


Figure 4.24 DSC of HA-CS KET physical mix and positively charged PECNs pre-wash loaded with increasing concentrations of KET. a) 0.4 mg/mg KET loading, b) 0.2 mg/mg KET loading, c) 0.1 mg/mg KET loading, d) 0.2 mg/mg KET and HA:CS PECNs physical mix.

No visible drug melting peak was seen in the thermograms of positively charged KET loaded HA:CS PECNs post-wash which was in agreement with the PXRD results for post-wash KET loaded in positively charged PECNs (**Figure 4.22, 4.25**). This suggests that KET may form a complex with the polymers and not crystallise, it may be amorphous or that the magnitude of the event is too small to measure.

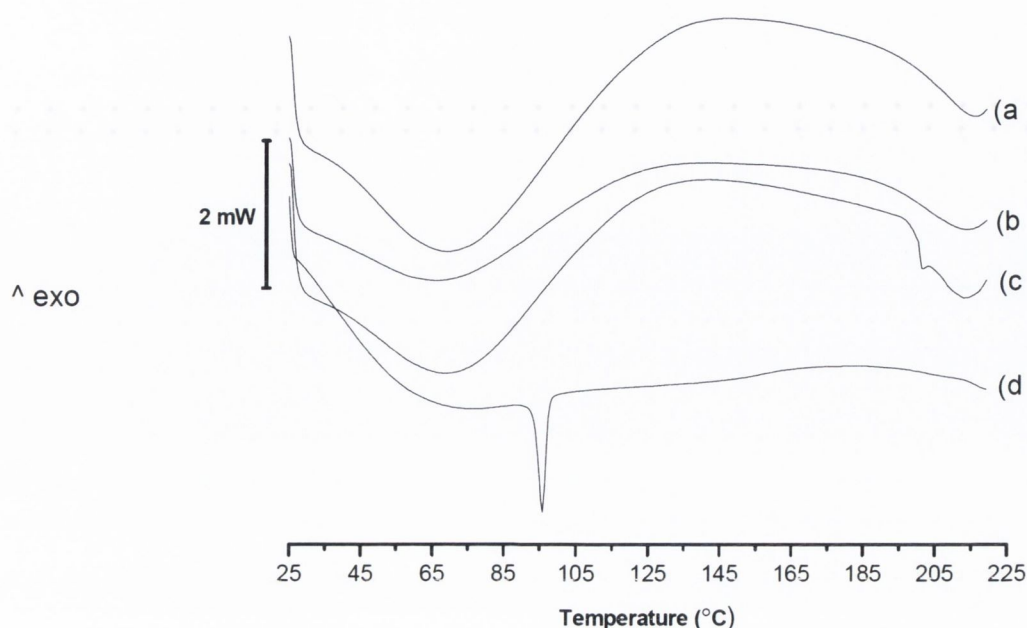


Figure 4.25 DSC of HA-CS KET physical mix and positively charged PECNs post-wash loaded with increasing concentrations of KET. a) 0.4 mg/mg KET loading, b) 0.2 mg/mg KET loading, c) 0.1 mg/mg KET loading, d) 0.2 mg/mg KET and HA:CS physical mix

When the negatively charged KET loaded PECN samples pre-wash were subjected to DSC analysis, no melting endotherm was seen at a concentration of 0.1 mg/mg. There was a melting peak visible for the 0.2 mg/mg concentration as well as for the 0.4 mg/mg, thus confirming the partially crystalline nature of these systems as seen by PXRD (**Figure 4.26**). For 0.4 mg/mg the melting point was 89.37 ± 0.21 °C and the enthalpy of fusion was 2.72 J/g, for KET 0.2 mg/mg the melting point was 87.04 ± 1.08 °C and the enthalpy of fusion was 1.69 J/g.

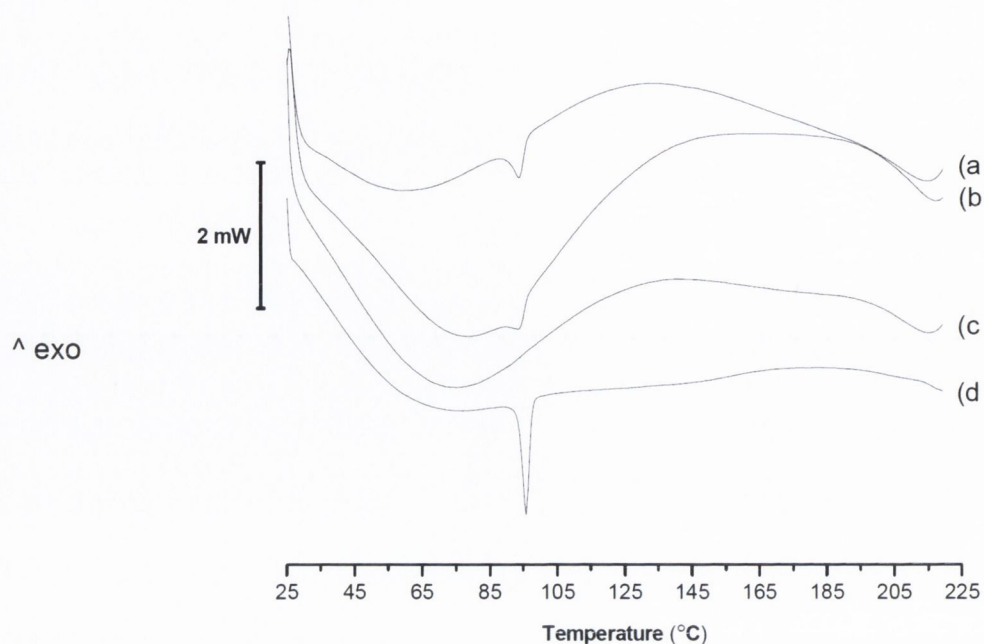


Figure 4.26 DSC of HA-CS KET physical mix and negatively charged PECNs pre-wash loaded with increasing concentrations of KET. a) 0.4 mg/mg KET loading b) 0.2 mg/mg KET loading, c) 0.1 mg/mg KET loading, d) 0.2 mg/mg KET and HA:CS physical mix.

No visible drug melting peak was seen in thermograms of negatively charged KET loaded PECNs post-wash, which was in agreement with the PXRD results for post-wash KET loaded in negatively charged PECNs (**Figures 4.23 and 4.27**).

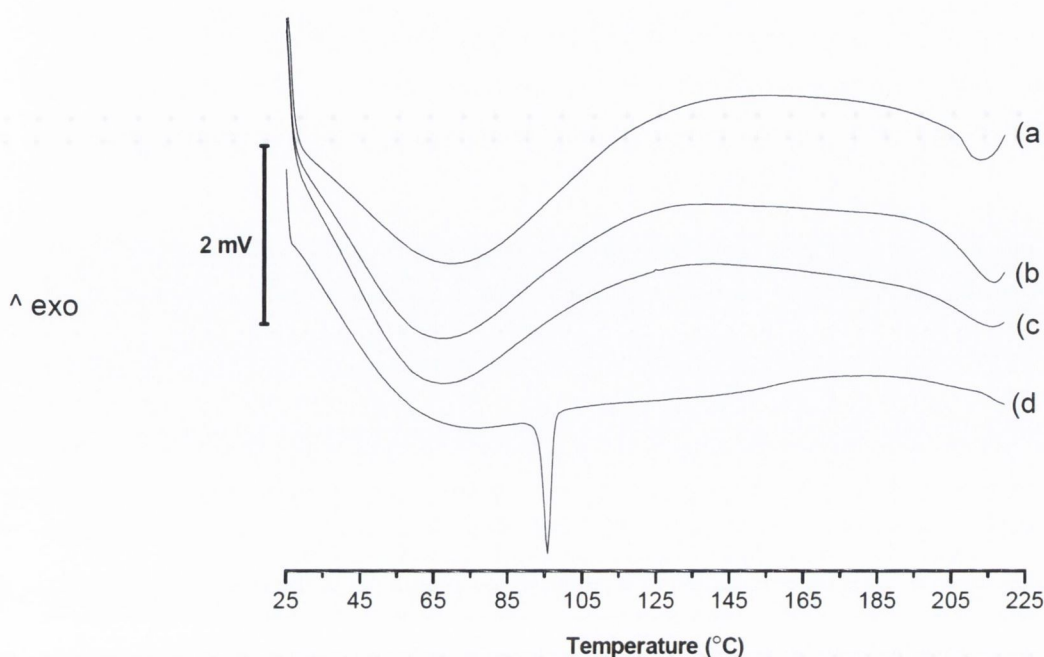


Figure 4.27 DSC of HA-CS KET physical mix and negatively charged PECNs post-wash loaded with increasing concentrations of KET. a) 0.4 mg/mg KET loading, b) 0.2 mg/mg KET loading, c) 0.1 mg/mg KET loading, d) 0.2 mg/mg KET and HA:CS physical mix.

The washing step, according to the results, appears to remove free KET and loosely bound KET so that only the more tightly bound drug remains, in doing so the drug crystals are removed. The melting point of KET pre-wash was lowered by the presence of the polymers in the HA:CS PECNs indicating that there was an interaction with the polymers in the PECNs. The KET melting point in the physical mix with the polymers was 94.77 ± 0.26 °C. For KET positively charged HA:CS PECNs the melting point depression here was not significantly different for 0.2mg/mg KET (90.65 ± 1.75 °C) and 0.4 mg/mg KET (90.19 ± 1.11 °C). Although these crystals were removed by the washing step the crystals still displayed some interaction with the polymer, which is evident by the lowered melting point.

The negatively charged KET loaded HA:CS PECNs had higher DL and showed a greater melting point depression than the positively charged KET loaded HA:CS PECNs. The melting point depression was greater for the lower

concentration KET 0.2 mg/mg (87.04 ± 1.08 °C) than the higher concentration KET 0.4 mg/mg (89.37 ± 0.21 °C). This highlights that the interaction between the drug and polymers in the negatively charged system may be stronger than in positively charged HA:CS PECNs given its higher DL and larger depression of the melting point (Chiu and Prenner 2011). These crystals were removed by the washing step which suggests that the drug crystals have a weak interaction with the HA:CS PECNs. If the drug was present but not interacting with the polymers at all there would not be a decrease in the melting point of the drug when the sample was tested by DSC as was seen for the KET and polymer physical mix.

4.3.3.3 Miscibility of KET in HA and CS Polymers

Drug miscibility with the polymers indicates how well the drug and polymers will mix when in a liquid (amorphous) state. The hypothesis was that if the drug has higher miscibility in the polymer it is more likely to precipitate dispersed in the polymers in an amorphous form than to crystallise out. DSC could have experimentally determined the miscibility but this would have required heating the polymers to the point where they would be unstable. CS undergoes thermal degradation in the temperature range 200 – 400 °C (de Britto and Campana-Filho 2007) while HA degrades at 276 °C (Villette et al. 2002). Also, the T_g of KET is low 41.7 ± 2.0 °C so it is difficult to detect (Blasi et al. 2006). An estimation of KET miscibility with the polymers was therefore assessed by calculating the Hildebrand solubility parameters. This parameter indicates the degree of interaction between materials and is particularly useful for polymers. Two substances that have similar parameter values are likely to be miscible. If the difference in the Hildebrand solubility parameter (δ) < 7 MPa^{1/2} the polymer and drug were found to be miscible and if the $\delta > 10$ MPa^{1/2} the polymer and drug were likely to be immiscible. (Burke 1984; Greenhalgh et al. 1999) (**Table 4.13**). Miscibility in this case relates to solubility of amorphous KET in the amorphous dry polymers HA and CS. To support the estimated miscibility, optical microscopy was used to see how many phases were present and if any crystals were formed. If the drug is soluble just a single, uniform phase was observed under the optical microscope.

$$\delta = \sqrt{\frac{\Delta H_v - RT}{V_m}} \text{ (Eq 4.2)}$$

Equation 4.2 Hildebrand Equation to determine miscibility of KET with the polymers HA and CS by estimating the Hildebrand solubility parameter. The parameter is calculated by the square root of the cohesive energy density, which is equal to the heat of vaporization (ΔH_v) minus gas constant (R) times the temperature (T in Kelvin) divided by molar volume V_m .

Table 4.13 Estimates of Hildebrand solubility parameters (δ) for each of the components used to prepare the HA:CS PECNs.

Constituent	δ (MPa ^{1/2})	$\Delta\delta$ (drug-polymer)
HA	19.8	0.4
CS	20.0	0.2
KET	20.2	-

The results showed that KET should have high affinity for the polymers, HA and CS, as the difference in solubility parameters between KET and these polymers is low; 0.4 for KET and HA and 0.2 for KET and CS (**Table 4.13**). This indicates good miscibility between the components.

The optical microscope was used as a tool to investigate the drug miscibility with the polymers and give a visual representation of the combination (**Figures 4.28 – 4.34**). The number of phases apparent on drying can indicate whether the drug had mixed with the polymers or existed as a separate phase.

There were differences in the behaviour of the individual solutions of the polymers on drying (**Figures 4.28 and 4.29**). Additionally, these individual solutions dropped onto separate glass slides dried very differently to the unloaded HA:CS PECNs. While HA solution dried as one even phase, a fractal pattern was seen in the drying of CS solution which disappeared when the combination was dried as HA:CS PECNs (**Figure 4.30**) probably due to the interaction between the polymers preventing the formation of this fractal pattern (**Figure 4.28 - 4.34**). Such fractal growth pattern on drying of CS had previously reported in the in the literature, for instance Hu et al. (2007)

When KET dissolved in ethanol (1 mg/89.3 μ l) was added to 14 mls water without the presence of HA and CS polymer solutions or when KET was added in ethanol to a dispersion of HA:CS PECNs even at the highest concentration 0.4 mg/ml only one phase was seen. This supported the data from previous miscibility estimations that KET can mix with the polymers in one phase.

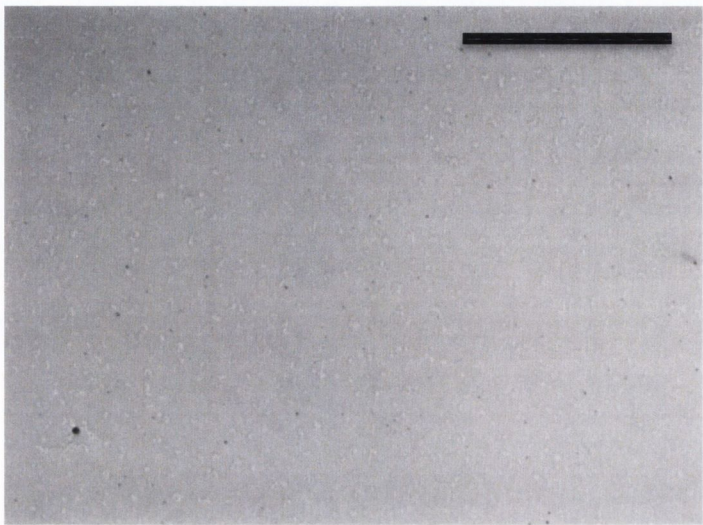


Figure 4.28 HA solution (0.1% w/v) air dried on a glass slide and seen by optical microscopy. Scale bar = 20 μ m.

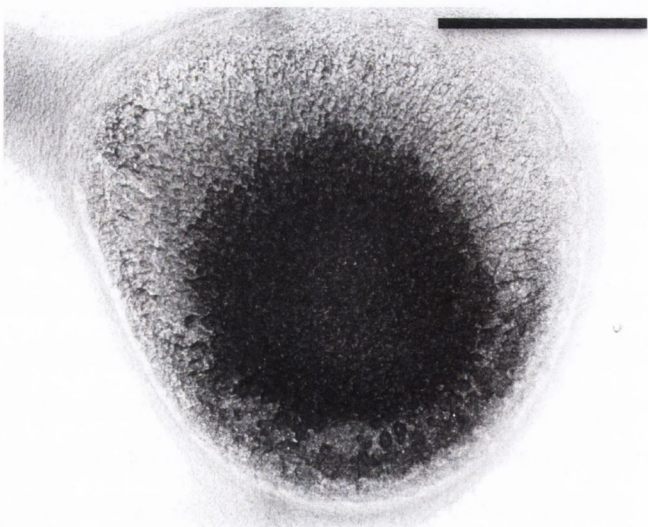


Figure 4.29 CS solution (0.1% w/v) air dried on a glass slide and seen by optical microscopy. Scale bar = 20 μ m.

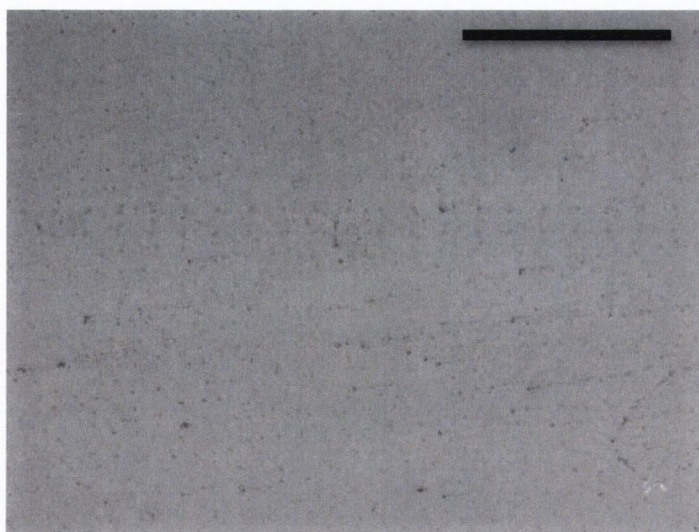


Figure 4.30 0.1% w/v HA:CS PECNs air dried on a glass slide. Scale bar = 20 μm .



Figure 4.31 0.2 mg/ml KET in ethanol solution (1 mg/89.3 μl) added to 14 mls of water and air dried on a glass slide. Scale bar = 20 μm .

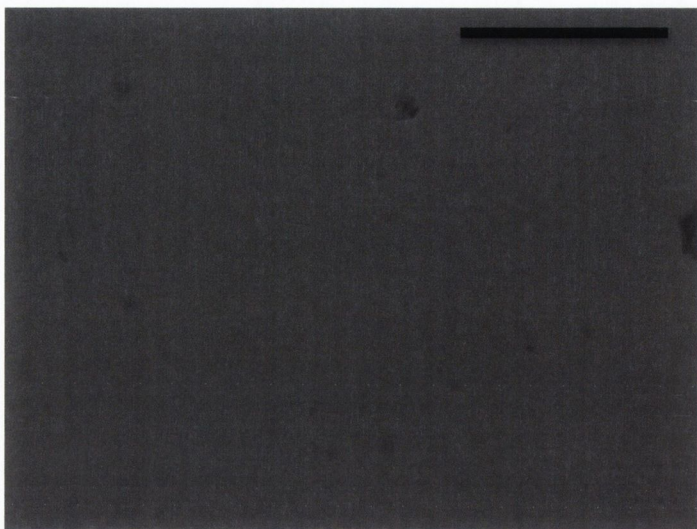


Figure 4.32 0.2 mg/ml KET loaded 7.5:6.5 HA:CS PECNs air dried on a glass slide. Scale bar = 20 μm .

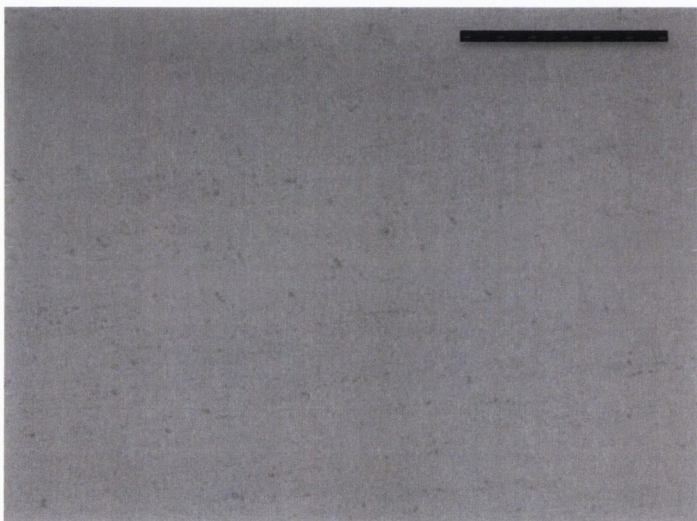


Figure 4.33 0.4 mg/ml KET in ethanol solution (1 mg/89.3 μl) added to 14 mls of water and air dried on a glass slide. Scale bar = 20 μm .

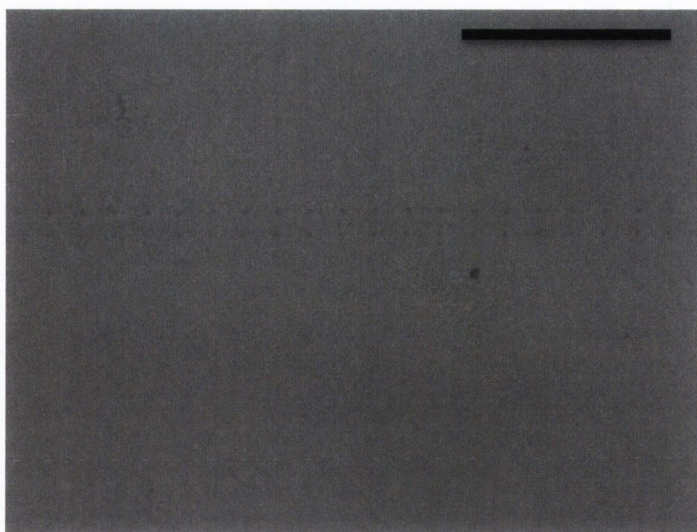


Figure 4.34 0.4 mg/ml KET loaded 7.5:6.5 HA:CS PECNs dried on a glass slide. Scale bar = 20 μm .

4.4 Determination of KET-loaded PECN Structure and the Solid-State Nature of KET in the HA:CS PECNs

A number of factors determine the quantity and strength of drug binding in the HA:CS PECNs including the strength and location of ionic sites, polymer chain rigidity, component chemistries, pH, temperature, ionic strength, mixing intensity (Hartig et al. 2007). As multiple factors influence the structure of these PECNs different steps of the formulation process were broken down to look more closely at their influence on the final formulation. These factors for the KET loaded HA:CS PECNs were DL, polymer ratio (which determined both weight ratio and charge ratio), solubility of KET in polymer solution and miscibility of KET in polymers.

DL was higher for KET loaded negatively charged HA:CS PECNs (10:4) than for positively charged KET loaded HA:CS PECNs (7.5:6.5) (**Table 4.9 and 4.10**). This means that pre-wash both positively and negatively charged PECNs displayed the same DL but post-wash they had different amounts of drug present when they were being tested by DSC and PXRD.

Table 4.14 Comparison of key physicochemical properties of KET loaded HA:CS PECNs (positively charged – 7.5:6.5 and negatively charged – 10:4).

HA:CS weight ratio	Theoretical KET loading (pre wash) (mg/mg NPs)	Actual KET loading (post wash) (mg/mg NPs)	Quantity of KET by DSC (pre-wash) (mg/mg NPs)	Quantity of KET by DSC (post-wash) (mg/mg NPs)	% saturation of + charge (pre-wash)	% saturation of + charge (post-wash)	Quantity of KET released at 60 min (mg/mg NPs)
7.5:6.5	0.1	0.038	0	0	67.3	59.1	0.037 ± 0.001
7.5:6.5	0.2	0.053	0.002	0	81.9	56.8	0.039 ± 0.015
7.5:6.5	0.4	0.059	0.040	0	111.2	58.2	0.057 ± 0.032
10:4	0.1	0.086	0	0	137.9	133.6	0.086 ± 0.001
10:4	0.2	0.114	0.050	0	161.7	142.4	0.114 ± 0.001
10:4	0.4	0.187	0.081	0	209.3	151.2	0.186 ± 0.001

Looking at the pre and post wash PXRD and DSC traces for each HA:CS PECN sample it was possible to elucidate how the drug may be associated with the HA:CS PECNs. No KET melting endotherms were seen by DSC analysis for the positively charged HA:CS PECNs (7.5:6.5) post-wash at the measured KET concentrations but pre-wash melting peaks were observed at 0.4 mg/mg and 0.2 mg/mg. The post-wash drug content for positively charged KET loaded HA:CS PECNs at 0.4 mg/mg addition was 0.059 mg/ml and the solubility of KET in the HA:CS 0.1% w/v PECNs was measured as 0.13 ± 0.01 mg/ml. The quantity of KET loaded (post-wash) was below the solubility of KET in the polymer solutions while the KET content was above the solubility in the pre-wash samples with a theoretical DL of 0.4 mg/ml. The KET solubility value 0.13 ± 0.01 mg/ml in the positively charged 0.1 % w/v HA:CS PECN dispersion means that for theoretical KET loading of 0.1 mg/ml, the entire KET could dissolve completely, but for the higher theoretical KET loadings of 0.2 mg/mg and 0.4 mg/mg, KET is used in excess of the drugs solubility. At 0.2 mg/mg the KET is above the solubility limit by 0.7 mg/ml but for 0.4 mg/mg it is above by 2.7 mg/ml. This meant that KET may have precipitated as crystals for the systems with theoretical DL of 0.4 mg/mg and 0.2 mg/mg as seen in the melting peak observed by DSC (**Figure 4.25**) and the Bragg peaks seen using PXRD (**Figure 4.23**) pre-wash for these samples. If the tightly associated drug precipitated in an amorphous state during formation it is possible that the rest of the KET not loaded post wash or present as crystals pre wash had dissolved in the solution and not been incorporated into HA:CS PECNs.

The amount of drug present as crystals, quantified using DSC, for 0.4mg/mg KET was 0.040 mg/mg and for 0.2 mg/mg KET it was 0.002 mg/mg indicating that only a small amount of the drug loaded had precipitated out as crystals (**Table 4.14**). Given that these crystals were not present post washing it is assumed that the crystals were loosely associated with the HA:CS PECNs.

The polymer ratio can be described in two ways: in terms of the weight ratio between the polymers and in terms of the charge ratio between the number of charges on the polymers; both of these are recorded in **Table 4.12**. These ratios are important in the formation of the KET loaded HA:CS PECNs. For the pre-wash positively charged KET loaded HA:CS PECNs at the 0.4 mg/mg KET concentration it was calculated that according to the charge ratio the negative charges from HA and KET combined would have saturated the positive CS charges by 111.2% and for KET concentration 0.2 mg/mg the positive CS

charges were saturated by the negative charges from HA and KET at 81.9% in the case of complete HA and KET binding. Since in reality this was not observed it can be concluded that complete HA and KET binding did not occur. The KET loaded HA:CS PECNs all had a positive ZP, therefore a positively charged surface which indicates that the KET and HA had not bound completely to the CS to the point of saturating the positive charge or neutralising it. At a KET concentration pre-wash of 0.1 mg/mg the charge ratio was 67.3% and here no precipitation was seen. This indicates that KET loaded in the HA:CS PECNs in the amorphous form occurs when the positive charge is still in excess in the system. No precipitation was seen for charge ratio values below 67.3% and post wash the HA and KET saturation of CS based on the actual drug loaded for KET 0.4 mg/mg and KET 0.2 mg/mg dropped to 58.2% and 56.8%, respectively. Only a minimal decrease was seen for KET 0.1 mg/mg to 59.1%. It seems from the data presented that as the charge ratio approached 1 the system began to become less able to bind the KET molecules as one polymer was required in excess to stabilise the HA:CS PECNs.

Conducting a similar investigation for negatively charged KET loaded HA:CS PECNs, KET had very similar solubility of 0.14 ± 0.01 mg/ml in the negatively charged HA:CS PECN dispersion. Despite the closeness of KET solubility in positive or negative HA:CS PECN dispersions, the negatively charged KET PECNs had higher actual DL than positively charged KET-loaded PECNs (**Table 4.14**).

It is hypothesised that for the positively charged HA:CS PECNs as the KET concentration added increased from 0.1 mg/mg to 0.4 mg/mg the negative charges increase which would affect the overall balance of charge between positive and negative charges in the positive HA:CS PECN system. For positively charged KET loaded HA:CS PECNs the positive charge is required to be in excess to physically stabilise the system from collapse due to Brownian motion.

The pattern seen for charge ratios for negatively charged KET loaded HA:CS PECNs had the negative charge in excess. These HA:CS PECNs had higher DLs post wash than the positively charged PECNs despite the same pre wash loading. Looking at the charge ratios the pre-wash saturation values were further from 1.0 than the post-wash values and the drop between the saturation values

pre and post wash was smaller than the drops seen for positively charged HA:CS PECNs at KET 0.4 mg/mg and KET 0.2 mg/mg. Here precipitation was seen pre wash for KET 0.4 mg/mg which dropped in charge ratio from 209.3 to 151.2 and for 0.2 mg/mg charge ratio changed from 161.7 to 142.4 and precipitation was not seen for 0.1 mg/mg which dropped minimally from 137.9 to 133.6 post-wash. No crystalline peaks were observed post wash. Both positively charged and negatively charged HA:CS systems appeared to be most stable when the charge in excess was close to 50% higher than the opposite charge.

DSC and PXRD data was used to estimate how much of the drug was incorporated into the KET loaded HA:CS PECNs complexed as crystals associated with the PECNs. Post-wash the negatively charged KET loaded HA:CS PECNs had no crystalline drug present so the crystals may have disassociated during the washing step. Pre-wash crystals of KET were present for higher loading concentrations of 0.2 mg/mg and 0.4 mg/mg for the negatively charged KET loaded HA:CS PECNs. The amount of drug present as crystals, quantified using DSC, for 0.4mg/mg KET was 0.081 mg/mg and for 0.2 mg/mg KET it was 0.050 mg/mg. At 0.1 mg/mg pre-wash there were no KET crystals detected so the drug may not have precipitated out at this concentration due to its solubility in the medium. The quantity of crystalline drug present pre-wash was higher for the negatively charged KET loaded HA:CS PECNs than for the positively charged. KET loaded HA:CS PECNs. Post wash the amount of KET loaded for negatively charged HA:CS PECNs was higher than for positively charged HA:CS PECNs post wash. The post-wash drug content for negatively charged KET loaded HA:CS PECNs at 0.4 mg/mg addition was 0.187 mg/ml, at 0.2 m/mg addition was 0.114 mg/ml, and at 0.1 mg/mg was 0.086 mg/ml. All of these DL values were below the solubility of KET except at 0.4 mg/mg. This could mean that the 0.4 mg/mg formulation was supersaturated with KET or that there was a small amount of drug crystals present that were not detected. More drug may have associated with the negative KET loaded HA:CS PECNs due to the higher pH value of the dispersion resulting in a greater amount of KET being ionised in the system for incorporation. It is also possible that hydrophobic interactions played a part in the stabilisation of these systems between KET and the HA chains. Similar interactions were seen for Luoa et al. (2000) who reported that HA in aqueous solution forms a random coil – coil structure with hydrophilic and hydrophobic strands. They found a linear relationship between the hydrophobicity of drug molecule and its release rate from their HA hydrogel films

with the more hydrophobic drugs being released slower due to hydrophobic interactions with HA. These interactions could have been responsible for the higher DL seen for the negative KET loaded HA:CS systems. Post-washing the crystals were removed so the drug present was complexed with the HA:CS system strongly enough to remain bound through the centrifugation process. The remaining drug post-wash may have been mixed with the polymers and have precipitated with them in an amorphous form or it may have been present as a salt complex with CS.

The release studies showed that complete release occurred for the negatively charged HA:CS PECNs. For positively charged KET loaded HA:CS PECNs complete release was not seen at all concentrations which may have been due to drug being tightly complexed and non-dissociable from the excess CS molecules.

4.5 Conclusions

HA:CS PECNs may be formed with a positive charge at a weight ratio of 7.5:6.5 or with a negative charge at a weight ratio of 10:4. The charge did not have a large influence on HA:CS PECN size with both negative and positive PECNs having sizes within a narrow size range of 165 – 173 nm. The ZP of these HA:CS PECNs showed that both were stable despite being oppositely charged +33.2 and -29.4 mV.

Different parameters that may influence the formation of these HA:CS PECNs were investigated: polymer weight ratio, pH of HA, solvent choice and it was concluded that the optimal polymer weight ratio for positively charged HA:CS PECNs was 7.5:6.5 and that the optimal polymer weight ratio for negatively charged HA:CS PECNs was 10:4. No pH adjustment of HA solution was implemented as altering the pH did not show enough benefits for the labour involved in altering the pH and ethanol was chosen as the solvent of choice as it had low toxicity and is miscible with water. The positively charged HA:CS PECNs showed charged neutralisation of pH 7 and the negatively charged had an isoelectric point at pH 3.2 meaning that at these pH values the HA:CS PECNs are expected to be least stable.

The empty HA:CS PECNs showed extended stability over a 14 day period with the HA:CS PECNs remaining intact for the duration of the study but some decrease in the particle size for positively charged HA:CS PECNs was observed.

HA:CS PECNs were successfully loaded with KET both as positive and negative HA:CS PECNs. As with the empty carriers the parameters involved in their formation were investigated; polymer weight ratio, pH of HA, solvent choice. These investigations resulted in the conclusion that for KET loaded HA:CS PECNs 7.5:6.5 was the optimal weight ratio for positively charged particles and 10:4 was the optimal weight ratio for negatively charged particles. Ethanol was again the most suitable solvent and the pH of HA did not show advantages when altered from pH 5.7.

Three different concentrations of drug were tested for loading. These were 0.1 mg/mg, 0.2 mg/m and 0.4 mg/mg. The DL at each concentration was higher for negatively charged KET loaded HA:CS PECNs than for positively charged KET loaded HA:CS PECNs possibly due to hydrophobic interactions occurring between KET and the HA chains. This was concluded because if the loading was based on electrostatic interactions then it would have been expected that the positively charged HA:CS PECNs would have higher loading because KET is negatively charged. There was no linear relationship seen between the amount of drug loaded and the amount of drug added suggesting that the HA:CS PECN systems have only a limited capacity to bind drug. This may have been due to the charge ratios of the HA:CS PECN systems as stability was only seen within a narrow range of charge ratio values. KET loaded HA:CS PECNs formed best when the ratio of NEG:POS charges was 2:1 or 1:2 with the least chance of stable HA:CS PECN formation at a polymer ratio of 1.

The presence of drug altered the charge ratio of the PECNs from that seen for the empty HA:CS carriers. The isoelectric point for positively charged HA:CS PECNs was pH 6.8 while that for negatively charged PECNs was pH 4.5. This difference reflects the addition of drug to the systems.

These systems remained stable for a 14 day but a decrease in the particle size was observed for positively charged KET loaded HA:CS PECNs but there were no significant changes in the PDI.

Rapid drug release was observed from these systems with all the KET being released within 60 mins. This was the case regardless of the charge or the concentration of drug loaded. The release medium did not have a major impact on the release pattern despite having different pH values and ionic strength.

The solid state properties of KET were investigated in the HA:CS PECNs to learn about how it was present in the HA:CS PECNs. Pre-wash some loosely bound drug was present as drug crystals which were observed as a melting point during DSC analysis for 0.4mg/mg and 0.2 mg/mg KET concentration for both positively and negatively charged KET loaded HA:CS PECNs. This drug may have been loosely bound as crystals or nanocrystals while the remaining drug was assumed to have formed a complex with the polymers or to be in an amorphous state. The reason it is estimated that the drug was present as nanocrystals as opposed to crystals was due to the lack of Bragg peaks when tested by PXRD. While some Bragg peaks were seen pre-washing at 0.4 mg/mg and 0.2 mg/mg for negatively charged KET loaded HA:CS PECNs (which had a higher drug content) they were not seen for positively charged KET loaded HA:CS PECNs. Post washing the remaining drug was either amorphous or in a complex with the polymers as no melting peaks were observed using DSC and there were no diffraction peaks present on PXRD.

KET showed good miscibility with the polymers as only one phase was seen when the polymers and KET were mixed in solution and allowed to dry. This may support the hypothesis that the drug would precipitate with the polymers in HA:CS formation in an amorphous state or as a complex.

**The Optimisation and
Characterisation of Indometacin
Loaded Hyaluronic Acid:Chitosan
Polyelectrolyte Nanoparticles**

Chapter 5

5.1 Introduction

This chapter focuses on the preparation of HA:CS polyelectrolyte nanoparticle complexes (PECNs) loaded with IND, a hydrophobic (BCS class II) NSAID. The previous chapter detailed the formation of HA:CS PECNs and loading of these systems with KET, which is another hydrophobic, NSAID. IND is less soluble than KET in water (Bodmeier and Chen 1989) and has more complex solid-state properties partially due to its polymorphism (Surwase et al. 2013). While the British Pharmacopoeia states that KET and IND are both practically insoluble in water, IND is also sparingly soluble in ethanol while KET is freely soluble in ethanol and thus the preparation of these particles from KET loaded HA:CS PECNs varied only in the total ethanol content.

This chapter aimed to look at the behaviour of IND loaded in HA:CS PECNs to observe the drug's solid-state and association behaviour. It was considered important to use a second model drug from the with similar properties to KET to observe differences that may arise between different drugs due to solubility differences and variations in the solid-state nature of the drugs.

5.2 The Development and Characterisation of IND loaded HA:CS PECNs

5.2.1 Optimisation of the IND Loaded HA:CS PECN Formulation Variables

5.2.1.1 Solubility of IND in Aqueous Solutions

IND is classified by the BCS as a class II drug meaning that it has low solubility and high permeability. The British Pharmacopoeia (2013) states that IND is practically insoluble in water and sparingly soluble in alcohol.

The solubility of IND in the various solvents/solutions can be ranked in order of increasing solubility: 0.1% w/v CS < 0.1% w/v 7.5:6.5 HA:CS PECNs < 0.1% w/v 10:4 HA:CS PECNs < water < 0.1% w/v HA < ethanol (**Table 5.1**). With the exception of ethanol the solubility of IND increased as the pH of the aqueous dissolution medium increased this is to be expected due to the acidic nature of

the IND (IND pKa = 4.5) (**Figure 5.1**). The solubility of IND in the polymer solutions was compared with the solubility of IND in water at the corresponding pH values pre and post IND addition as predicted using the Henderson-Hasselbalch equation (**Eq.4.1, Table 5.1**). The solubility of IND was predicted at the pH both pre and post IND addition because the actual solubility in the 0.1% w/v HA and 0.1% w/v CS solutions for IND loaded HA:CS PECNs will be in a range between the pre and post IND addition solubility prediction. This is because the experimental solubility value is based on a saturated IND solution. The predicted solubility of IND in water at the corresponding pH values to the pH values of the component solutions showed no notable differences from the actual measured solubility of IND in the component solutions (**Table 5.1**). The actual solubility of IND in water at pH 5.5 was 0.031 ± 0.004 mg/ml, which showed no significant differences from that predicted for water at the same pH (0.028 mg/ml). The predicted solubility of IND at pH 5.7, 0.040 mg/ml was the same as the actual solubility measured in 0.1% w/v HA solution (pH 5.7), at 0.041 ± 0.002 mg/ml). For 0.1% w/v CS solution the predicted solubility (0.004 mg/ml) in water at pH 4.2 was the same as the actual solubility in 0.1% w/v CS solution (0.003 ± 0.002 mg/ml). The similarity between actual and predicted solubility for IND indicated that pH is strongly influencing the drug solubility in the polymer solutions. It was expected that if the drug was interacting with the polymers it would have influenced the solubility of IND independent of the pH in these solutions but pH appeared to be the primary influence.

The actual solubility of IND in aqueous dispersions (0.1% w/v) of the positively charged HA:CS 7.5:6.5 and the negatively charged HA:CS 10:4 PECNS showed no significant differences to the solubility values predicted at the equivalent pH. This inferred that pH of IND in the HA:CS PECNs was strongly influenced by pH of the dissolution medium without strong additional interactions with the polymers.

Table 5.1 Solubility of IND in the constituents of the PECN formulations. pH is given both pre-addition of IND and post-dissolution of IND. The predicted solubility of IND at the pre-dissolution pH calculated based on the Henderson-Hasselbalch equation (**Eq 4.1**) is also given.

Solvent	pH of solution pre-solubility studies	Measured Solubility (mg/ml)	pH of solution post-solubility studies	Predicted Solubility in Aqueous Solution at pre-solubility study pH (mg/ml)	Predicted Solubility in Aqueous Solution at post-solubility study pH (mg/ml)
Ethanol		20.880 ± 1.130			
Water	5.9	0.031 ± 0.004	5.5	0.070	0.028
0.1% HA solution	6.2	0.041 ± 0.002	5.7	0.130	0.040
0.1% CS solution	4.0	0.003 ± 0.002	4.2	0.003	0.004
0.1% 7.5:6.5 HA:CS PECNs	4.5	0.005 ± 0.001	4.6	0.220	0.050
0.1% 10:4 HA:CS PECNs	5.7	0.029 ± 0.003	4.8	0.040	0.060

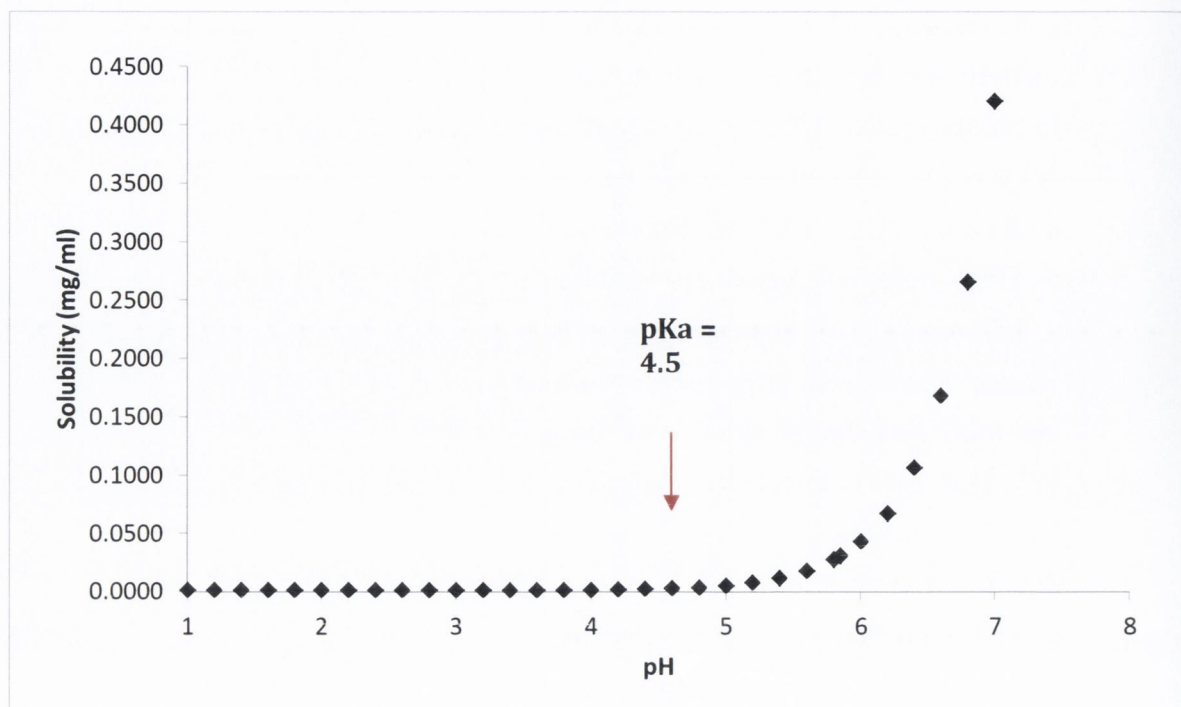


Figure 5.1 The predicted solubility of IND with increasing pH as calculated using the Henderson Hasselbalch equation. Once the pH = pKa of 4.5 for IND is reached the solubility rises rapidly.

An ionisation profile was constructed to give an overview of the % ionisation of the components of the system: IND, HA and CS (**Figure 5.2**). Since IND loaded HA:CS PECNs are formed primarily based on electrostatic interactions, the initial ionisation of the components is important to take into account when preparing the PECNs. IND reached 100% ionisation at pH 8 but it was 99% ionised from pH 6.5. CS was 100% ionised up to pH 3 and remained 99% ionised until pH 4.5 at which point the ionisation started to decline and HA solution was not predicted to be 100% ionised until pH 6.4 but it was 99% ionised from pH 4.9. Therefore, if the pH of the system remained close to pH 5, as was the case for the positively charged HA:CS 7.5:6.5 samples and the 10:4 HA:CS negatively charged PECN samples, all components would have a high degree of ionisation above 80%.

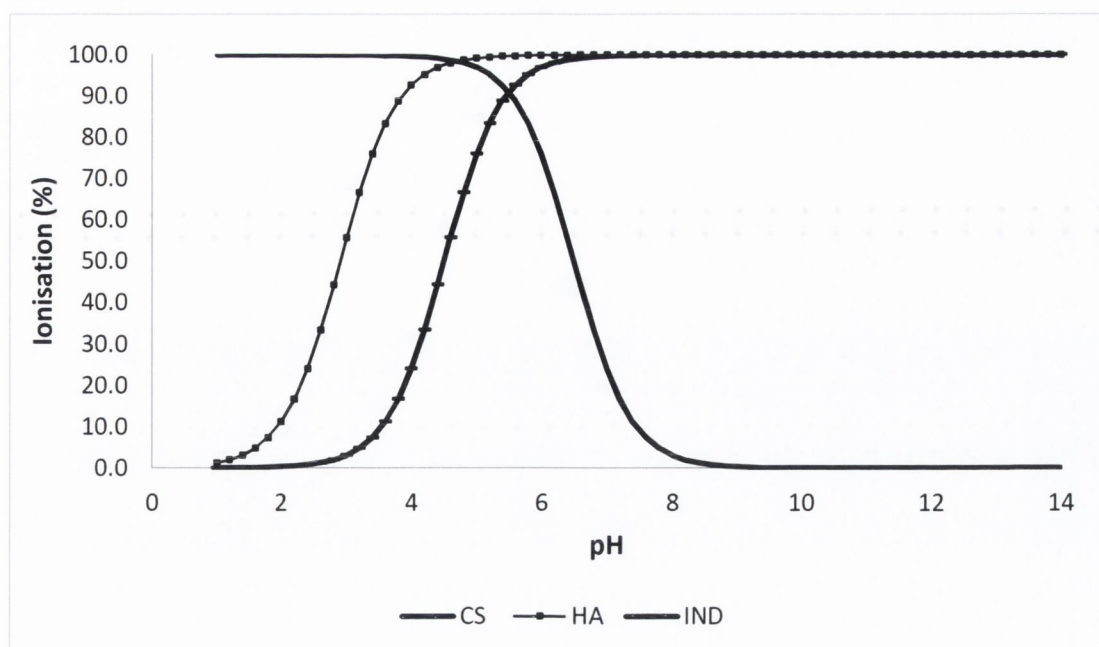


Figure 5.2 Ionisation % profile versus pH for the components of IND HA:CS PECNs.

5.2.1.2 Varying HA:CS Polymer Ratio

The polymer ratio was varied to study how the IND loaded HA:CS PECNs changed when the polymer ratios used were varied. IND was used at a concentration of 0.2 mg/mg for these studies, as this concentration was the middle of the range of concentrations tested for these studies. Varying the polymer ratio of HA:CS revealed that stable HA:CS PECNs were only formed within a narrow range of values which were the same as those seen for KET loaded PECNs and empty PECNs (**Table 5.2**). Varying the polymer ratio proved it was possible to form HA:CS PECNs with a positive charge and HA:CS PECNs with a negative charge. To form positively charged IND loaded HA:CS PECNs weight ratios (w/w) of HA:CS between 8:6 and 7:7 showed reproducible results with low PDI values and small particle size. Only at a weight ratio of HA:CS 10:4 negatively charged IND loaded HA:CS PECNs were successfully formed with a low particle size and low PDI. Outside of these ratios the IND loaded PECNs aggregated or were more polydisperse.

The addition of IND decreased pH of the PECN suspensions, due to the presence of ionised drug but the optimal ratios for positively charged and negatively charged PECN formation were the same as those for unloaded

HA:CS PECNs showing that the drug did not impact considerably on the formation of the HA:CS PECN systems. The pH of suspensions containing unloaded HA:CS PECNs with a HA:CS w/w ratio of 7.5:6.5 was 4.4, while that for the IND-loaded HACS PECNs at the same polymer weight ratio was 3.8. For the IND loaded HA:CS PECNs with ratios indicating higher HA content (10:4 w/w) the pH was 4.6 while unloaded HA:CS PECNs with a HA:CS w/w ratio of 10:4 had a pH of 5.8.

The ZP of the IND loaded HA:CS PECNs increased sharply from the HA:CS 10:4 (w/w) ratio until the HA:CS charge balance became closer to 1:1. When the weight ratio reached 7.5:6.5 w/w the ZP began to decrease again despite the increase in CS. This could be explained by looking at the charge ratios for the weight ratios tested. At HA:CS 10:4 weight ratio the charge ratio was 1.59, which decreased to 0.64 at the HA:CS weight ratio of 7.5:6.5 become unstable at the HA:CS weight ratio 9:5 with a charge ratio of 1.15. After this point (7.5:6.5) the charge ratio dropped further to 0.32 at the HA:CS (5:9) weight ratio. The reduction in charge ratio was greater between 10:4 and 7.5:6.5 than it was from 7.5:6.5 to 5:9. This shows that as the concentration of CS was increasing and HA was reducing the negative charges (from both IND and HA) were becoming saturated with CS to the point that CS was in excess and the dominant charge. Once this point was reached, further increases in CS and decreases in HA did not greatly alter the charge ratio. This is thought to occur as HA and IND had bound to CS at their maximum capacity based on electrostatic interactions. This was reflected in the smaller change in the charge ratio after 7.6:6.5 and in the fact that the ZP did not become more positively charged. The ZP decreased in charge most likely due to the particle surface changing due to excess IND or the size of the NPs increasing. The CS molecule has two charges/unit while the HA only has one/unit. Once the IND loaded HA:CS PECNs reached their optimal positive charge composition, additional CS may not have had free HA to interact with.

At the 9:5 weight ratio the charge ratio was 1.15, which was the closest ratio to 1.0 of all the combinations tested and these were unstable. IND-loaded HA:CS PECNs did not follow the same pattern at the 9:5 weight ratio as KET-loaded or unloaded PECNs. IND loaded HA:CS PECNs had a ZP of -10 mV, at the 9:5 weight ratio which was closer to neutral than the desired colloidal stability values of at least ± 30 mV but was more negatively charged than the equivalent weight

ratio for HA:CS PECNs unloaded and KET loaded HA:CS PECNs. While it is unclear exactly what caused the difference in ZP it is most likely to be related to the solubility of IND which is lower than KET and so its tendency to precipitate would be greater. KET loaded HA:CS PECNs are more likely to follow the behaviour of unloaded HA:CS PECNs as KET dissolves and mixes more easily with this system

The charge ratios were calculated as another way to identify stable ratios for combining these HA and CS polymers with drugs. The optimal charge ratios for IND loaded HA:CS PECNs were 1.59 at a HA:CS weight ratio of 10:4 and 0.64 for 7.6:6.5. According to these results one polymer is required to be in excess in order to stabilise the PECN system as if the charge ratio became closer to 1 then poor stability was seen with the least stable system at a weight ratio of 9:5 HA:CS having a charge ratio of 1.15

Table 5.2 The ratios of polymers were varied to note the effect on formulation of these NPs. HA and CS were combined at different weight ratios. The final concentration of polymers was always 0.1% w/v.

HA:CS w/w	pH	Size (nm)	PDI	ZP (mV)	Mass Ratio HA:CS:IND	Charge Ratio
10:4	4.6	212 ± 22	0.126 ± 0.052	- 32 ± 1	2.50	1.59
9:5	4.6	282 ± 35	0.354 ± 0.076	- 10 ± 24	1.80	1.15
8:6	4.0	182 ± 3	0.226 ± 0.083	+ 31 ± 4	1.33	0.78
7.5:6.5	3.8	196 ± 1	0.150 ± 0.090	+ 38 ± 3	1.15	0.64
7:7	3.8	204 ± 9	0.221 ± 0.090	+ 35 ± 3	1.00	0.56
6:8	3.8	267 ± 26	0.280 ± 0.043	+ 30 ± 1	0.81	0.45
5:9	3.9	5631 ± 5319	0.820 ± 0.452	+ 24 ± 10	0.56	0.32

5.2.1.3 Varying the pH of HA Solution

HA was prepared at different pH values to see if the degree of ionisation could be tailored to improve the physical properties of IND loaded HA:CS PECNs. A profile of HA pH versus PECN size (nm), ZP (mV) and PDI was constructed for IND loaded HA:CS PECNs at 7.5:6.5 w/w. The ratio was kept constant so that

only the pH of HA was varying, and thus its degree of ionisation (**Table 5.3**). HA is most stable at neutral pH (Liao, et al. 2005) and naturally dissolves to give a solution of pH 5.7.

While IND loaded HA:CS PECNs formed from HA solutions at pH 2 and 4, they visibly aggregated in under 20 minutes and were highly polydisperse. IND is less than 20% ionised at pH 2 and 4 but since this is true for both drugs (IND and KET), it does not explain the difference in formation. Since IND has very limited solubility at pH 2 (0.0013 mg/ml) and pH 4 (0.0017 mg/ml), this may account for the lack of physical stability for the HA:CS PECN formed at pH 2 and pH 4.

Despite the addition of HA at varying pH values, the resulting pH of the PECN dispersion varied only slightly. This could be because CS had the ability to neutralise the increasing negative charge.. HA solution at pH 5.7 and 8 was the most desirable pH for IND loaded PECNs.

Table 5.3 Effect of varying pH of HA solution on formation of IND-loaded HA:CS PECNs.

HA pH	% Ionised HA	% Ionised IND	pH of NP Suspension	pKa CS	% ionised CS	pKa HA	% ionised HA	Size (nm)	PDI	ZP (mV)
2	9	0.3	2.2	6.5	100	2.9	16.6	579 ± 43	0.78 ± 0.06	+ 36 ± 2
4	91	24.0	4.4	6.5	99.2	2.9	96.9	315 ± 55	0.49 ± 0.03	+ 26 ± 2
5.7	100	94.1	4.5	6.5	99.0	2.9	97.5	218 ± 2	0.19 ± 0.01	+ 35 ± 1
8	100	100.0	4.7	6.5	98.4	2.9	98.4	219 ± 1	0.14 ± 0.01	+ 31 ± 1
10	100	100.0	5.3	6.5	94.1	2.9	99.6	200 ± 3	0.15 ± 0.02	+ 28 ± 1

5.2.1.4 Varying Solvent for IND

The solvent was varied to see if changing solvent would have a notable impact on the physical properties of IND loaded HA:CS PECNs. The experiments were carried out using IND 0.2 mg/mg positively charged HA:CS PECNs. Positively charged IND loaded HA:CS 7.5:6.5 PECNs, which were formed using different solvents did not result in notable variations their physical properties (**Table 5.4**).

Table 5.4 The properties of IND loaded HA:CS 7.5:6.5 w/w PECNs formed using different solvents to dissolve the drug.

Solvent	Size (nm)	PDI	ZP (mV)
Ethanol	217 ± 2	0.13 ± 0.05	+ 34 ± 1
Ethyl acetate	198 ± 8	0.21 ± 0.02	+ 34 ± 1
Acetone	235 ± 30	0.29 ± 0.02	+ 35 ± 1
DMSO	196 ± 1	0.15 ± 0.04	+ 38 ± 1

The variation in size of the IND loaded HA:CS PECNs when different solvents were used showed significant differences ($p=0.048$). The IND loaded HA:CS PECNs produced using ethanol as the solvent had an average size at 217 ± 2 nm, using ethyl acetate the average size was at 198 ± 8 nm, using acetone it was 235 ± 30 nm and using DMSO it was at 196 ± 1 nm. The amount of solvent used was very low at a % v/v ratio of 1:50.9 for solvent:polymer solutions and although the variation in size was significant the differences in size did not span a wide size range (196 – 235 nm). Acetone showed the largest variation for size of the formed IND loaded HA:CS PECNs. This could be due to the fact that acetone has the highest vapour pressure of all the solvents used. For this reason acetone may have evaporated the quickest during formation resulting in more variation in the size of the IND loaded HA:CS PECNs formed than the other samples and also a higher standard deviation showing that they were difficult to reproduce.

The ZP values for IND loaded HA:CS PECNs were significantly different ($p=0.004$) but the range was narrow spanning only 4 mV so this was not expected to have a large influence on the behaviour of the formed IND loaded HA:CS PECNs. The ZP values observed were 34 ± 1 mV for ethanol, 34 ± 1 mV for ethyl acetate, 38 ± 1 mV for DMSO and 35 ± 1 mV for acetone (**Table 5.4**).

Significant differences were observed for the PDI of the IND loaded HA:CS PECNs ($p=0.002$). Given the very low aqueous solubility of IND in water it may precipitate quickly on addition to water. The solvent could influence the rate and extent of IND precipitated and thus could lead to increased polydispersity and variation in physicochemical properties. Acetone showed the highest PDI value at 0.29 ± 0.02 mV and it was the solvent with the highest vapour pressure meaning it was most likely to evaporate fastest during addition. Ethyl acetate also had a high PDI at 0.21 ± 0.02 mV and it is the solvent with the lowest miscibility with water (Godfrey, 1972).

Ethanol was chosen as the solvent of choice, similar to the HA:CS formulations with KET (**Chapter 4.3.1.2**).

5.2.1.5 Varying Drug Concentration and Drug Loading

IND loaded HA:CS PECNs were prepared successfully with the inclusion of IND at two different concentrations: 0.1 mg/mg (10% DL) and 0.2 mg/mg (20% DL). Attempts were made to load IND at 0.4 mg/mg (40% DL) but this resulted in rapid precipitation of visible aggregates during formation. For positively charged 0.4 mg/mg IND loaded HA:CS PECNs these aggregates were visible within 10 minutes of formation while for negatively charged IND loaded HA:CS PECNs aggregates were obvious immediately. From this behaviour it was clear that IND loaded HA:CS systems were too saturated with IND at the 0.4 mg/mg concentration for HA:CS PECN formation and excess IND had precipitated before it had the opportunity to bind. It could have also been the case that the IND loaded HA:CS PECNs did form but since the IND saturation was high the IND loaded HA:CS PECNs became physically unstable. The solubility of IND was lower in the component solutions than any of the attempted DL concentrations (0.1 mg/mg, 0.2 mg/mg and 0.4 mg/mg) as seen in **Table 5.1**. IND added at a concentration of 0.4 mg/mg was at least 10 times greater than the solubility of IND in any of the components of the HA:CS PECNs (**Table 5.1**). Positively charged IND loaded PECNs showed an increase in size upon addition of IND (**Table 5.5**).

The average particle size for positively charged HA:CS PECNs increased from 173 ± 2 nm for empty HA:CS PECN carriers, to 184 ± 3 nm at 0.1 mg/mg DL,

196 ± 1 nm at 0.2 mg/mg and 397 ± 3 nm at 0.4 mg/mg and the PDI had risen to approximately 0.3 at the 0.4 mg/mg concentration. The particle size rose for positively charged HA:CS PECNs in the same pattern as has been noted for KET loaded HA:CS PECNs which could suggest a common method of formation between hydrophobic drug and the polymers in these PECNs. Given the differences in solubility between IND and KET, the common rise in particle size seen with the addition of these drugs is likely to be due to their ionisation profile. IND and KET have similar pKa values: IND = 4.5 and KET = 4.23. The weight ratio of HA:CS was lower in the positively charged HA:CS PECNs than in the negatively charged HA:CS PECNs. As the positive charge from CS, is dominant in the positively charged HA:CS PECNs it is likely that IND or KET interaction with the system is based on the charge interaction with CS by electrostatic interactions. KET and IND are negatively charged and so would interact by charge with a positively charged polymer such as CS. IND loaded positively charged HA:CS PECNs were not physically stable at higher loading values (0.4 mg/mg) in comparison to KET loaded where 0.4 mg/mg showed similar stability as the lower loading samples (0.1 mg/mg and 0.2 mg/mg). There is a large difference in the solubility of IND and KET in water as well as in the polymer solutions and so the difference in stability at high concentration may be due to precipitation of free IND from the system at a concentration where KET would have been dissolved in the HA. The difference in solubility for IND and KET in the HA solution is 0.041±0.002 mg/ml for IND and 0.17±0.003 mg/ml for KET (**Tables 4.5 and 5.1**), which is approximately four times less for IND than KET. This means that for KET loading up to 0.2 mg/mg all of the KET loaded would be soluble in the HA polymer while even for 0.1 mg/mg IND loading the drug would be less than completely soluble in the system. This highlights an inherent difference in the formation of IND PECNs and is the most likely explanation for the lower stability and DL capacity.

Negatively charged IND loaded HA:CS PECNs were different in terms of physical properties to positively charged IND loaded HA:CS PECNs as no correlation between the particle size and theoretical amount of drug loaded was seen on addition of IND (**Table 5.6**). The mean particle size was 200±1 nm for 0.1 mg/mg loading and 186±1 nm for 0.2 mg/mg loading. Negatively charged HA:CS PECNs at IND 0.4 mg/mg showed even lower stability than positively charged HA:CS PECNs IND 0.4 mg/mg as the precipitation of large visible

particles was apparent immediately at formation. The solubility of IND in 0.1 % w/v HA solution is 0.17 ± 0.003 mg/ml at a theoretical drug loading of 0.1 mg/mg and 0.2 mg/mg almost all of the IND may have dissolved in the HA solution prior to precipitation. This illustrates the importance of charge in the binding of IND to the PECN complex as if IND incorporation was entirely related to solubility the IND 0.4 mg/mg would have been expected to display greater physical stability than positively charged HA:CS PECNs as the pH was higher and thus more IND could dissolve.

The charge ratio (total negative:positive charges) did not fluctuate greatly between different IND DL concentrations for both the negatively (0.1 mg/mg 1.563, 0.2 mg/mg 1.592 and 0.4 mg/mg 1.847) and positively charged (0.1 mg/mg 0.650, 0.2 mg/mg 0.650 and 0.4 mg/mg 0.658) HA:CS PECNs so the IND may have not been able to compete with the excess negative charge from HA and had a limit to its association in the HA:CS PECNs systems resulting in precipitation at IND 0.4 mg/mg (**Table 5.10**).

The ZP of the positively charged IND loaded HA:CS PECNs increased with DL from a ZP value of $+33 \pm 1$ mV for the empty system, which increased to $+38 \pm 3$ mV at IND 0.2 mg/mg while negatively charged NPs had a ZP of -32 ± 5 mV for the empty system, which decreased with increasing drug content to -36 ± 1 mV at 0.2 mg/mg (0.4 mg/mg not mentioned as the particles did not form at all). This change in ZP may be due to the contribution of IND to the charged groups on the surface of the particles. The ZP for IND, when precipitated without polymers from ethanolic solution, was close to neutral (10 ± 3 mV). This ZP was seen to vary during measurements so it is unclear how the IND charge contributed to the overall surface charge of the HA:CS PECNs.

All IND loaded HA:CS PECNs displayed a narrow monomodal size distribution as indicated by the low polydispersity values except for the 0.4 mg/mg IND sample. The PDI rose as the drug content increased for both positively and negatively charged IND loaded HA:CS PECNs.

Table 5.5 Properties of positively charged IND loaded HA:CS (7.5:6.5) PECNs.

Polymer/Drug	Size (nm)	ZP (mV)	PDI	EE (% w/w)	DL (%w/w)	Drug Conc (mg/mg)
HA-CS Empty	173 ± 2	+ 33 ± 1	0.102 ± 0.017	-	-	-
IND 0.1 mg/mg	184 ± 3	+ 34 ± 1	0.120 ± 0.030	32.8 ± 6.8	3.3 ± 0.7	0.035
IND 0.2 mg/mg	196 ± 1	+ 38 ± 3	0.242 ± 0.054	62.5 ± 2.4	13.1 ± 0.5	0.132
IND 0.4 mg/mg	397 ± 3	+ 46 ± 1	0.300 ± 0.071	N/A	N/A	N/A

Table 5.6 Properties of negatively charged IND loaded HA:CS (10:4) PECNs.

Polymer/Drug	Size (nm)	ZP (mV)	PDI	EE (% w/w)	DL (% w/w)	Drug Conc (mg/mg)
HA-CS Empty	192 ± 1	- 32 ± 5	0.116 ± 0.020	-	-	-
IND 0.1 mg/mg	200 ± 1	- 32 ± 1	0.100 ± 0.015	26.4 ± 5.7	2.6 ± 0.6	0.026
IND 0.2 mg/mg	186 ± 1	- 36 ± 1	0.142 ± 0.050	17.6 ± 1.7	3.5 ± 0.3	0.068
IND 0.4 mg/mg	N/A	N/A	N/A	N/A	N/A	N/A

5.2.1.6 Precipitation of IND in an Aqueous Environment

Free IND was added to water as ethanolic solutions (1.02% w/v) to see how IND would behave when added to water using the same method as was used to make IND loaded HA:CS PECNs except without the polymers being present. Adding IND ethanolic solution to water without the presence of HA and CS polymers, in the same volume as used to form the HA:CS PECNs, showed erratic particle size results with IND NPs and microparticles formed meaning there was high polydispersity (**Table 5.7**). Irregular sized colloidal suspensions were formed post addition of IND with some populations of particles present in the nano-range. When the dispersion of IND in water was visually observed, IND dispersion showed very poor stability over time (see **Section 5.2.2.3**).

As mentioned in **Section 5.2.1.1** IND has an aqueous solubility of 0.031 ± 0.001 mg/ml. Therefore when IND was added at a concentration of 0.1 mg/ml, 0.2 mg/ml and 0.4 mg/ml precipitation of the drug would be expected for all samples. It is difficult to conclude on the true parameter values (size, ZP) for IND NPs in

the dispersion because of the high polydispersity observed. Another problem when interpreting this measurement is that the IND concentration is low and thus size measurements are not very accurate because of the low count rate. .

As was observed that IND can form a suspension itself this may contribute to the loading values obtained for IND loaded HA:CS PECNs as part of the suspension could be colloidal IND, which is not associated with the polymers. IND dissolved in ethanol alone had a positive ZP (10 ± 3 mV). The size and ZP distributions for free IND were erratic, while the IND loaded HA:CS PECN samples showed a monomodal size and ZP distribution (**Figures 5.3 - 5.6**). The differences between IND dispersions and IND loaded HA:CS PECNs indicate that the drug is being stabilised by association with the PECNs as observed in the stability studies (see **Section 5.2.2.3**).

In order to increase the drug solubility and thus to increase the chance of IND physically interacting with the polymers to form PECNs, it was decided to use a cosolvent for IND. IND is soluble in ethanol and so the IND was completely dissolved when it was being added as ethanolic solution. This meant that IND could bind to the CS polymer chains by electrostatic interactions, which is the basis of PECNs formation (Thünemann et al. 2004), or by precipitation. The ionised IND in solution can compete with HA for CS binding sites. Lankalapalli and Kolapalli (2009) described how an increase in the ionic strength of a solution of polyelectrolytes can influence the PECN formation as the small ions compete for binding sites on the polymer chains.

Table 5.7 Size distribution profile for ethanolic drug solution on addition to water, without addition of polymers. Size measured by DLS.

Sample	PDI	Pk 1 Int (nm)	Pk 2 Int (nm)	Pk 1 Int (%)	Pk 2 Int (%)
Indo 0.2 mg/mg	0.23	282 ± 18	4293 ± 734	92	8

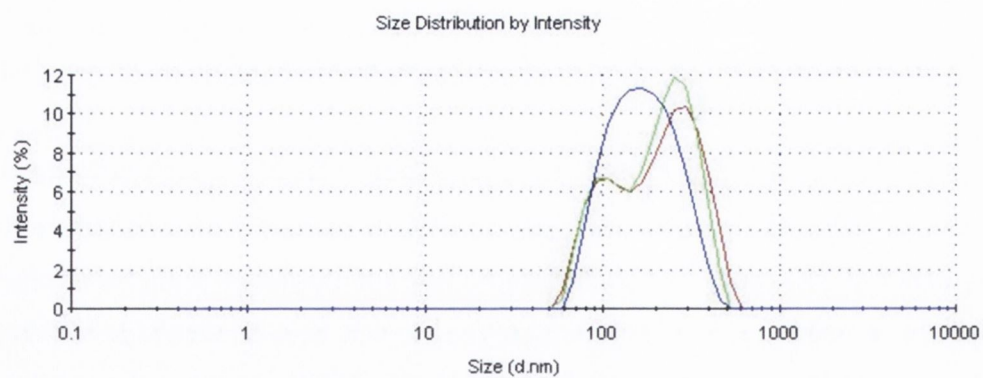


Figure 5.3 Particle size distribution of free IND precipitate, no PECNs.

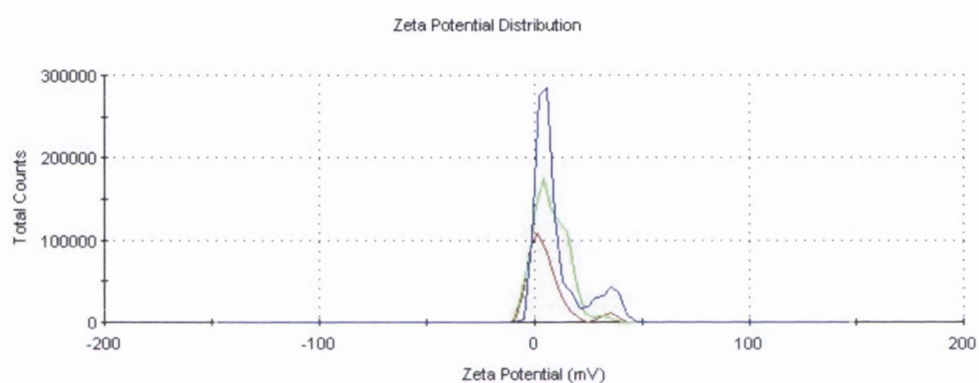


Figure 5.4 Zeta potential distribution of free IND precipitate, no PECNs

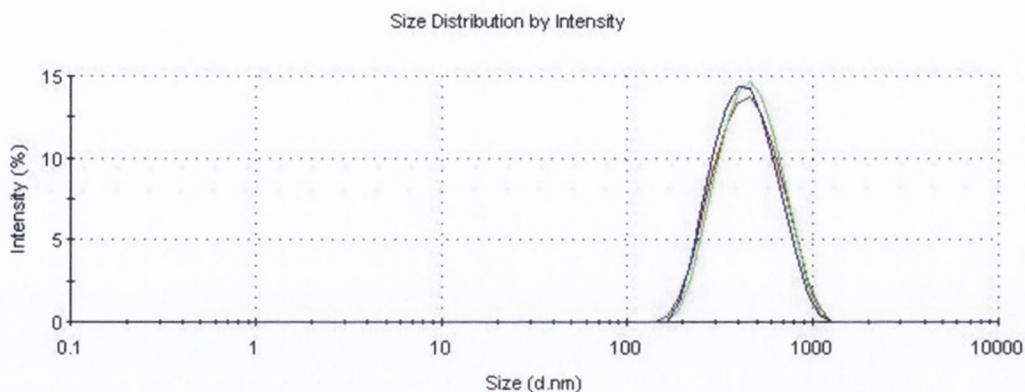


Figure 5.5 Particle size distribution of HA:CS PECNs, positively charged, with 0.2 mg/mg IND loading

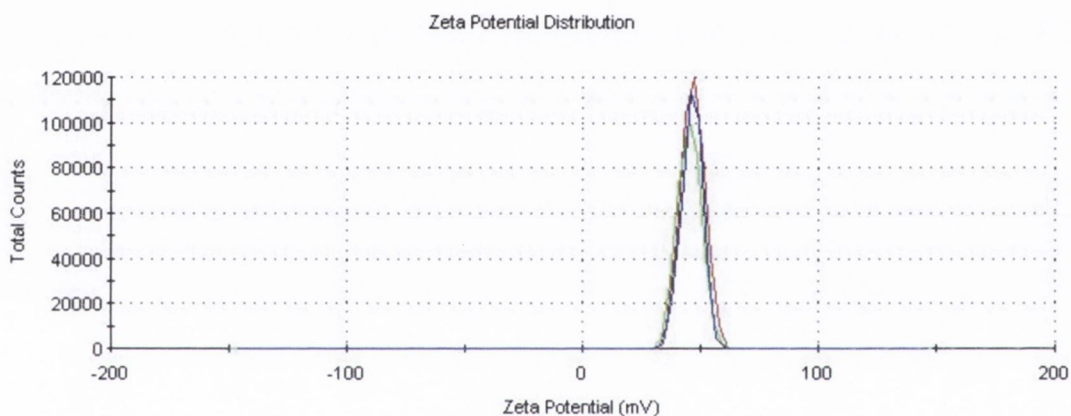


Figure 5.6 Zeta potential distribution of HA:CS PECNs, positively charged, with 0.2 mg/mg IND loading

5.2.1.7 Varying Stirring Speed and Time

Stirring speed and stirring time were important considerations in the formulation of IND loaded HA:CS PECNs due to the poor solubility of IND. The stirring speed and time stirred were tested for positively charged IND loaded HA:CS PECNs at an IND concentration of 0.2 mg/mg. IND loaded 7.5:6.5 w/w HA:CS PECNs were stirred at two speeds 600 rpm and 1200 rpm and were stirred at each of these different speeds for 10 seconds, 20 seconds, 1 minute, 10 minutes and 60 minutes. There was no significant difference between the samples stirred at 600 rpm and those at 1200 rpm in terms of particle size (nm) ($p=0.523$) but there were significant differences seen between the different stirring times. There was

so significant difference seen between 10 and 20 seconds, between 10 seconds and 1 minute or between 20 seconds and 1 minute but significant differences were seen between 10 minutes and all other stirring times ($p<0.05$) and between 60 minutes and all other stirring times ($p<0.05$). This was seen in **Table 5.8** as the particle size began to rise with stirring time from PECNs around 200 nm to those at approximately 300 nm. This means that the extra stirring time may be increasing the potential for the IND loaded HA:CS PECNs to come into contact and aggregate and for IND precipitation. This behaviour was not observed for KET loaded HA:CS PECNs and empty PECNs with the stirring speed or time having no significant effect on the particle size (nm) (data not shown). As the IND ethanolic solution is a more saturated solution than KET ethanolic solution, this could be the reason there was aggregation because when testing this by dissolving IND in ethanol at the same ethanol saturation as KET it did not aggregate at any at the tested speeds and time combinations. IND ethanolic solution for the experiments had to be prepared at a lower concentration and higher saturation value than KET since the addition of a large volume of ethanol was found to aggregate all samples. IND requires more optimisation of conditions for preparation because of its lower solubility in ethanol, thus higher saturation compared to KET

Table 5.8 The effect of stirring speed and time on the size (nm) of IND 0.2 mg/mg loaded HA:CS PECNs.

Time	10sec	20sec	1min	10min	1hr
Size at 600rpm	188 ± 17	206 ± 23	199 ± 43	295 ± 64	351 ± 57
Size at 1200rpm	215 ± 54	207 ± 32	270 ± 42	228 ± 39	310 ± 79

5.2.1.8 Summary of IND Loaded HA:CS PECNs Composition

Having combined IND with the HA:CS PECNs, their physical characteristics were investigated. The particle size of positively charged IND loaded HA:CS 7.5:6.5 PECNs increased following addition of drug. Unloaded HA:CS 7.5:6.5 had an average size of 173 ± 2 nm, which increased as the drug content increased from 184 ± 3 nm at IND 0.1 mg/mg, 196 ± 1 nm at IND 0.2 mg/mg to 398 ± 3 nm at IND 0.4 mg/mg. Negatively charged IND loaded HA:CS 10:4 PECNs did not

show a correlation between the particle size of the IND loaded HA:CS PECNs and the IND content. Less variation was seen in the particle size with increasing IND addition than for the positively charged IND loaded HA:CS PECNs: 192 ± 1 nm for empty PECNs, 186 ± 1 nm at IND 0.1 mg/mg, and 200 ± 1 nm at IND 0.2 mg/mg (**Table 5.5 and 5.6**). IND loaded HA:CS PECNs with a positive charge showed a larger particle size and higher PDI values.. For the IND 0.4 mg/mg positively charged HA:CS PECNs, aggregation was observed within 10 mins. Negatively charged HA:CS PECNs loaded with IND 0.4 mg/mg would not form as aggregation was seen immediately. This negatively charged sample may have been more unstable due to the large presence of negatively charged HA molecules reducing both binding opportunities for IND and the solubility of IND by lowering the pH for formation. The differences in the size changes for positively and negatively charged HA:CS PECNs loaded with IND may be due to physical stability or the strength of drug binding.

DL observed for negatively charged IND loaded HA:CS 10:4 PECNs did not show significant differences than the IND loaded positively charged PECNs at a concentration of 0.1 mg/mg ($p=0.108$) but there were significant differences seen at 0.2 mg/mg ($p<0.001$) (**Tables 5.5 and 5.6**). IND loading at 0.2 mg/mg was higher for positively charged HA:CS PECNs than negatively charged HA:CS PECNs indicating that positively charged HA:CS PECNs had a higher capacity to bind IND.

5.2.2 Characterisation of the IND loaded HA:CS PECN

5.2.2.1 Final Chosen Formulations

The optimal empty PECN formulations HA:CS 7.5:6.5 w/w and 10:4 w/w were chosen as the most physically stable formulations when loaded with IND to represent the positively charged formulation and negatively charged formulation, respectively. This was verified by the previous optimisation studies (**Section 5.2.1**). **Table 5.9** summarises the properties of these optimal IND loaded HA:CS PECNs used. The specific weight ratio and charge ratio of HA:CS is crucial for physically stable HA:CS PECN formation. Positively charged IND loaded HA:CS 7.5:6.5 PECNs were prepared and negatively charged IND loaded HA:CS 10:4

by varying the polymer weight ratio. The optimal formulations had a charge ratio with one polymer in excess close to the charge ratio of 2:1 or 1:2 for the number of negative charges:number of positive charges as summarised in **Table 5.10**. IND loaded HA:CS PECNs would only form at an initial DL of 0.1 mg/mg or 0.2 mg/mg. IND 0.4 mg/mg did not result in stable IND loaded HA:CS PECNs. The charge ratios (total negative:positive charges) are similar for each drug loaded value: positively charged IND 0.1 mg/mg 0.650, IND 0.2 mg/mg 0.650, negatively charged IND 0.1 mg/mg 1.563, IND 0.2 mg/mg 1.592.

Table 5.9 Summary of the constitution of all HA-CS NPs prepared. Positive and negatively charged are shown. + = positive charge, - = negative charge.

Sample	0.1% w/v HA (mls)	0.1% w/v CS (mls)	EtOH Solution (mls)	Added Drug Conc (mg/mg polymer)	HA:CS ratio (w/w)	EtOH: Polymer Solution Ratio (v/v)	Drug: EtOH Ratio (w/v)
IND 0.1 +	7.5	6.5	0.138	0.1	1:0.87	1:103.8	1:98.2
IND 0.2 +	7.5	6.5	0.275	0.2	1:0.87	1:50.9	1:98.2
IND 0.4 +	7.5	6.5	0.550	0.4	1:0.87	1:25.4	1:98.2
IND 0.1 -	10	4	0.138	0.1	1:0.4	1:103.8	1:98.2
IND 0.2 -	10	4	0.275	0.2	1:0.4	1:50.9	1:98.2
IND 0.4 -	10	4	0.550	0.4	1:0.4	1:25.4	1:98.2

Table 5.10 Summary of the ratios of formation of the IND loaded HA:CS PECNs including the mass ratio (MR) and the charge mass ratio (CMR) calculated as number of negative charges (HA+ IND) divided by the number of positive charges.

Sample HA:CS	HA (mls)	CS (mls)	HA (mg/ml)	CS (mg/ml)	Drug (mg/ml)	HA/CS/IND MR	pH Sample	n - HA	n+ CS	n- Drug	CMR HA:CS:Drug	CMR (Neg/Pos)
IND 0.1 +	7.5	6.5	0.54	0.46	0.10	5.4: 4.6 :1.0	3.9	0.00129	0.00268	0.000057	22.6 : 47.1 : 1.00	0.650
IND 0.2 +	7.5	6.5	0.54	0.46	0.20	2.7: 2.3 :1.0	3.8	0.00126	0.00268	0.000095	13.3 : 28.2 : 1.00	0.650
IND 0.4 +	7.5	6.5	0.54	0.46	0.40	1.4: 1.2 :1.0	3.7	0.00122	0.00268	0.00015	8.20 : 18.1 : 1.00	0.658
IND 0.1 -	10	4	0.71	0.29	0.10	7.1: 2.3: 1.0	4.3	0.00182	0.00164	0.00012	15.8 : 14.3 : 1.00	1.563
IND 0.2 -	10	4	0.71	0.29	0.20	3.6 :1.5 :1.0	4.3	0.00181	0.00164	0.00022	8.37 : 7.58 : 1.00	1.592

5.2.2.2 Isoelectric Point Determination

The surface charge (ZP) of positively charged IND loaded HA:CS PECNs (weight ratio of 7.5:6.5) was neutralised at approximately pH 6.4 (**Figure 5.7**). A sharp increase in the particle size of IND loaded HA:CS PECNs was observed around the isoelectric point, which verified the necessity for stabilisation of surface charge in order to maintain intact IND loaded HA:CS PECNs. Following the increase in size, the IND loaded HA:CS PECNs never returned to their original size. For positively charged IND loaded HA:CS PECNs the size increase was more pronounced than for empty positively charged HA-CS PECNs (pH 7.0). At high PDI values the Zetasizer Nano ZS instrument does not accurately predict size due to the quality of the sample being too poor. The polydispersity levels at the isoelectric point allowed measurements to be made for empty HA:CS PECNs but this was not possible for IND loaded HA:CS PECNs as the polydispersity was too high. This may be due to disassociation of IND from the HA:CS PECNs to form a polydisperse colloidal dispersion.

For negatively charged IND loaded 10:4 HA:CS PECNs the isoelectric point was at a lower pH value than for positively charged IND loaded HA:CS PECNs. Empty HA:CS PECNs had an isoelectric point at approximately 3.8 but negatively charged PECNs IND isoelectric point was approximately pH 3 indicating that the isoelectric point had been lowered in the presence of IND as was seen for the positively charged IND loaded HA:CS PECNs (**Figure 5.8**).

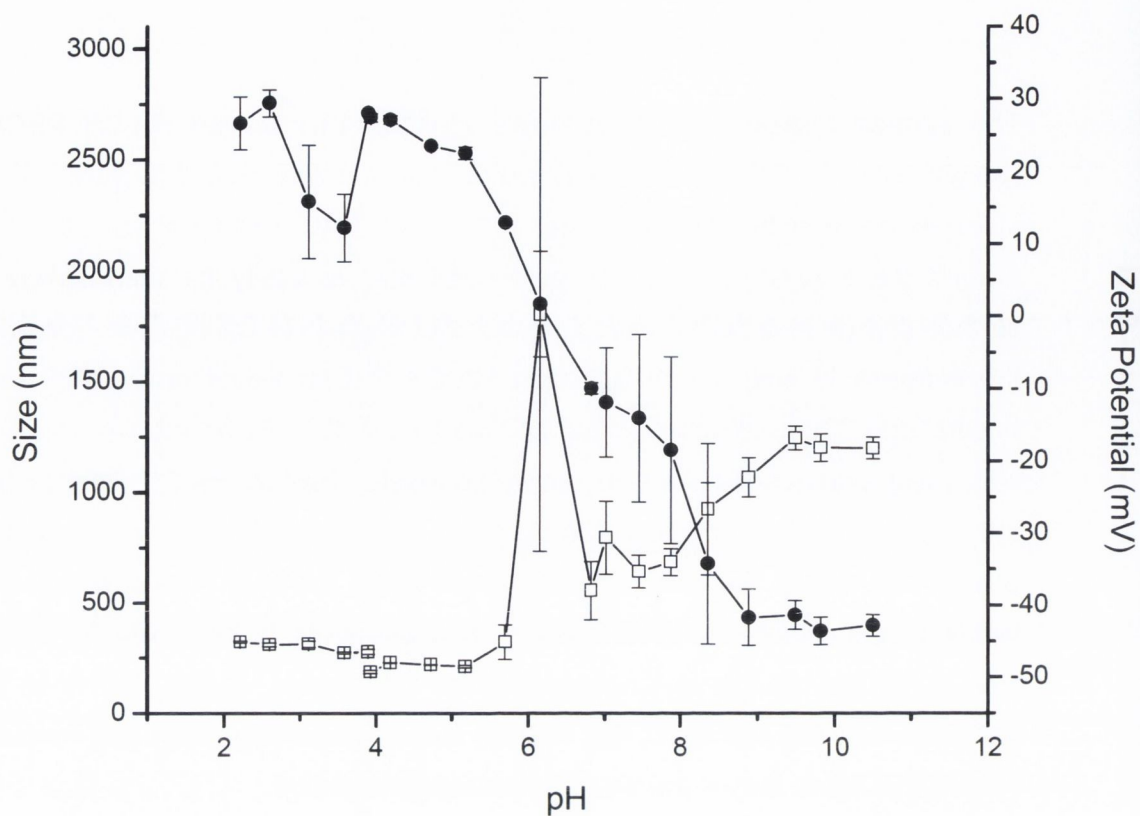


Figure 5.7 Isoelectric point titration for positively charged IND 0.2 mg/mg loaded HA:CS PECNs. ● = ZP (mV) and □ = size (nm).

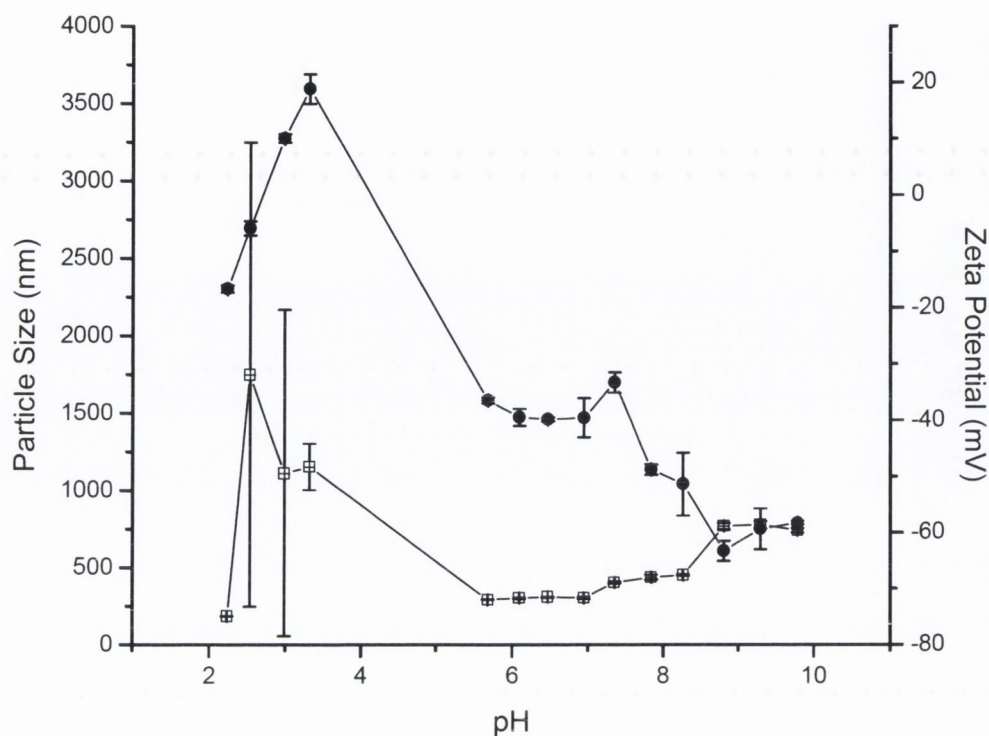


Figure 5.8 Isoelectric point titration for negatively charged IND 0.2 mg/mg HA:CS PECNs. ● = ZP (mV) and □ = size (nm).

5.2.2.3 Stability studies

The physical stability of IND loaded HA:CS PECNs with a positive or a negative charge was tested over a period of 14 days. All IND loaded HA:CS PECN formulations remained in the nano-range with low PDI over a 14 day period.

Positively charged HA:CS PECNs decreased in size, over 14 days but no precipitation was observed during this time (**Figure 5.9**). The average particle size for IND 0.1 mg/mg loaded HA:CS PECNs was 75% of the original size on day 14 and for 0.2 mg/mg IND it was 78% of the original starting size on day 14. Negatively charged IND loaded HA:CS PECNs displayed only minimal reductions in the particle size (**Figure 5.10**). IND 0.1 mg/mg HA:CS PECNs were 98% of their original size on day 14 and at 0.2 mg/mg the reduction in size of the particles was only to 99% of the original size on day 14.

Free IND (IND added as ethanolic solution to water, as described in **Section 5.2.1.6**) appears to have partly dissolved as the free drug suspension gave large

particle size values after 4 days and precipitation was visible on the bottom of the sample containers after 1 day. The ZP of free drug was erratic and varied widely indicating a lack of stability if the drug was suspended. The stability studies highlighted the importance of the polymers to stabilise IND in PECNs with negatively charged HA:CS PECNs being more stable.

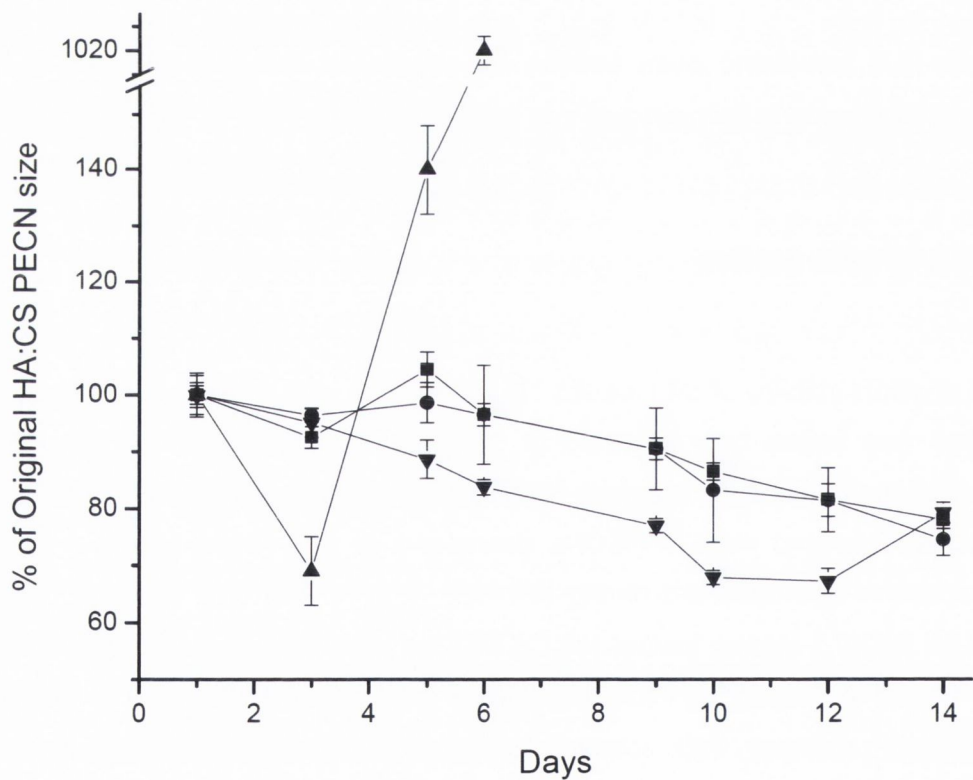


Figure 5.9 Positively charged HA:CS PECNs physical stability over 14 days reported in size (nm). ▲ = Free IND ethanolic solution, ● = HA:CS IND 0.1 mg/mg PECNs, ■ = HA:CS IND 0.2 mg/mg PECNs, ▼ = empty HA:CS PECNs

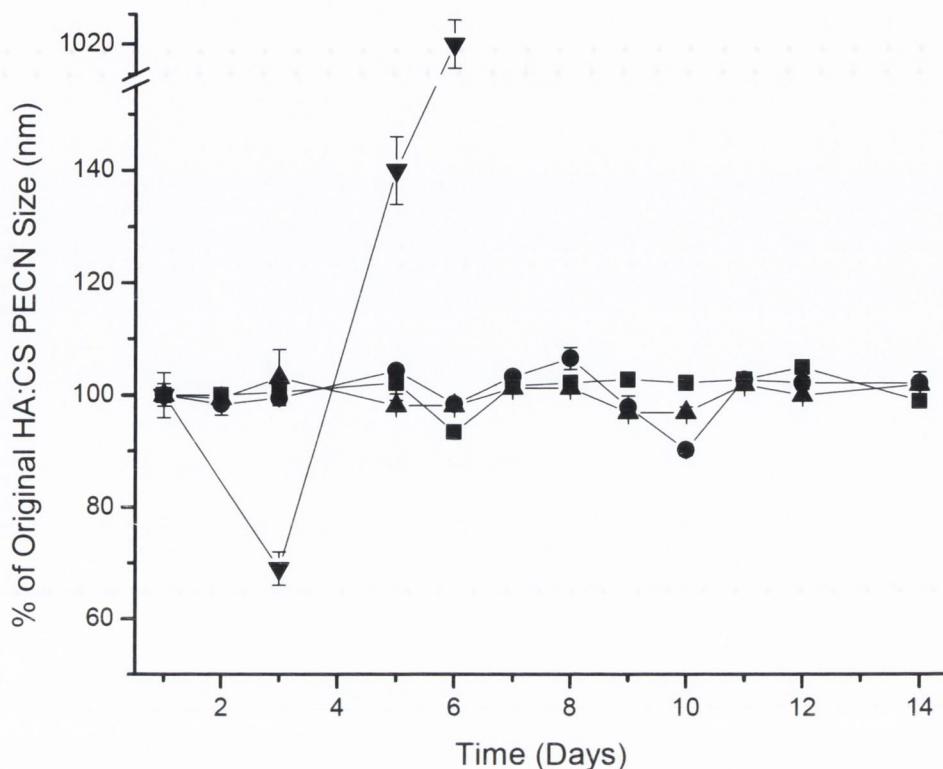


Figure 5.10 Negatively charged HA:CS PECNs physical stability over 14 days reported in size (nm). ▼ = Free IND ethanolic solution, ■ = HA:CS IND 0.1 mg/mg PECNs, ● = HA:CS IND 0.2 mg/mg PECNs, ▲ = Empty HA:CS PECNs.

The ZP values showed only very minor decreases over the 14 day period and so the HA:CS PECNs retained their ability to repel each other in the dispersion over the duration of study. Minimal differences were observed between the positively charged HA:CS PECN and the negatively charged HA:CS PECNs. For positively charged IND loaded HA:CS PECNs at 0.1 mg/mg the range for ZP +32 mV to +45 mV from day 1 to day 14, and for 0.2 mg/mg was +30 mV to +44 mV on day 1 to day 14. For negatively charged IND loaded HA:CS PECNs at 0.1 mg/mg the range for ZP was -25 mV to -32 mV over the 14 days, and for 0.2 mg/mg -26 mV to -31 mV. The change in zeta-potential was greater for the positively charged HA:CS PECNs than for the negatively charged HA:CS PECNs which is most likely associated with the change in particle structure as the size changed.

The drug content in the supernatant for positively charged IND 0.2 mg/mg HA:CS PECNs on day 1 was 2.5 ± 0.4 % of the IND loaded (the actual IND loading was treated as 100%) and on day 14 it was 11.2 ± 2.1 %. For negatively charged IND 0.2 mg/mg HA:CS PECNs on day 1 there was 1.7 ± 0.8 % and on day 14 was 8.2 ± 4.3 % of the actual IND loaded present in the supernatant. This shows that there was some drug leakage over the 14 day period into the surrounding dispersion medium with higher leakage for positively charged HA:CS PECNs than negatively charged HA:CS PECNs. This again supports the negatively charged HA:CS PECNs as more reliable carriers for IND.

5.2.2.4 Morphology –TEM

The TEM images for positively charged HA:CS PECNs loaded with IND at 0.2 mg/mg showed that the particle shape was not spherical and the size was close to that measured by DLS (**Figure 5.11**). It was difficult to distinguish between these HA:CS PECNs and those empty or loaded with KET. IND 0.2 mg/mg loaded HA:CS PECNs which were negatively charged were more spherical than the other HA:CS PECNs and the size was comparable to DLS (**Figure 5.12**).

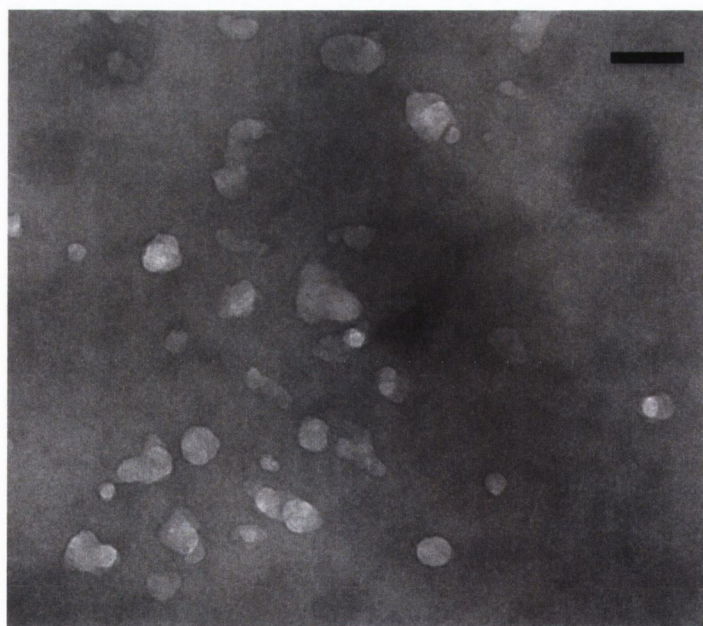


Figure 5.11 TEM image of positively charged IND 0.2 mg/mg HA:CS PECNs. Scale bar = 100 nm.

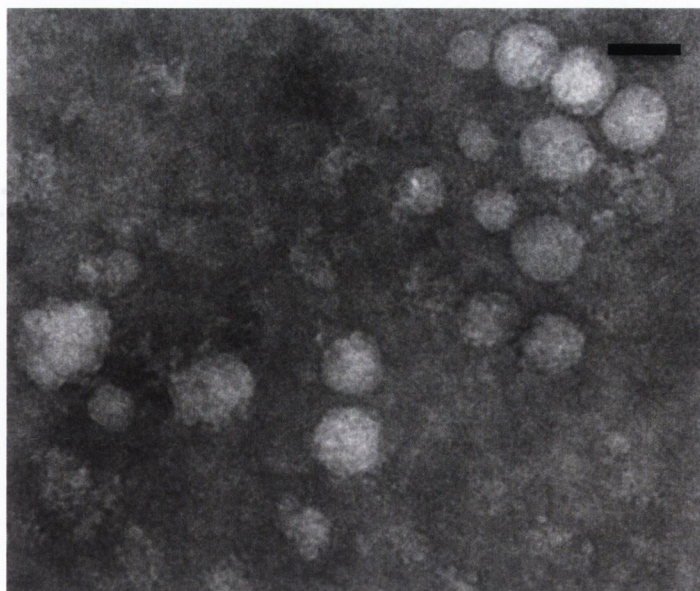


Figure 5.12 TEM image of negatively charged IND 0.2 mg/mg HA:CS PECNs. Scale bar = 100 nm.

5.2.2.5 Release studies

Like with KET HA:CS PECNs it was decided to test IND release in three difference release media: PBS buffer, 0.01M HCl and deionised water. As the isoelectric point of positive IND loaded HA:CS PECNs was pH 6.4 (**Figure 5.7**) and the isoelectric point of negative IND loaded PECNs was pH 3 (**Figure 5.8**) it was anticipated that the HA:CS PECNs would behave differently in different media. Release studies showed that IND loaded HA:CS PECN systems are fast release systems with all samples reaching 100% release between 30-60 min.

The first release medium investigated was PBS buffer. The greatest release was observed for the positively charged IND (0.2 mg/mg) loaded HA:CS PECNs as they released the majority of their IND within 30 minutes with gradual release observed to this point. This same release pattern was observed for IND 0.1 mg/mg positively charged HA:CS PECNs, which released their drug content gradually and at 30 min all the IND was released. In contrast to this the negatively charged IND loaded HA:CS PECNs released most of their drug content within 10 minutes. Complete drug release was observed for all of the

HA:CS PECNs in this medium. PBS has a higher ionic strength than water and had pH 7.2. At pH 7.2 the solubility of IND was calculated to be 0.666 mg/ml. Positively charged IND loaded HA:CS PECNs were expected to be more unstable in this medium than negatively charged PECNs as they would have experienced charge neutralisation around this pH. Evidence of this was not seen with both negative and positive IND loaded HA:CS PECNs releasing their drug content rapidly (**Figure 5.13**).

0.01 M HCl was the second release medium used and it highlighted the effect of pH on the release from HA:CS PECNs (**Figure 5.14**). When 0.01 M HCl was used as the release medium it was expected that the negatively charged IND loaded HA:CS PECNs would have lower stability as their isoelectric point was at pH 3. In this release medium IND was expected to have very poor solubility (0.0013 mg/ml) as the pKa for IND is 4.5 and it was only 0.1% ionised. The amount of IND released for all samples was lower than the loaded amount of IND. Negatively charged IND loaded HA:CS PECNs at 0.2 mg/mg loading showed the highest amount of drug released here which could be due to the instability of negatively charged IND loaded HA:CS PECNs at this low pH. If the particle structure was altered at this pH the drug may have released quickly. The release from positively charged IND loaded HA:CS PECNs was not significantly different for IND 0.1 mg/mg or IND 0.2 mg/mg despite different DLs indicating that only a specific amount of IND was capable of being released from these IND loaded HA:CS PECNs in 0.01M HCl. IND negatively charged HA:CS PECNs at 0.1 mg/mg released the lowest amount of drug which could be due to initial DL being the lowest or the fact that if the negatively charged HA:CS PECNs aggregated at this pH the small amount of drug may have been trapped inside the aggregates. At higher drug concentration it is possible that more IND was associated with the surface and thus released quickly when aggregation was occurring. It was observed that instead of dissolving into solution the IND precipitated as insoluble crystals in this medium.

Finally, deionised water was used to test if the ionic content of PBS was affecting IND release (**Figure 5.15**). For water again a lower amount of IND was released than was loaded into the HA:CS PECNs (**Figure 5.15**). At the post-dissolution pH of 4.5 both negatively and positively charged IND loaded HA:CS PECNs were expected to be reasonably physically stable and the resulting release profiles showed no strong differences between the quantity of drug released

between the IND loaded HA:CS PECNs of the same loading despite opposite charges. This could be an indication that the IND loaded HA:CS PECNs were all stabilised here and that the amount of IND released was being regulated by the pH of the surrounding medium. Positively charged IND loaded HA:CS PECNs showed faster initial release for IND 0.2 mg/mg loading but the final amount of IND released was similar to IND 0.2 mg/mg negatively charged HA:CS PECNs. The higher initial release may be due to the positively charged HA:CS PECNs having a greater amount of IND loaded.

For all IND loaded HA:CS PECNs it appeared that the medium was having a strong effect on the release profile from these PECNs. This was likely to be due to the very poor solubility of IND meaning that the conditions for its release were crucial to obtaining full IND release. Given that these HA:CS PECNs released very little IND in HCl solution it is likely that these particles will not release much of their loaded IND in the stomach if consumed orally.

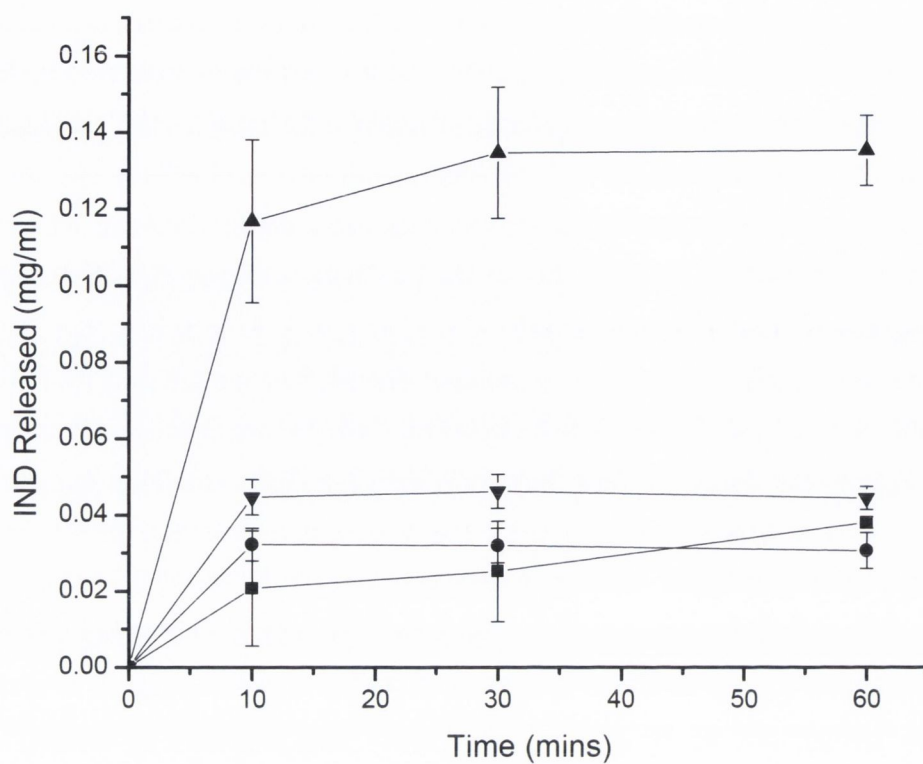


Figure 5.13 Release studies in PBS for IND loaded HA:CS PECNs. ▲ = IND 0.2 mg/mg positively charged, ▼ = IND 0.2 mg/mg negatively charged, ■ = IND 0.1 mg/mg positively charged, ● = IND 0.1 mg/mg negatively charged.

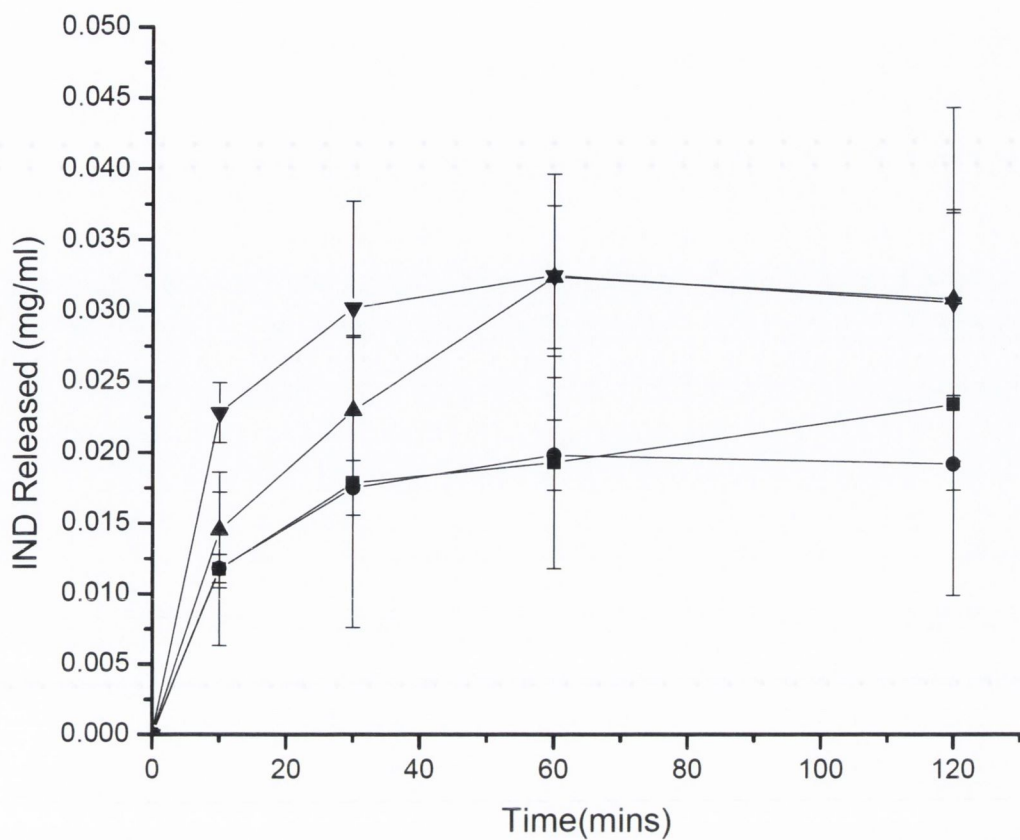


Figure 5.15 Release studies in water for IND loaded HA:CS PECNs. ▲ = IND 0.2 mg/mg positively charged, ▼ = IND 0.2 mg/mg negatively charged, ■ = IND 0.1 mg/mg positively charged, ● = IND 0.1 mg/mg negatively charged.

5.2.3 Investigations into the Solid-State Properties of IND in IND Loaded HA:CS PECNs

5.2.3.1 Introduction

IND is known to have many polymorphic forms with five currently identified (Surwase, 2013). This means IND is capable of precipitating from solution to form NPs in different physical forms including an amorphous form. The studies conducted in this current section (5.2.3) aimed to learn about the precipitation behaviour of IND while forming HA:CS PECNs. For insoluble drugs it was expected that IND would interact with the PECNs by electrostatic interactions with the CS polymer due to the electrostatic interaction between IND's negative and CS's positive charge, it may have dissolved and mixed with the polymers before precipitating in an amorphous state or it may have precipitated as nanocrystals stabilised by the polymers. For IND these nanocrystals may be in different polymorphic forms and may be associated with the polymers or exist independently in sample. IND may also be present in more than one of these forms within the same sample. It is important to know the solid-state of IND as this will be implicated in its storage conditions, stability and bioavailability (Newman and Byrne 2003).

5.2.3.2 PXRD and DSC Analysis

PXRD and DSC analysis were used as techniques to observe the behaviour of IND in HA:CS PECNs. Both pre-wash and post-wash samples were investigated as pre-wash IND loaded HA:CS PECN samples had the all of the IND added still present in the sample with the HA and CS polymers while the post-wash IND loaded HA:CS PECNs had only tightly bound IND remaining as both free IND and loosely bound IND had been removed during the washing step. IND loaded HA:CS PECNs at 0.4 mg/mg were not reliably physically stable. IND 0.4 mg/mg samples were tested during the solid-state analysis as crystallisation and as saturation was visibly obvious for these samples they were used for comparison.

PXRD analysis was carried out first as the solid-state properties observed would not be influenced by heat as in the DSC analysis. Heat could contribute to changes in the solid-state of IND in the HA:CS PECNs. In the pre-wash positively charged IND loaded HA:CS PECNs visible Bragg peaks of crystalline IND were visible in the PXRD analysis for IND at the higher concentration 0.4 mg/mg indicating the presence of IND crystals in these samples (**Figure 5.16**). This crystallisation was visible with time for the 0.4 mg/mg IND samples so the presence of Bragg peaks was expected here. It is possible that the absence of visible Bragg peaks in the pre-wash positively charged IND loaded HA:CS PECNs at the IND concentrations of 0.1 mg/mg and 0.2 mg/mg, despite the fact that these DLs were much higher than the IND solubility values (**Table 5.1**), was due to the IND being amorphous or it may have been present in a nano-crystalline state with the drug crystals too small to be detected by the PXRD analysis. Amorphous IND was reported to have solubility values higher than those seen for crystalline IND in any of its polymorphic forms by Hancock and Parkes (2000).

PXRD analysis showed that the surfaces of positively charged post-wash IND loaded HA:CS PECNs were also crystalline for IND 0.4 mg/mg, while IND 0.2 mg/mg and IND 0.1 mg/mg still did not show Bragg peaks indicating crystallinity (**Figure 5.16**). There was a peak noted at 32.2° 2θ for two samples IND 0.1 mg/mg HA:CS PECNs post-wash and IND 0.2 mg/mg HA:CS PECN post-wash, which was due to salt formation with the sodium ions from the sodium hyaluronate used to prepare the samples. This peak was previously noted by Nickels et al. (1949).

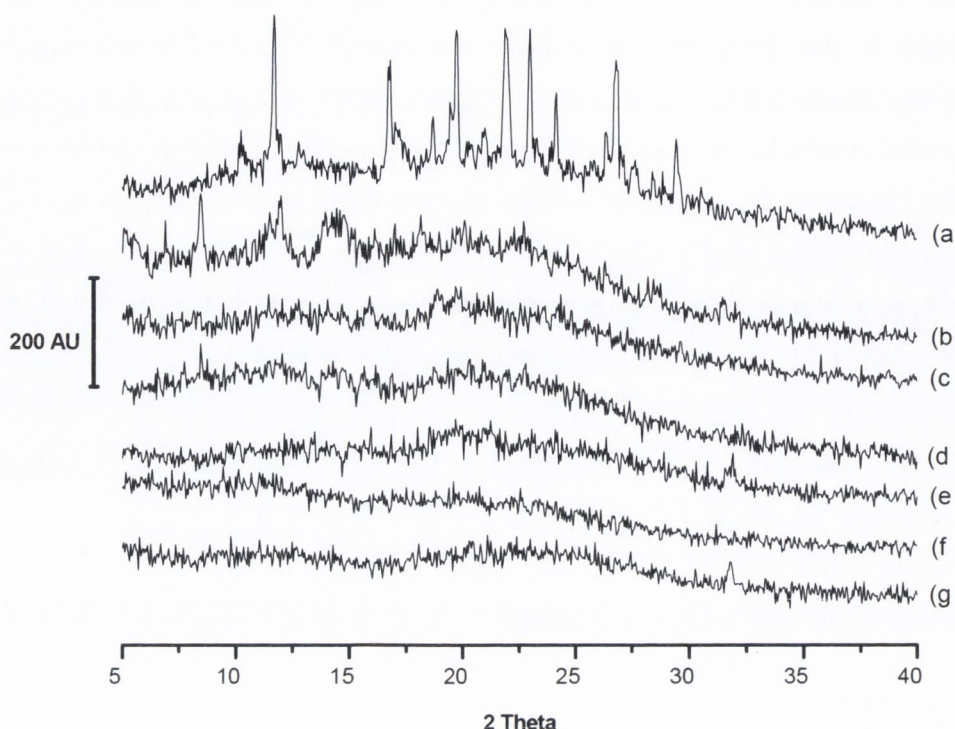


Figure 5.16 PXRD of HA:CS IND loaded PECNs **positively** charged and a polymer/drug physical mix. **a)** IND 0.2 mg/mg physical mix with HA and CS polymers, **b)** IND 0.4 mg/mg PECNs pre-wash **c)** IND 0.4 mg/mg PECNs post-wash, **d)** IND 0.2 mg/mg PECNs pre-wash, **e)** IND 0.2 mg/mg PECNs post-wash, **f)** IND 0.1 mg/mg PECNs pre-wash, **g)** IND 0.1 mg/mg PECNs post-wash.

Pre-wash negatively charged IND loaded HA:CS PECNs did not show Bragg peaks of crystalline IND for the samples at any of the IND concentrations tested (**Figure 5.17**). The negatively charged pre-wash IND loaded HA:CS PECNs did not display crystallinity as was seen for the positively charged pre-wash IND 0.4 mg/mg loaded HA:CS PECNs perhaps due to the fact that negatively charged IND 0.4 mg/mg loaded HA:CS PECNs showed lower physical stability on preparation and IND may have precipitated out and did not remain associated with PECNs.

PXRD analysis showed Bragg peaks for the negatively charged post-wash IND loaded HA:CS PECNs at 0.4 mg/mg and there was an indication that there were some peaks present at IND concentration 0.2 mg/mg and at 0.1 mg/mg. This indicated that drug crystals were present for each of these samples after the

washing process despite the fact that unbound IND and loosely bound IND was expected to have been removed by the process. The suggestion here is that the centrifugation process involved in washing stimulated crystal growth due the agitation of the dispersion. Since this was not observed for the positively charged IND loaded HA:CS PECNs, post-wash IND binding may have been stronger for positively charged HA:CS PECNs. It could be expected that because IND is acidic it would be more tightly bound to the HA:CS PECNs with a positive charge. It may also indicate that the drug was bound in different forms as the IND present in the negatively charged HA:CS PECNs was more unstable and crystallised during agitation while this was not the case for the IND present in the equivalent positively charged samples.

The difference in behaviour between the positively charged and negatively charged IND loaded HA:CS PECNs could also be indicative of the variations in types of drug binding within these PECNs. Considering the similarity in DL for IND post-wash, for all samples except positively charged IND 0.2 mg/mg PECNs, the same amount of drug could be bound by different mechanisms. The crystallised drug may have been stabilised by the polymers while the rest of the drug present would be expected to be in an amorphous state or complexed with the polymers. Any excess drug that is not tightly bound may have precipitated out as free crystals post addition of the drug. The difference between the IND loaded HA:CS PECNs was the weight ratio of HA:CS moving from 7.5:6.5 w/w for positively charged to 10:4 w/w for negatively charged PECNs. This indicated that the polymer ratio was influencing the type of association between IND and the HA and CS polymers involved in IND loaded HA:CS PECN formation.

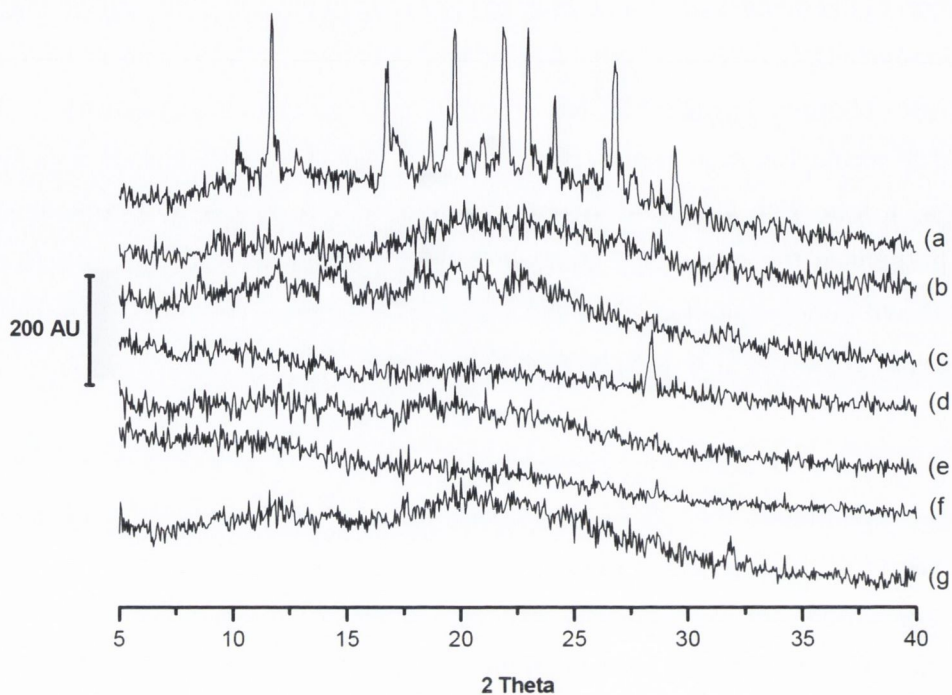


Figure 5.17 PXRD of HA:CS IND loaded PECNs **negatively** charged and a polymer/drug physical mix. **a)** IND 0.2 mg/mg physical mix with HA and CS polymers, **b)** IND 0.4 mg/mg PECNs pre-wash, **c)** IND 0.4 mg/mg PECNs post-wash, **d)** IND 0.2 mg/mg PECNs pre-wash, **e)** IND 0.2 mg/mg PECNs post-wash, **f)** IND 0.1 mg/mg PECNs pre-wash, **g)** IND 0.1 mg/mg PECNs post-wash.

DSC was used to clarify the data from PXRD about the solid-state of IND in the HA:CS PECNs. Tests were again carried out both on samples pre-wash and post-wash for positive and negatively charged HA:CS PECNs loaded with IND at all concentrations 0.1 mg/mg, 0.2 mg/mg, 0.4 mg/mg. IND has a melting point in the range 158°C – 162°C according to the BP. The melting point of free IND used for these experiments was experimentally determined to be 159.7 ± 0.05 °C with an enthalpy of fusion -103.0 ± 6.01 J/g. The melting point of IND in the physical mix with the polymers HA and CS (0.2 mg IND/1 mg polymers) was at 159.6 ± 0.8 °C. There was no significant difference between the melting point temperatures ($p=0.026$) indicating that the polymers were not lowering the melting point of IND by interaction within the physical mix.

In pre-wash positively charged IND loaded HA:CS PECNs there was a melting peak present at 137.60 ± 3.00 °C with an enthalpy of fusion -1.50 ± 0.70 J/g for the IND concentration of 0.4 mg/mg. For the IND loaded concentration of 0.2 mg/mg pre-wash the melting point was 145.9 ± 1.3 °C and the enthalpy of fusion was -0.47 ± 0.13 J/g. Samples with the lower drug concentration of 0.1 mg/mg did not show melting peaks (**Figure 5.18**). This indicates that at the higher concentration (0.4 mg/mg) IND had precipitated out in a crystalline form, which had been confirmed by PXRD analysis (**Figure 5.16**). Since crystals were not detected by PXRD for IND 0.2 mg/mg, IND could have been in an amorphous form in these samples and crystallised on heating, as amorphous IND has a T_g at 43.7 °C (Aceves-Hernandez et al. 2008), or the crystals may have been in the nano-size range and not detectable by PXRD.

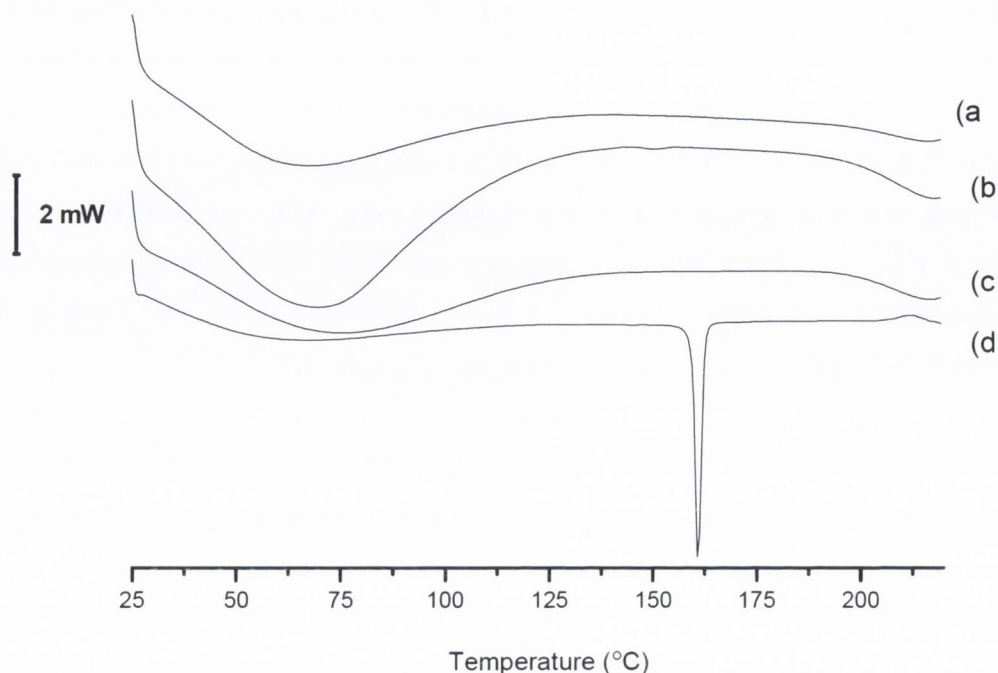


Figure 5.18 DSC of HA-CS IND physical mix and positively charged PECNs pre-wash loaded with increasing concentrations of IND. **a)** 0.4 mg/mg IND loading, **b)** 0.2 mg/mg IND loading, **c)** 0.1 mg/mg IND loading, **d)** 0.2 mg/mg IND and HA:CS PECNs physical mix.

Once the IND loaded HA:CS PECNs were washed melting peaks were visible at all concentrations of IND used: 0.4 mg/mg, 0.2 mg/mg and 0.1 mg/mg. These melting peaks were very small in comparison to the free, crystalline IND. For the IND 0.4 mg/mg the melting point was 141.76 ± 4.05 °C and the enthalpy of fusion was -2.62 ± 1.84 J/g, IND 0.2 mg/mg PECN system had a melting point at 143.0 ± 6.00 °C and the enthalpy of fusion was -2.80 ± 2.40 J/g, for IND 0.1 mg/mg the melting point was 149.80 ± 4.5 °C and the enthalpy of fusion was -2.60 ± 2.50 J/g. The PXRD results for post-wash IND loaded in positively charged PECNs had only shown the presence of IND crystals for the 0.4 mg/mg samples and so at the lower concentrations it is likely that the IND crystals were either formed during heating of amorphous IND or the crystal size was in the nano-size range and so could not be detected by PXRD (**Figure 5.19, 5.16**). This suggests that IND may form a complex with the polymers and not crystallise

but instead remain amorphous or else it is possible that the magnitude of the crystallisation event is too small to measure.

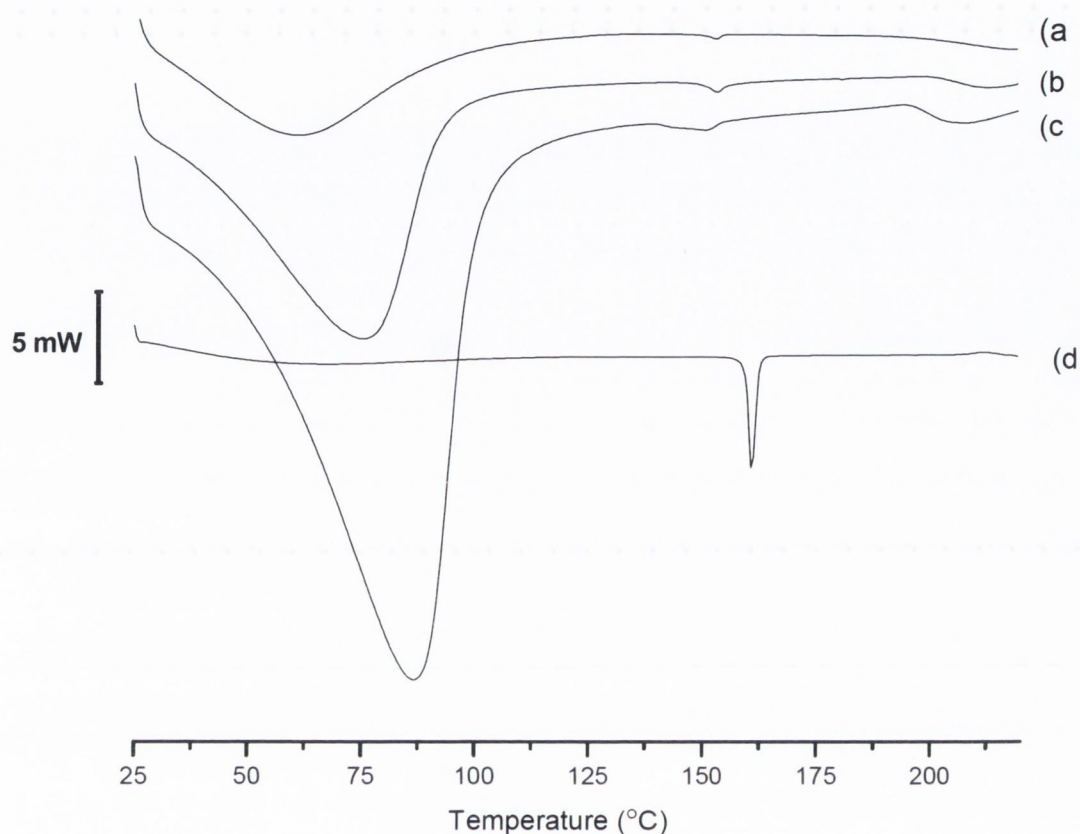


Figure 5.19 DSC of HA-CS IND physical mix and positively charged PECNs post-wash loaded with increasing concentrations of IND. **a)** 0.4 mg/mg IND loading, **b)** 0.2 mg/mg IND loading, **c)** 0.1 mg/mg IND loading, **d)** 0.2 mg/mg IND and HA:CS physical mix.

When the negatively charged IND loaded PECN samples pre-wash were subjected to DSC analysis, melting endotherms were visible at all concentrations 0.1 mg/mg, 0.2 mg/mg and 0.4 mg/mg. Although Bragg peaks for IND crystals had not been clearly defined for any of these drug concentrations, the DSC traces clearly showed IND melting points (**Figure 5.20**). For 0.4 mg/mg the melting point was 140.30 ± 2.4 °C and the enthalpy of fusion was -13.00 ± 2.6 J/g, for IND 0.2 mg/mg the melting point was 138.21 ± 4.0 °C and the enthalpy of fusion was -3.25 ± 1.9 J/g and for IND 0.1 mg/mg the melting point was 137.49 ± 0.11 °C and the enthalpy of fusion was -1.41 ± 0.32 J/g.

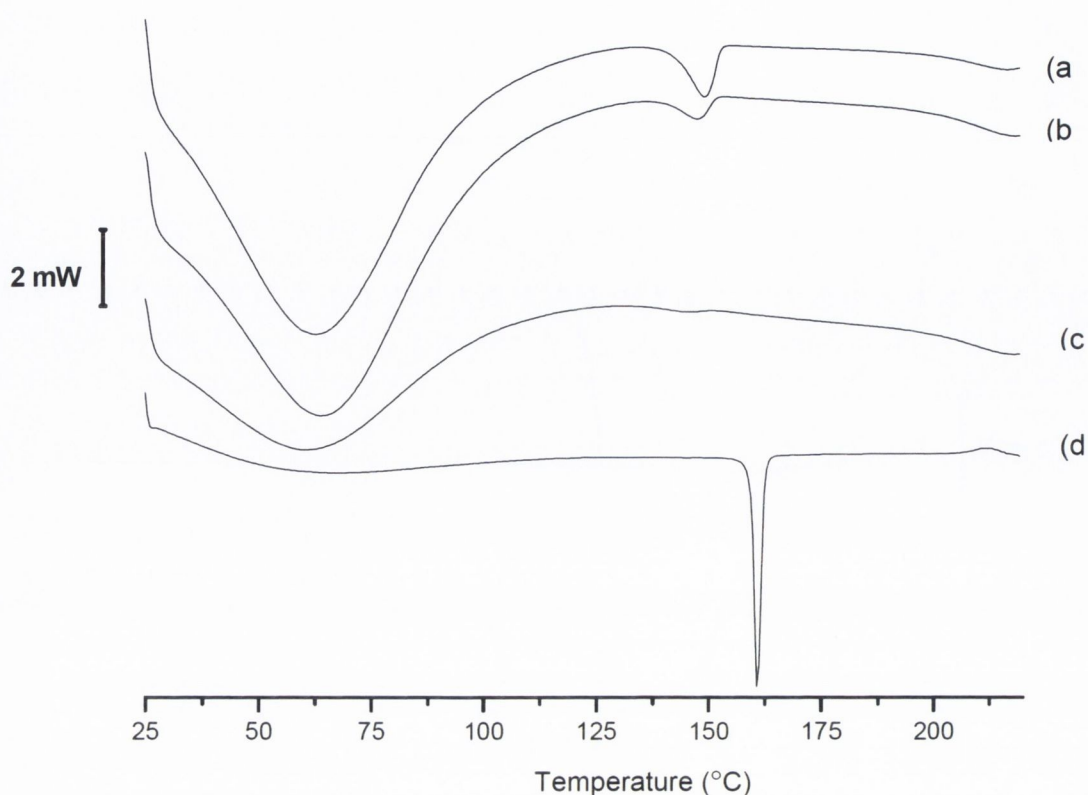


Figure 5.20 DSC of HA-CS IND physical mix and negatively charged PECNs pre-wash loaded with increasing concentrations of IND. **a)** 0.4 mg/mg IND loading **b)** 0.2 mg/mg IND loading, **c)** 0.1 mg/mg IND loading, **d)** 0.2 mg/mg IND and HA:CS physical mix.

Negatively charged IND loaded HA:CS PECNs post-wash showed melting peaks at all concentrations of IND 0.4 mg/mg, 0.2 mg/mg and 0.1 mg/mg, which was in agreement with the PXRD results for post-wash IND loaded in negatively charged PECNs (**Figures 5.17 and 5.21**). IND 0.4 mg/mg the melting point was 144.26 ± 0.73 °C and the enthalpy of fusion was -1.72 ± 1.52 J/g, IND 0.2 mg/mg the melting point was 141.8 ± 5.3 °C and the enthalpy of fusion was -3.17 ± 3.1 J/g, IND 0.1 mg/mg the melting point was 141.26 ± 0.25 °C and the enthalpy of fusion was -0.05 ± 0.03 J/g. PXRD analysis had only shown definable Bragg peaks for the 0.4 mg/mg indicating a low concentration of IND crystals present or that the crystals were nanocrystalline. This was the opposite to the observations for the positively charged IND loaded HA:CS PECNs as for these the crystallinity appeared to decrease with washing indicating that crystalline drug was

physically removed in the washing process but for negatively charged IND loaded HA:CS PECNs the melting points were present post-washing so the crystal growth could have been stimulated by the washing process. The enthalpy of fusion values decreased post washing and thus there was most likely loosely bound or unbound IND crystals removed during washing.

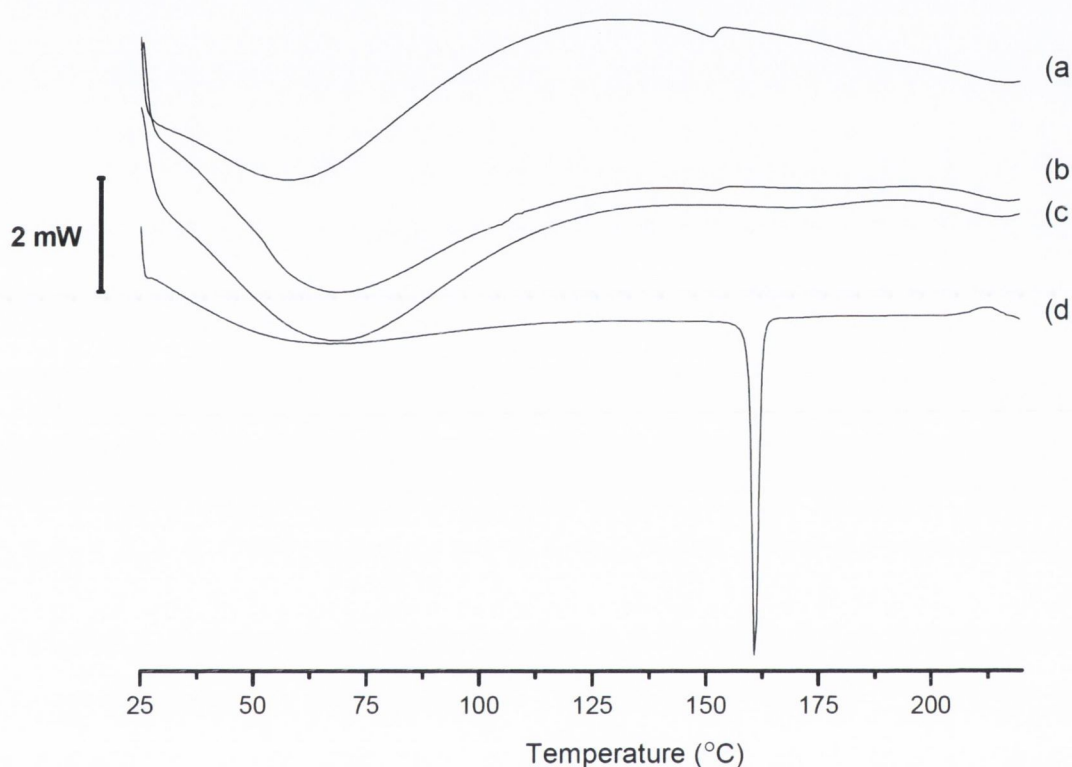


Figure 5.21 DSC of HA-CS IND physical mix and negatively charged PECNs post-wash loaded with increasing concentrations of IND. **a)** 0.4 mg/mg IND loading, **b)** 0.2 mg/mg IND loading, **c)** 0.1 mg/mg IND loading, **d)** 0.2 mg/mg IND and HA:CS physical mix.

5.2.3.3 Miscibility of IND in HA and CS Polymers

Drug miscibility with the polymers indicates how well the drug and polymers will mix when in a liquid (amorphous) state. The hypothesis was that if IND has

higher miscibility with the polymers it is more likely to precipitate dispersed in the polymers in an amorphous form than to crystallise out. Miscibility in this case relates to solubility of amorphous IND in the amorphous dry polymers HA and CS. To support the estimated miscibility, optical microscopy was used to see how many phases were present and if any crystals were formed. If the drug is miscible just a single, uniform phase is observed under the optical microscope.

Table 5.11 Estimates of Hildebrand solubility parameters (δ) (Eq 4.2) for each of the components used to prepare the HA:CS PECNs.

Constituent	δ (MPa ^{1/2})	$\Delta\delta$ (drug-polymer)
HA	19.8	4.2
CS	20.0	4.0
IND	24.0	-

The results showed that IND should be miscible with the polymers HA and CS as the difference in solubility parameters between IND and these polymers is low: 4.2 for IND and HA and 4.0 for IND and CS (Table 5.11).

The optical microscope was used as a tool to investigate the drug miscibility with the polymers and give a visual representation of the combination (Figures 5.22 – 5.25). The number of phases apparent on drying can indicate whether the drug had mixed with the polymers or exists as a separate phase. IND at 0.2 mg/mg was chosen and IND at 0.4 mg/mg was used for comparison as crystallisation was observed at this IND concentration.

When IND dissolved in ethanol (1 mg/98.2 μ l) was added to 14 mls water without the presence of HA and CS polymer solutions large visible crystals precipitated out of solution. These crystals were larger for 0.4 mg/mg than for 0.2 mg/mg. The free IND crystals appeared to grow in clusters and as the concentration of IND increased to 0.4 mg/mg these clusters were larger.

The behaviour of IND upon addition of IND to the HA:CS showed obvious crystal growth with smaller crystals seen for IND 0.2 mg/mg than for IND 0.4 mg/mg. It was noted that the IND crystals precipitating with the polymers did not form large clusters as was observed for free IND. The polymers appeared to keep IND crystals from growing and reduced their attraction for each other. It was not possible to estimate the fraction of IND that had crystallised and the fraction that was in an amorphous state.

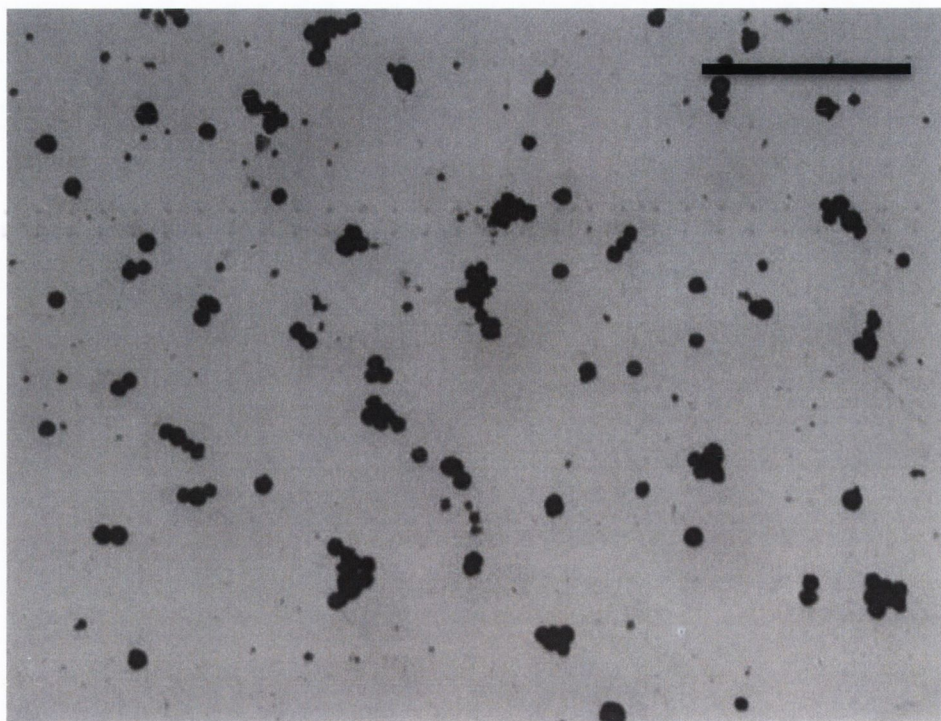


Figure 5.22 0.2 mg/ml IND in ethanol solution (1mg/98.2 μ l) added to water and air dried on a glass slide. Scale bar = 20 μ m.

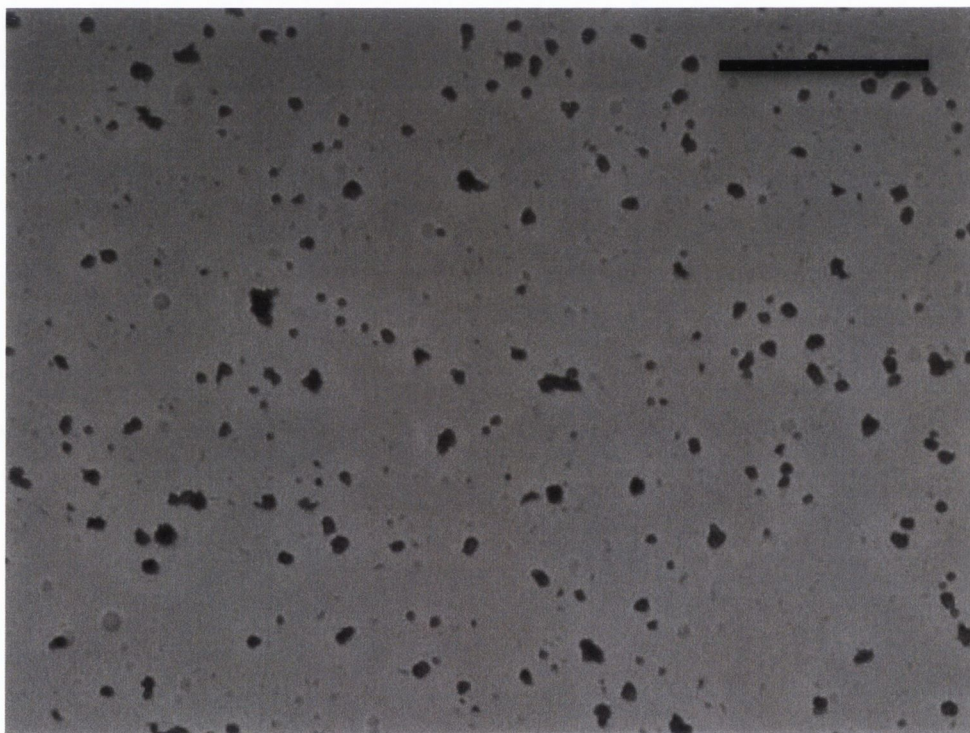


Figure 5.23 0.2 mg/ml IND loaded 7.5:6.5 HA:CS dispersion (weight ratio) and air dried on a glass slide. Scale bar = 20 μ m.

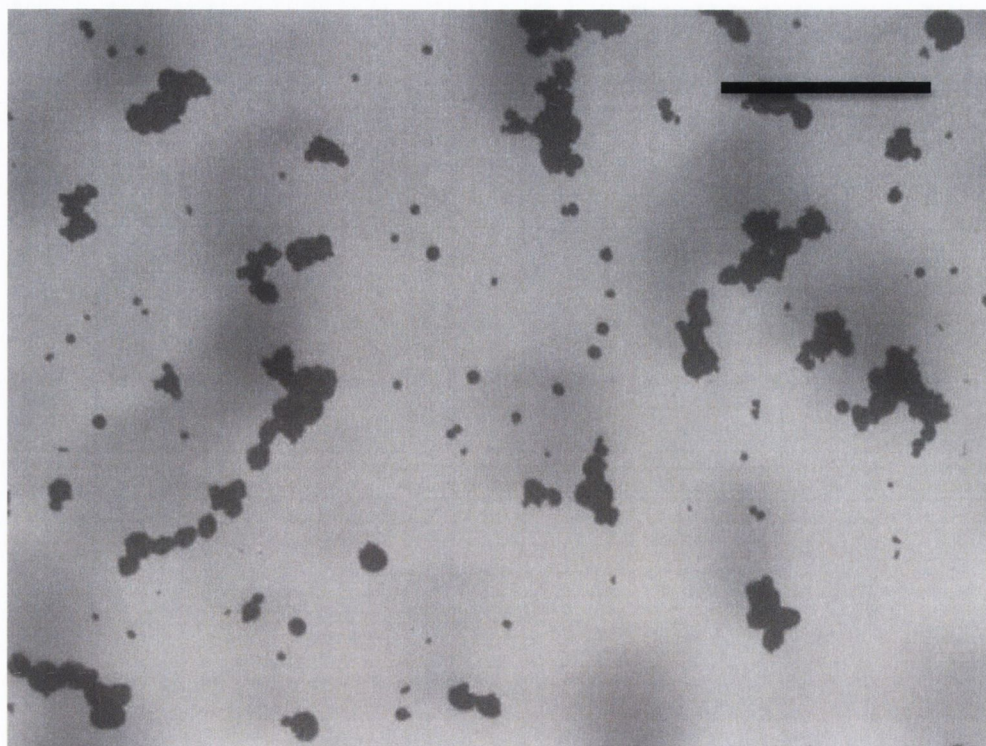


Figure 5.24 0.4 mg/ml IND in ethanol solution (1mg/98.2 μ l) added to water and air dried on a glass slide. Scale bar = 20 μ m.

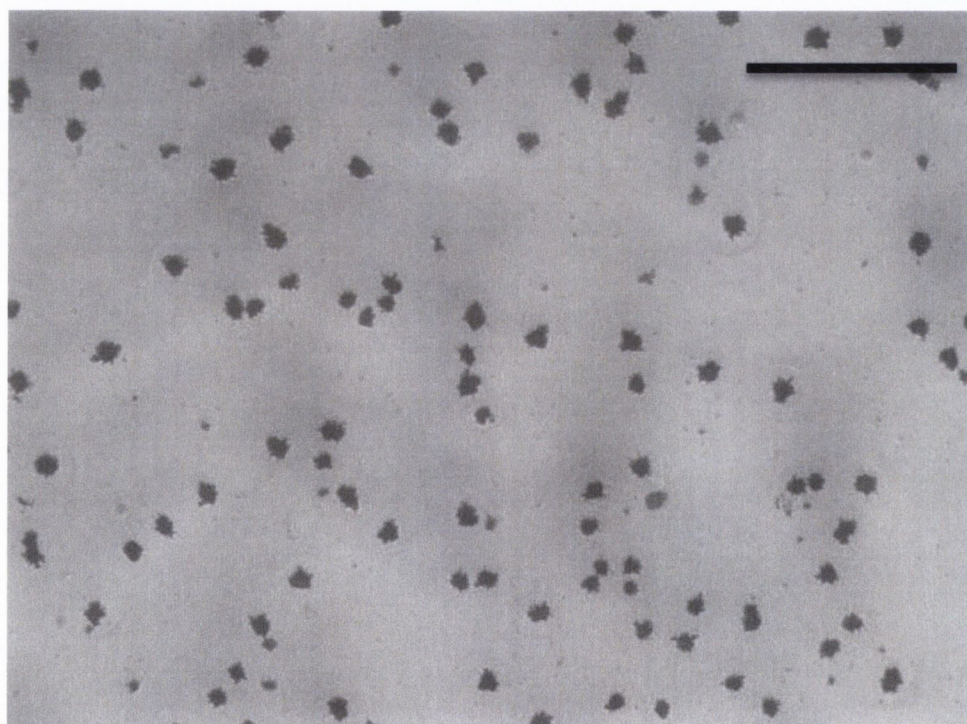


Figure 5.25 0.4 mg/ml IND loaded 7.5:6.5 HA:CS dispersion (w/w) and air dried on a glass slide. Scale bar = 20 μ m.

It was decided to look more closely at how IND precipitated from the different components of the HA:CS PECN systems to see if one of the polymers was affecting the crystallisation of IND in particular. PXRD and DSC scans were taken of IND precipitated from ethanolic solution (1 mg/98.2 μ l) in different solutions – water, 0.1% HA solution, 0.1% CS solution and HA:CS PECNs at 0.2 mg/mg and 0.4 mg/mg. IND commonly crystallises in the γ form, as this is the most stable and least soluble polymorph of IND (Hancock and Parkes 2000).

The PXRD traces confirmed that free IND (as supplied) was present in the γ form with its PXRD trace resembling the γ form reported in literature (Andronis et al. 2007, Savolainen et al. 2007, Lin 2002) (**Figure 5.26**). The PXRD traces for IND precipitated from other HA:CS PECN components: 0.1% w/v HA solution, 0.1% w/v CS solution and HA:CS PECNs, all showed differences from the PXRD trace obtained for free IND precipitated from water. When IND was precipitated in water and in 0.1% w/v HA solution, the peaks were clearly those of the α form as described by other research groups (Andronis et al. 2007, Savolainen et al. 2007, Lin 2002, Surwese et al. 2013). IND 0.2 mg/ml precipitated from 0.1% w/v CS solution showing a pattern of Bragg peaks that was very close to the α form, but the peaks were not as pronounced so there may have been some amorphous IND or other IND polymorphs present in this sample. Positively charged 0.2 mg/mg IND loaded HA:CS PECNs pre-wash precipitated IND with some Bragg peaks indicating IND crystallinity. The peaks observed for IND precipitated from this sample were not exactly the same as the γ form or the α form. For IND 0.2 mg/ml and IND 0.4 mg/ml post-wash the Bragg peaks were much less visible than in the other samples. Due to the removal of excess IND during washing the IND content was lower for these samples the degree of crystallinity was greater at IND concentration of 0.4 mg/mg than 0.2 mg/mg. The crystals present may also have been in a nanocrystalline state making them difficult to detect using PXRD measurements.

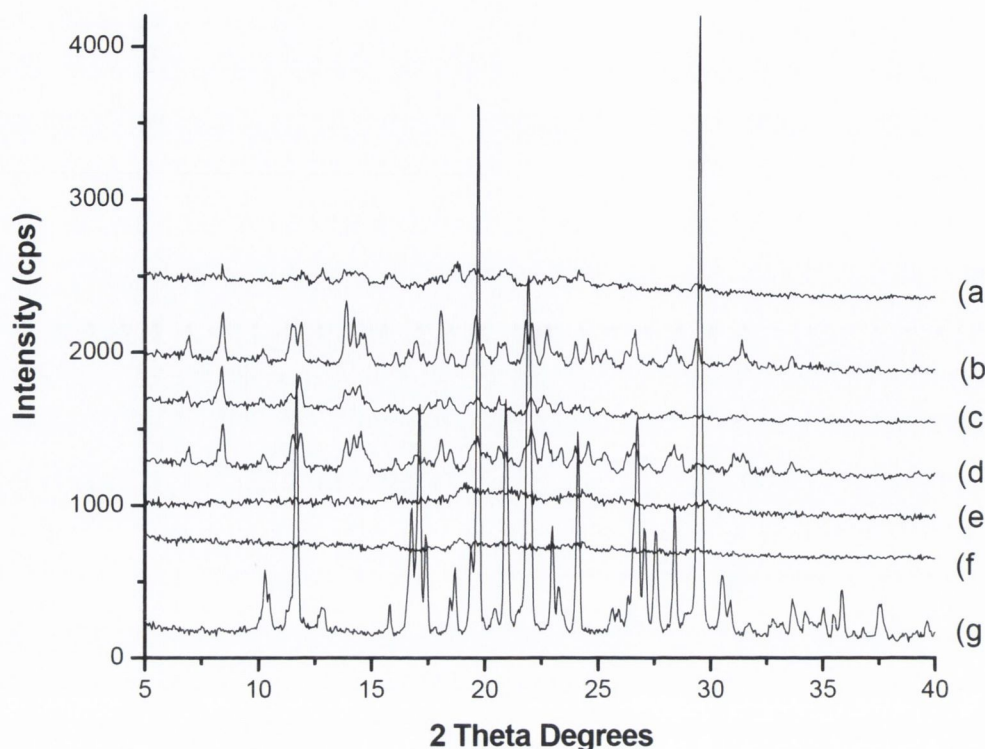


Figure 5.26 PXRD patterns of **a)** IND (0.2 mg/mg) loaded HA:CS PECNs (+ charge) pre-wash, **b)** IND 0.2 mg/ml precipitated from water, **c)** IND 0.2 mg/mg precipitated from 0.1 % w/v CS solution, **d)** IND 0.2 mg/ml precipitated from 0.1 % w/v HA solution, **e)** IND 0.4 mg/mg HA:CS PECNs (+ charge) post-wash, **f)** IND 0.2 mg/mg HA:CS PECNs (+ charge) post-wash, **g)** free IND as supplied.

The DSC traces were used to learn more about the solid-state of IND, as precipitated from the different components (**Figure 5.27**, **Table 5.12**). Free IND precipitated in water from ethanolic solution was present in the α form and the γ form according to the DSC results, which, given the results observed using PXRD, meant that the α form most likely converted to the γ form during heating. In 0.1 % w/v CS solution IND precipitated in the α form from ethanol and the sample had an onset of melting at 145.6 °C followed by a conversion to the γ form. The conversion of IND from the α form to the γ form on heating for IND when precipitated in 0.1% w/v CS solution was not as pronounced as was the case when IND was precipitated from water alone so it is likely that the polymer prevented this conversion to some extent (**Figure 5.27**). For IND precipitated from 0.1% w/v HA solution the area of the melting peaks associated with the α form and the γ form was less than when IND is precipitated from water or 0.1 % w/v CS solution. Less conversion from the α form and the γ form on heating was

observed in 0.1% w/v HA also suggesting that it is possible that some of the IND was in an amorphous state and also that HA was preventing the conversion of IND from the α form and the γ polymorphic form.

Looking at the IND loaded HA:CS PECNs at IND 0.2 mg/mg with a positive charge pre-washing showed very interesting behaviour for IND as it appeared to change between many polymorphs of IND during the heating process. This could be due to the fact that IND was present both associated with the HA:CS PECNs and free in suspension stabilised by the HA:CS PECNs. Comparing the IND melting peaks to those observed by Surwase et al. (2013) it would appear that IND was initially in the amorphous form and converted to the ζ , then α form and finally the γ form during the heating process.

When IND loaded HA:CS PECNs post-wash with 0.2 mg/mg of IND were tested using DSC, a small melting peak at 146.0°C was present. This was indicative of the α state of IND being present in the IND loaded HA:CS PECNs. There was no presence of the γ polymorphic form and so the polymers may have been preventing this conversion (e.g. HA) or the crystals with γ polymorphic form were removed by washing.

This shows that strict testing should be performed on PECNs loaded with drugs as there can be much variation in the forms of drug present associated with these delicately formed PECNs.

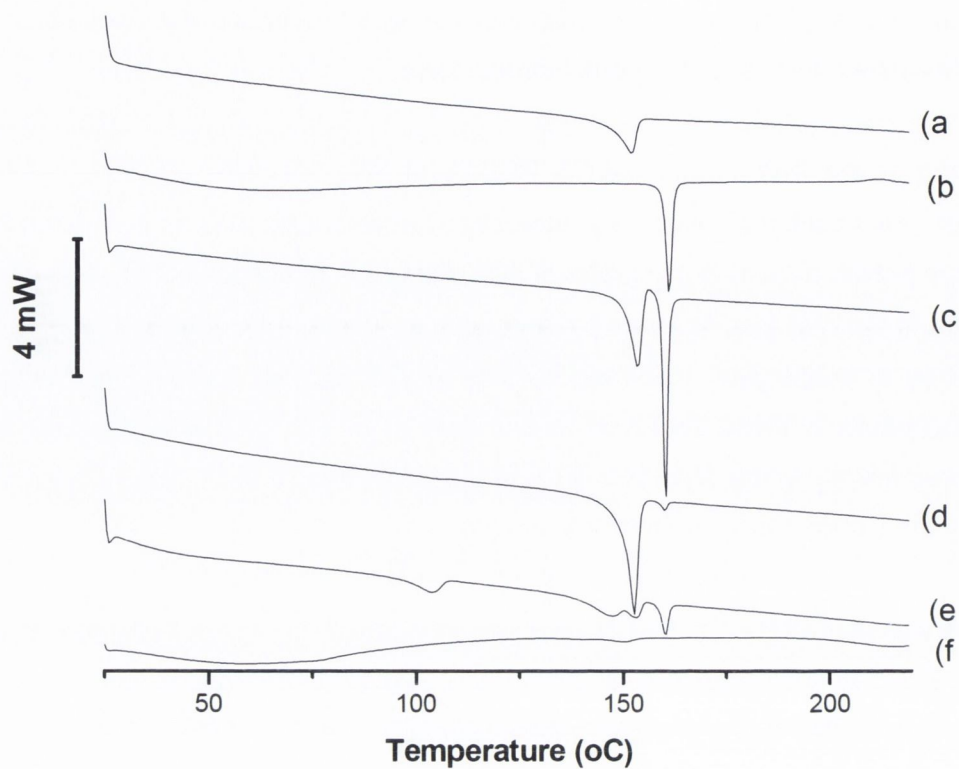


Figure 5.27 DSC traces for **a)** IND 0.2 mg/ml precipitated from 0.1% w/v HA solution, **b)** IND 0.2 mg/mg physical mix with the polymers HA and CS, **c)** IND 0.2 mg/ml precipitated from water, **d)** IND 0.2 mg/mg precipitated from 0.1% w/v CS solution, **e)** IND (0.2 mg/mg) loaded HA:CS PECNs (+ charge) pre-wash, **f)** IND 0.2 mg/mg HA:CS PECNs (+ charge) post wash.

Table 5.12 Melting points and heat of endothermic processes for DSC scans of IND 0.2 mg/mg precipitated from water, 0.1% w/v HA solution and 0.1% CS solution and IND 0.2 mg/mg as occurring in positively charged HA:CS PECNs pre and post washing.

System	Onset of endothermic process (°C)	Heat of endothermic process (J/g)
IND 0.2 mg/mg in Water	147.1 ± 0.40	42.9 ± 9.9
	156.9 ± 0.5	58.4 ± 1.9
IND 0.2 mg/mg in 0.1% w/v CS solution	145.6 ± 2.5	45.4 ± 17.6
	156.5 ± 1.1	24.5 ± 3.2
IND 0.2 mg/mg in 0.1% w/v HA solution	148.1 ± 2.4	2.6 ± 0.3
	160.2 ± 1.5	0.01 ± 0.1
IND 0.2 mg/mg loaded HA:CS PECNs (+ charge) pre-wash	97.0 ± 0.9	14.6 ± 0.1
	140.8 ± 0.2	8.8 ± 0.6
	150.7 ± 0.3	5.0 ± 0.7
	158.3 ± 0.2	11.1 ± 0.3
IND 0.2 mg/mg loaded HA:CS PECNs (+ charge) post-wash	146.0 ± 6.0	2.0 ± 2.3

5.2.3.4 Studies on IND Crystal Growth in HA:CS PECN Components

Studies on IND crystal growth in HA:CS PECN components were carried out using IND as it was interesting to look at the particle/crystal growth of IND in the components of the HA:CS PECNs given the variable results seen when IND was precipitated from HA:CS PECN component solutions.

IND ethanolic solution (1 mg/ 98.2 ul) was added to water, the individual polymer solutions (0.1% w/v HA and 0.1% w/v CS) and in the IND loaded HA:CS PECNs post formation all at a concentration of 0.1 mg/ml and 0.2 mg/ml as it was possible to form physically stable IND loaded HA:CS PECNs at these concentrations. DLS was used to track changes in the sample as crystals began to grow. The size increase and the polydispersity for these PECNs are represented in **Figure 5.28 - 5.31**. IND ethanolic solution (1 mg/ 98.2 ul) added

to water (0.2 mg IND/ml) showed rapid crystal precipitation with large particles appearing within 20 minutes of addition and rapidly sedimenting. When IND ethanolic solution (1 mg/ 98.2 ul) was added to 0.1% HA solution IND also began to crystallise rapidly forming large clusters of crystals which rapidly sedimented within 20 minutes. IND ethanolic solution (1 mg/ 98.2 ul) was added to 0.1% w/v CS solution took longer for IND crystals to form and grow. The sedimented material seen at the bottom of the nucleation vessel after 60 minutes was yellow in colour and had a much finer particle size than the clusters seen for 0.1% w/v HA solution. When IND loaded HA:CS PECNs were formed, they formed PECNs with a stable particle size and low polydispersity. When the IND 0.2 mg/mg loaded HA:CS PECNs pre-wash were monitored no crystal growth was seen at 90 minutes and post-wash good physical stability was maintained for 14 days (see **Section 5.2.2.3**). For the IND loaded HA:CS pre-wash samples at 90 minutes the particle size, ZP and PDI remained stable but the count rate began to rise indicating changes within the sample. This could be seen as IND crystal precipitation shortly after this time. This confirmed that IND in the HA:CS PECNs was more physically stable than IND alone with either of the polymers CS or HA. When IND precipitation was observed from the IND loaded HA:CS PECNs pre-wash it had a similar appearance to the yellow precipitate seen from 0.1% w/v CS solution than from 0.1% w/v HA solution or from water. The size increase and the PDI for IND 0.2 mg/mg HA:CS PECNs are represented in **Figure 5.28 - 5.29** The same pattern seen for IND 0.2 mg/mg was observed for the IND 0.1 mg/mg samples also indicating that the polymers were responsible for precipitation and not just the concentration of IND alone (**Figure 5.31 and 5.32**). **Figure 5.33** compares the count rate increase for empty positively charged HA:CS PECNs compared to the count rate for the positively charged HA:CS PECNs loaded with 0.1 mg/mg IND and 0.2 mg/mg IND. While the empty PECNs remained stable the count rate rose for the IND loaded PECNs with the count rate raising faster for IND 0.2 mg/mg due to the higher drug content. The rising count rate indicated changes within the sample and crystal growth. These samples were pre-wash so the full quantity of IND was present.

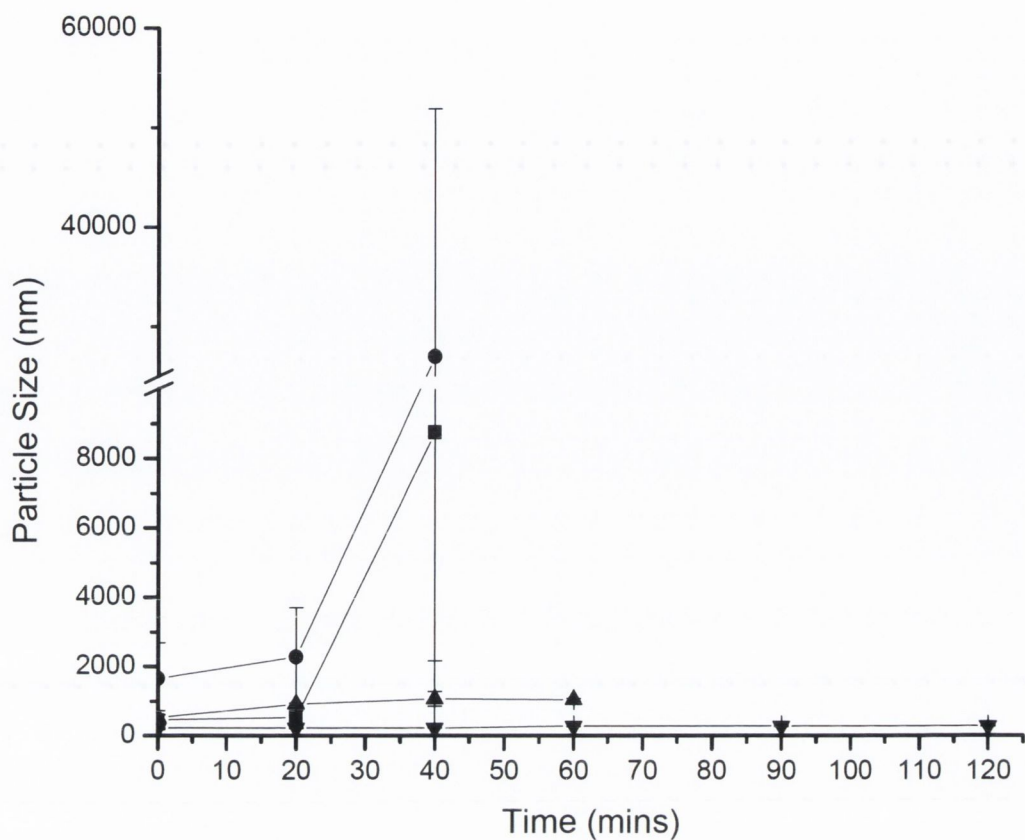


Figure 5.28 Particle size (nm) recorded over 120 mins for IND 0.2 mg/ml free in water and IND added to solutions of polymer 0.1% w/v HA and 0.1% w/v CS and HA:CS PECNs loaded with IND. ● = IND 0.2 mg/mg in 0.1 % w/v HA solution, ▲ = IND 0.2 mg/mg in 0.1% w/v CS solution, ▼ = IND 0.2 mg/mg in positively charged HA:CS PECNs, ■ = free IND 0.2 mg/mg

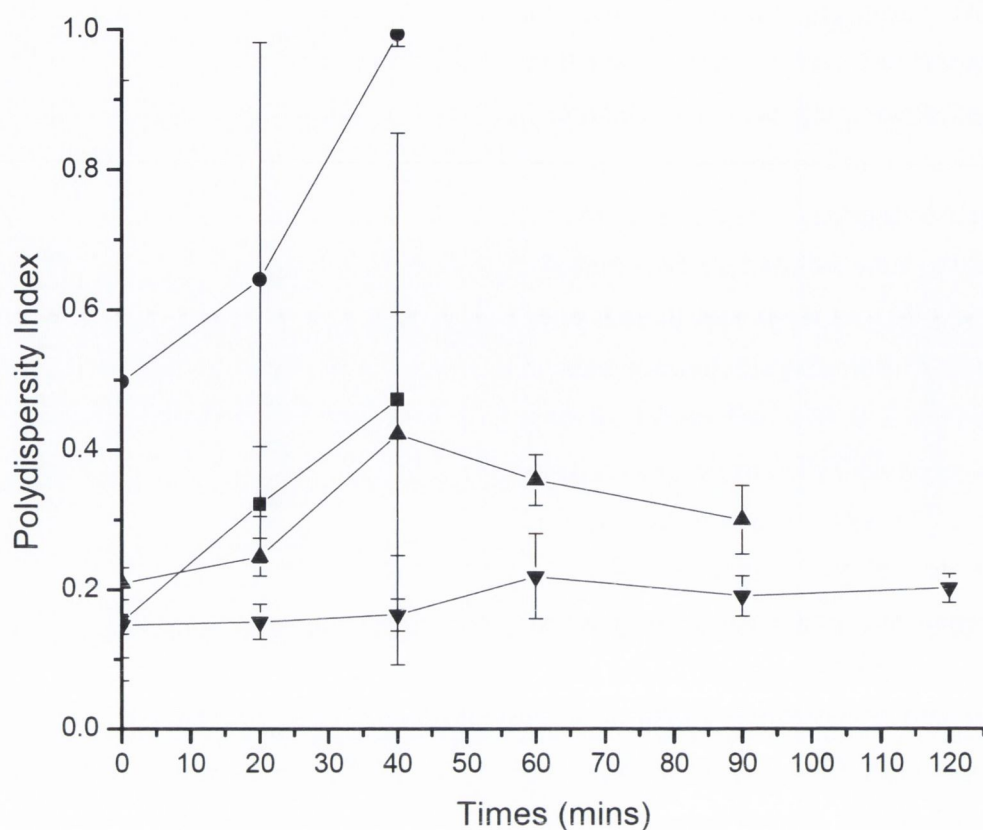


Figure 5.29 PDI recorded over 120 mins for IND 0.2 mg/ml free in water and IND added to solutions of polymer 0.1% w/v HA and 0.1% w/v CS and HA:CS PECNs loaded with IND. ● = IND 0.2 mg/mg in 0.1% w/v HA solution, ▲ = IND 0.2 mg/mg in 0.1% w/v CS solution, ▼ = IND 0.2 mg/mg in positively charged HA:CS PECNs, ■ = free IND 0.2 mg/mg.

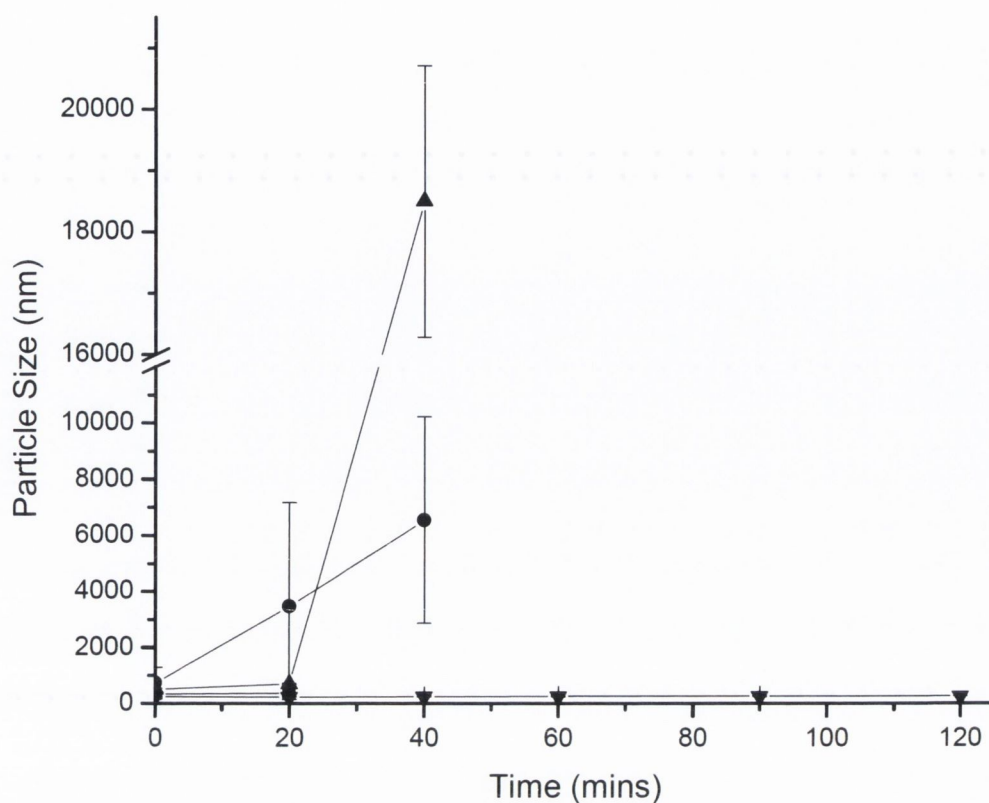


Figure 5.30 Particle size (nm) recorded over 120 mins for IND 0.1 mg/ml free in water and IND added to solutions of polymer 0.1% w/v HA and 0.1% w/v CS and HA:CS PECNs loaded with IND. ● = IND 0.1 mg/mg in 0.1% w/v HA solution, ▲ = IND 0.1 mg/mg in 0.1% w/v CS solution, ▼ = IND 0.1 mg/mg in positively charged HA:CS PECNs, ■ = free IND 0.1 mg/mg.

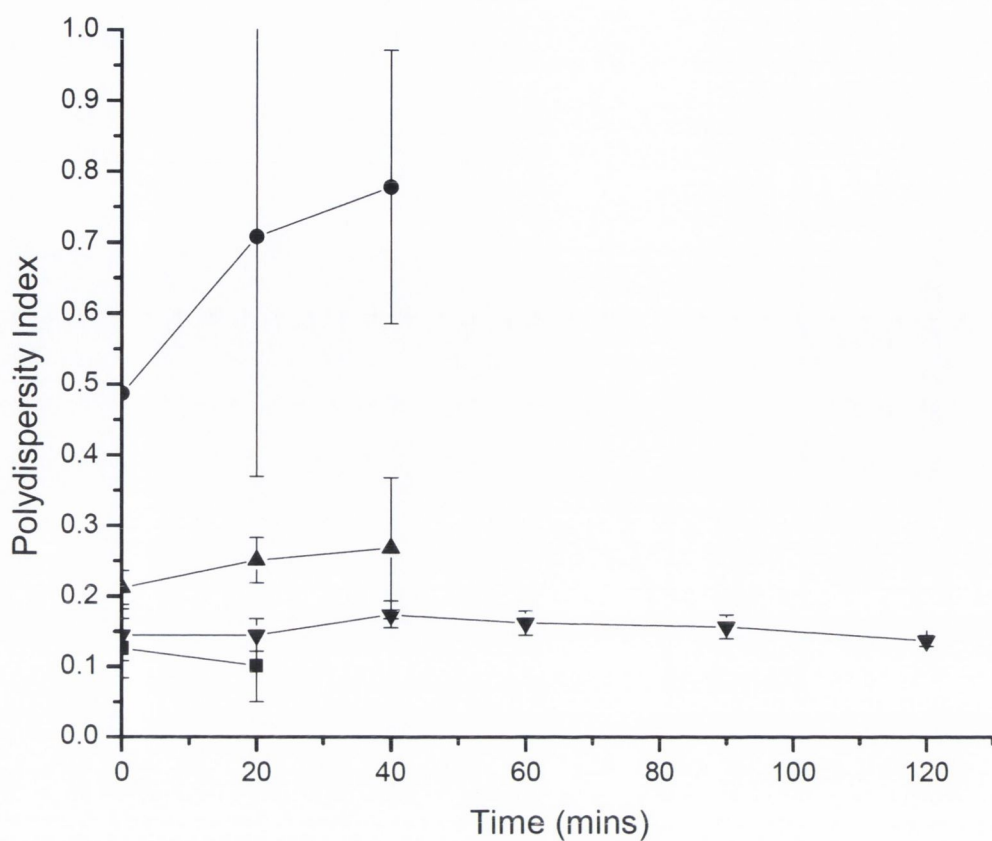


Figure 5.31 PDI recorded over 120 mins for IND 0.1 mg/ml free in water and IND added to solutions of polymer 0.1% w/v HA and 0.1% w/v CS and HA:CS PECNs loaded with IND. ● = IND 0.1 mg/mg in 0.1% w/v HA solution, ▲ = IND 0.1 mg/mg in 0.1% w/v CS solution, ▼ = IND 0.1 mg/mg in positively charged HA:CS PECNs, ■ = free IND 0.1 mg/mg.

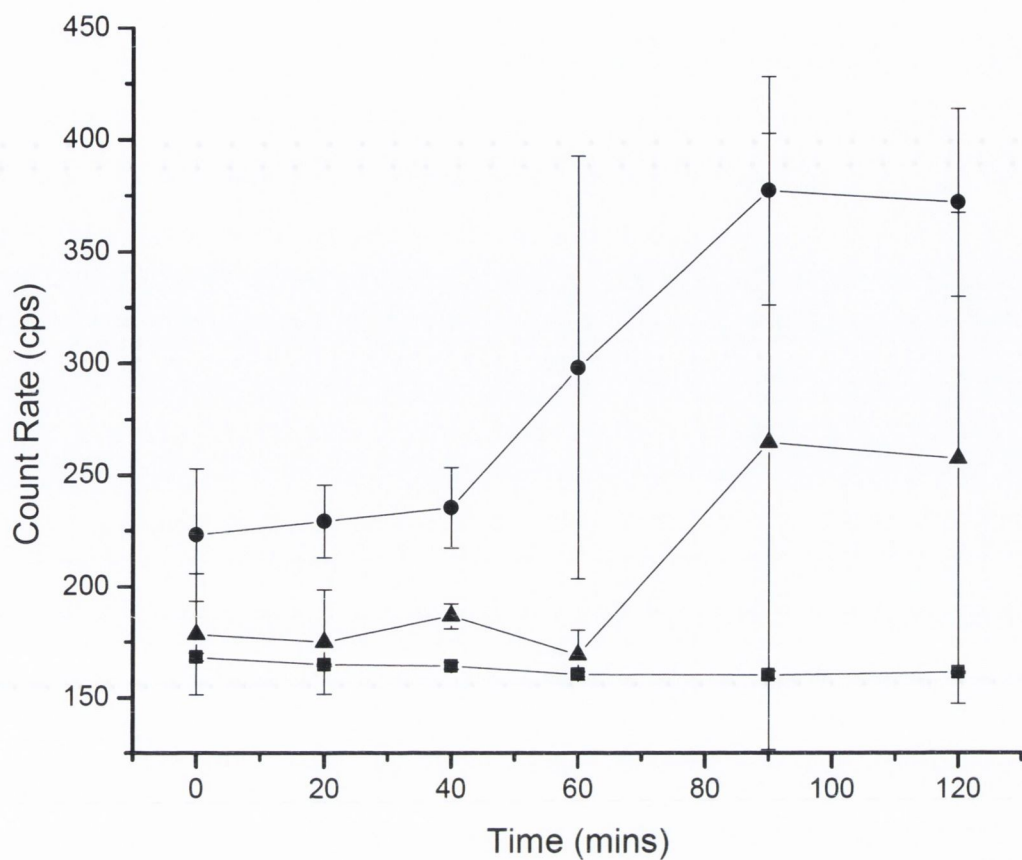


Figure 5.32 Count rate (cps) recorded over 120 mins period using DLS for positively charged HA:CS PECNs. ● = IND 0.2 mg/mg HA:CS PECNs, ▲ = IND 0.1 mg/mg HA:CS PECNs, ■ = Unloaded HA:CS PECNs.

5.3 Determination of IND-loaded HA:CS PECN Structure and the Solid-State Nature of IND in the HA:CS PECNs

The parameters describing IND association with the HA:CS polymers were taken into account to learn more about this association. The key factors for IND loaded HA:CS PECNs were DL, polymer ratio (which determined both weight ratio and charge ratio), solubility of IND in the polymer solutions, miscibility of IND in polymers and the solid-state behaviour of IND in the HA:CS PECNs (**Table 5.13**).

DL was higher for IND loaded positively charged PECNs (7.5:6.5 w/w) than for negatively charged IND loaded PECNs (**Table 5.13**). This means that pre-wash both positively and negatively charged HA:CS PECNs displayed the same DL but post-wash they had different amounts of drug present when they were being measured using DSC and PXRD. No significant differences in the DL values were observed between the positive and negative IND loaded HA:CS PECNs at 0.1 mg/mg DL, but there were significant differences in the DL at 0.2 mg/mg, this implies that the capacity of these HA:CS PECNs to load IND is lower for negatively charged than for positively charged HA:CS PECNs. Given that IND is an acidic drug with very low solubility it is assumed that electrostatic binding is important for the inclusion of IND in these HA:CS PECNs, while for KET the higher solubility in HA appeared to have a greater influence on the KET inclusion as greater loading was observed for negatively charged KET loaded HA:CS PECNs than positive.

Clear evidence for differences in drug binding between positively and negatively charged IND loaded HA:CS PECNs were observed during the solid-state analysis. Pre-wash positively charged IND loaded HA:CS PECNs showed DSC melting peaks at 0.4 mg/mg and 0.2 mg/mg and no peak at 0.1 mg/mg. Post-wash more clearly defined melting peaks were seen for all concentrations of IND loaded. Crystallisation for the lower IND 0.1 mg/mg concentration was induced by the agitation involved in the washing step and some of the crystalline IND for 0.2 mg/mg loading was removed but not all of the crystalline IND as had been observed for KET loaded HA:CS PECNs. This means that in the positively charged IND loaded HA:CS PECNs IND may have been present in an amorphous form to start or the drug may have complexed with CS (visible as a yellow precipitate in precipitation studies).

The post-wash drug content for positively charged IND loaded HA:CS PECNs at 0.1 mg/mg addition was 0.035 mg/mg and at 0.2 mg/mg addition was 0.133 mg/mg while the solubility of IND in the HA:CS PECNs polymer dispersion was measured as 0.005 ± 0.0001 mg/ml. Thus the quantity of IND loaded (post-wash) was above the solubility of IND in the polymer solutions for the positively charged samples so some of IND present was amorphous or else was not dissolved but most likely stabilised as crystals by the system. The crystallinity increased for these samples post-wash.

For the negatively charged HA:CS PECNs the degree of crystallisation reduced with the washing step. Pre-wash positively charged IND loaded HA:CS PECNs showed very small melting peaks at 0.4 mg/mg, 0.2 mg/mg and 0.1 mg/mg. Post-wash the melting peaks were still present but were less pronounced at all concentrations of IND loaded.

For negatively charged IND loaded HA:CS PECNs at 0.2 mg/mg addition the DL was 0.068 mg/mg post-wash and for 0.1 mg/mg it was 0.026 mg/mg. While the IND DL at 0.2 mg/mg was above the solubility of IND (0.029 ± 0.003 mg/ml) the IND 0.1 mg/mg DL was at the solubility saturation concentration and so most of the IND loaded at this concentration could have dissolved in the HA:CS PECN structure. This solubility at 0.1 mg/mg may have rendered these samples more stable to crystallisation during the washing step.

Pre-wash the negatively charged IND loaded HA:CS PECNs had a higher content of crystalline drug so this shows that the drug did not mix with the polymer as well as it did for the positively charged HA:CS PECNs so the drug seems to rely on the positive charge to bind. Post-wash in the negatively charged HA:CS PECNs and the positively charged HA:CS PECNs much of the crystalline drug was removed indicating that the initial crystals may not have bound or been loosely bound to the system.

The polymer ratio can be described in two ways: in terms of the weight ratio between the polymers and in terms of the charge ratio between the number of charges on the polymers, both of these are recorded in **Table 5.10**. These ratios are important in the formation of the IND loaded HA:CS PECNs. For the pre-wash positively charged IND loaded HA:CS PECNs at the 0.1 mg/mg IND concentration it was calculated that for the saturation of the negative charges

from HA and IND combined by the positive CS charges was 63.1%. For the IND concentration 0.2 mg/mg the pre-wash IND loaded into HA:CS PECNs positive CS charges were saturated by the negative charges from HA and IND at 73.5%. These IND loaded HA:CS PECNs all had a positive ZP, therefore a positively charged surface which indicates that the IND associated with the HA:CS PECNs by charge but not to the point of saturating the positive charge or neutralising it. Once these systems had been washed the charge ratio dropped with saturation of positive charges being 56.8% at IND 0.1 mg/mg and 59.1% at IND 0.2 mg/mg. Post wash the DL resulted in charge ratios for IND 0.1 mg/mg and IND 0.2 mg/mg that were much closer in value than pre-wash indicating that stability may only be possible in a narrow range of charge ratio values. For these positively charged IND loaded HA:CS PECNs the positive charge was in excess by almost 50%.

For negatively charged IND loaded HA:CS PECNs pre-wash for the IND concentration of 0.2 mg/mg the negative charges from HA and IND saturated the positive CS charges by 147.9% and 0.1 mg/mg was 133.6%. Post-wash these values dropped to 142.4% for 0.2 mg/mg IND and 131.0% for 0.1 mg/mg IND which were smaller reductions than had been observed for the negatively charged HA:CS PECNs and this may have contributed to their increased physical stability over the positively charged HA:CS PECNs.

Table 5.13. Comparison of key physicochemical properties of IND loaded HA:CS PECNs (positively charged: 7.5:6.5 and negatively charged: 10:4)

HA:CS weight ratio	Theoretical IND loading (pre wash) (mg/mg)	Actual IND loading (post wash) (mg/mg)	Quantity of IND by DSC (pre-wash) (mg/mg)	Quantity of IND by DSC (post-wash) (mg/mg)	% saturation of + charge (pre-wash)	% saturation of + charge (post-wash)	Quantity of IND released at 60 min (mg/mg)
7.5:6.5	0.1	0.035 ± 0.007	0	0.0228	63.1	59.1	0.038 ± 0.001
7.5:6.5	0.2	0.133 ± 0.005	0.0034	0.0769	73.5	56.8	0.138 ± 0.013
10:4	0.1	0.026 ± 0.006	0.0255	0.0010	133.6	131.0	0.032 ± 0.005
10:4	0.2	0.068 ± 0.003	0.0685	0.0120	147.9	142.4	0.046 ± 0.005

DSC and PXRD data (**Table 5.13**) was used to estimate how much of the drug was incorporated into the IND loaded HA:CS PECNs complexed as crystals associated with the HA:CS PECNs. Post-wash there was an increased content of crystalline IND present for the positively charged HA:CS PECNs loaded with IND so the crystals may have grown during the washing step. For negatively charged HA:CS PECNs a lower content of crystalline IND was post-wash. IND was present in the negatively charged HA:CS PECNs at lower concentration and if it was incorporated by dissolution in the polymers as was suspected for KET loaded HA:CS PECNs, then the physical stability of these negatively charged HA:CS PECNs may have been greater during the washing step.

The melting peaks for post-wash positively IND loaded HA:CS PECNs indicated that a greater quantity of IND was in a crystalline state than before the washing step. The washing step is likely to have induced crystallisation because the IND content was so high above the solubility limit. The positive charge remained in excess for these positively charged IND loaded HA:CS PECNs and this may have been the reason that the IND loaded HA:CS PECNs were stable with a higher DL than IND. The amount of crystalline IND present for IND 0.2 mg/mg loading post-wash was quantified and it was found to be 0.068 mg/mg which increased from 0.0034 mg/mg before the washing step. This meant that post-wash 77% of the IND present was crystalline. For 0.1 mg/mg IND addition the quantity of IND crystals pre-wash was 0 mg/ml and post-wash was 0.0228 mg/mg. This meant that the total crystalline drug for IND 0.1 mg/mg was 57% of the total loaded IND post-wash.

The melting peaks for post-wash negatively IND loaded HA:CS PECNs indicated that a lower quantity of IND was in a crystalline state than before the washing step. The washing step appears to have removed IND crystals as the crystalline IND content was higher before the washing step. The quantity of crystalline IND present pre-wash for IND 0.2 mg/mg loading was quantified and it was found to be 0.0769 mg/ml which decreased to 0.0120 mg/mg after the washing step. This meant that post-wash 18% of the IND loaded was crystalline. For 0.1 mg/mg IND the quantity of IND crystals pre-wash was 0.026 mg/mg and post-wash was 0.0010 mg/mg. This meant that the total crystalline drug for IND 0.1 mg/mg was 4% of the total loaded IND post-wash.

These results showed that IND was incorporated in HA:CS PECNs by different mechanisms than were observed for KET loaded HA:CS PECNs. The miscibility studies and precipitation studies for IND loaded HA:CS PECNs showed that IND was not miscible with the polymers to the same degree as KET with crystal growth observed. Some stabilising interactions were present as the IND crystals precipitate in the HA:CS PECNs were smaller and less clustered than for free IND precipitated in water. Given that more IND crystals were present for positively charged HA:CS PECNs one would question their use as IND was observed to have different polymorphic forms which may be present simultaneously or transition between types. These forms had different solubility values and thus this could affect the dissolution and delivery of IND. The physical stability of positively charged HA:CS PECNs was observed to be less than that of negatively charged HA:CS PECNs with a decrease in particle size observed over the 14 day period. Negatively charged HA:CS PECNs, despite having a lower drug loading, appeared to have more drug present in the amorphous form and showed greater physical stability over 14 days. For this reason, like with KET, the negatively charged HA:CS PECNs were preferred although their low limit for drug loading must be noted.

5.4 Conclusions

HA:CS PECNs were successfully loaded with IND both as positively and negatively charged HA:CS PECNs but many unique differences were observed compared to empty HA:CS PECNs or KET loaded HA:CS PECNs. As with the empty carriers the parameters involved in their formation were investigated: polymer weight ratio, pH of HA and solvent choice. These investigations resulted in the conclusion that for IND loaded HA:CS PECNs 7.5:6.5 was the optimal weight ratio for positively charged particles and 10:4 was the optimal weight ratio for negatively charged particles. Ethanol was again the most suitable solvent and the pH of HA did not show advantages when altered from pH 5.7. Stirring time was seen to affect the formation of IND loaded HA:CS PECNs with crystal growth being induced with time.

Like for KET, three different concentrations of drug were tested for loading. These were 0.1 mg/mg, 0.2 mg/m and 0.4 mg/mg. 0.4 mg/mg proved to be beyond the capacity for IND in these HA:CS PECNs with aggregation observed

soon after formation for positively charged HA:CS PECNs and aggregation observed within minutes for the negatively charged HA:CS PECNs. The DL at IND 0.1 mg/mg and IND 0.2 mg/mg was higher for positively charged IND loaded HA:CS PECNs than for negatively charged IND loaded HA:CS PECNs with a higher crystalline content of IND also being observed. This indicates that a greater degree of loaded IND in the positively charged HA:CS PECNs was based on electrostatic interactions.

For negatively charged IND loaded HA:CS PECNs the IND loaded was lower but also the crystalline IND loaded formed a lower percentage of the total DL for these HA:CS PECNs. This indicated that amorphous IND may have been present in these PECNs.

As was the case for KET loaded HA:CS PECNs the charge ratio of the HA:CS PECN systems showed stability only within a narrow range of charge ratio values. IND loaded HA:CS PECNs formed best when the ratio of NEG:POS charges was 2:1 or 1:2 with the least chance of stable HA:CS PECN formation close to a charge ratio of 1.

The isoelectric point for positively charged HA:CS PECNs was pH 6.4 while that for negatively charged PECNs was pH 3.0. This difference reflects the addition of IND to the systems.

The negatively charged IND loaded systems remained stable for a 14 day period but for positively charged HA:CS PECNs the particle size reduced to a notable degree over 14 days of storage.

Rapid drug release was observed from these systems with all the IND being released within 60 min. This was the case regardless of the charge or the concentration of drug loaded. The release medium did affect the IND release from HA:CS PECNs with each release medium displaying a different release profile. The greatest release of IND was seen in PBS where the concentration of IND released was close to the actual drug loading. In 0.01M HCl very low IND release was observed for all samples. Deionised water showed a release profile in which neither negatively nor positively charged HA:CS PECNs showed greater release. It was concluded that IND release in these media was dependent on medium pH.

The solid-state properties of IND were investigated in the HA:CS PECNs to learn about how it was present in the HA:CS PECNs. Pre-wash some loosely bound IND was present as drug crystals which were observed as a melting point during DSC analysis for 0.4 mg/mg and 0.2 mg/mg IND concentration for positively and negatively charged IND loaded HA:CS PECNs. This drug may have been loosely bound as crystals or nanocrystals while the remaining drug was assumed to have formed a complex with the polymers or to be in an amorphous state. While some Bragg peaks were seen pre-washing at 0.4 mg/mg and 0.2 mg/mg for negatively charged KET loaded HA:CS PECNs (which had a higher drug content) they were not seen for positively charged KET loaded HA:CS PECNs. Post wash the melting point of IND was observed at all IND concentrations (0.4 mg/mg, 0.2 mg/mg and 0.1 mg/mg) for positively charged HA:CS PECNs indicating that crystallisation had been induced during washing. For negatively charged IND loaded HA:CS PECNs the melting point values for IND were reduced after washing indicating that crystalline IND had been removed. The remaining non-crystalline IND was either amorphous or in a complex with the polymers as no melting peaks were observed using DSC and there were no diffraction peaks present on PXRD. PXRD and DSC complemented each other although DSC may have picked up on some polymorphic transitions for IND on heating.

IND did not show good miscibility with the polymers as two phases were seen when the polymers and IND were mixed in solution and allowed to dry. Crystalline IND precipitated out showing that IND had more limited miscibility than KET where only one phase had been seen. This was further explored using precipitation studies and by monitoring crystal growth when IND was added to the polymer solutions. IND was capable of precipitating in different polymorphic forms although there was some evidence that HA may prevent the conversion of the α form and the γ polymorphic form. Crystallisation studies showed that the combination of HA:CS polymers prevented IND crystallisation for longer than water, HA or CS solution.

Given that the crystalline forms of IND could add unpredictable complications to IND loaded HA:CS PECNs the negatively charged HA:CS PECNs with lower crystalline IND content was considered a more useful formulation

The Compatibility of Polymeric Nanocarriers Unloaded and Drug Loaded with Platelets

Chapter 6

6.1 Introduction

The aim of these studies was to evaluate the potential for the NCs, PLGA and HA:CS systems, to deliver the anti-platelet drugs IND and KET. This was conducted *in vitro* on human blood platelets using aggregometry and flow cytometry.

This current work followed on from studies published in the paper "Platelet compatibility of PLGA, CS and PLGA-CS nanoparticles" (Li et al. 2009). Li et al. (2009) discovered that empty NCs made from PLGA, and CS incubated with resting platelets at concentrations up to 500 µg/ml did not induce platelet aggregation (0 ± 2 % light transmittance). Very high concentrations of PLGA and CS NPs (100 µg/ml) weakly (10–40 %) inhibited collagen-induced aggregation and P-selectin expression.

Li et al. (2009) used aggregometry and flow cytometry to demonstrate that PLGA and CS–PLGA NPs inhibit collagen-induced platelet aggregation in a concentration-dependent manner with the maximal effect of 21 ± 3 % and 17 ± 4 %, respectively at 100 µg/ml. A significant inhibition of the collagen effect on platelet aggregation was achieved with PLGA concentrations of at least 10 µg/ml ($p < 0.05$ vs collagen) and all concentrations of CS–PLGA (0.01 µg/ml, 0.1 µg/ml, 1 µg/ml, 10 µg/ml and 100 µg/ml). In the current work the Protasan ultrapure CS CL113 was used. Li et al. (2009) used CS NPs made from other CS types, such as CS–F, CL113, CL213 and G213 with resting platelets. At concentrations ranging from 0.01 µg/ml to 100 µg/ml platelet aggregation was not induced (CL = chloride and G = glutamate, mw of CL113 = 50,000 - 150,000 g/mol and mw of CL213 = 150,000 - 400,000 g/mol). CL113, CL213 and G213 NPs maximally inhibited aggregation by 40 ± 5 %, 37 ± 25 % and 17 ± 9 %, respectively, at the concentration of 100 µg/ml. CL113 NPs effectively inhibited the collagen-induced platelet aggregation even at a concentration of 10 µg/ml ($p < 0.05$ vs collagen) and thus was significantly different than the other batches of CS NPs. For this reason it was decided to use CL113 in the current studies.

Ramtoola et al. (2011) looked at the ability of PLGA (Resomer RG 504 H) and CS (Sigma-Aldrich, mw of 240 kDa) coated PLGA NCs to inhibit ADP induced platelet aggregation. They found that at NP concentrations between 0.01 – 500

ug/ml incubation of platelets with CS-PLGA or PLGA NPs before the addition of ADP did not inhibit aggregation of platelets by ADP ($p > 0.05$). Although Li et al. (2009) reported that a high concentration of PLGA and CS particles (100 ug/ml) resulted in weak inhibition of aggregation (10–40 %). Ramtoola et al. (2011) concluded that unlike Li et al. (2009) their NPs did not interact with platelets to inhibit platelet aggregation.

6.2 Types of NCs Used For Aggregometry and Flow Cytometry

There were differences between the empty PLGA NPs prepared previously by Li et al. (2009) and those PLGA NPs used in this current study (**Table 6.1 and 6.2**). The methods used for preparation were the same but the NPs produced for this study had a smaller particle size. This may have been due to factors such as different batches of solvents and materials (polymer batches) used, the methods of centrifugation and operator differences. A direct comparison was therefore not possible between the PLGA NPs used in this current study and those prepared by Li et al. (2009).

CS was tested previously by Li et al. (2009) for compatibility with the platelets but the use of HA:CS PECNs was a novel experiment in the current studies. This formulation was chosen as it displayed rapid drug release (**Chapters 4 and 5**) while the PLGA NPs showed an initial burst release followed by slower release (**Chapter 3**). HA was used as the second polymer to form the polyelectrolyte complexes as it has been shown by Verheye et al. (2000) to reduce thrombus formation on stainless steel stents used as endovascular devices. Mitchell et al. (1994) also found HA solution to be efficacious in the prevention of pericardial adhesions and HA solution appeared to be safe in doses five times the amount needed to prevent adhesions.

It was decided to test PLGA NPs and HA:CS PECNs loaded with the NSAIDs KET and IND which are known to have anti-platelet activity (Leach and Thorburn 1982, Petrusiewicz et al. 2005, Schafer 2005 and Niemi et al. 2007) to see if the NP type would influence the drug activity and to investigate if these NPs could be a viable option for delivering these drugs.

Table 6.1. Physical properties of PLGA, CS–PLGA and CS NPs taken from “Platelet compatibility of PLGA, CS and PLGA-CS nanoparticles” (Li et al. 2009). CS – PLGA = PLGA - chitosan chloride NPs, CL113 = chitosan chloride, PLGA = Poly(lactic-co-glycolic) acid.

Type	Size (nm)	ZP (mV)
PLGA	640 ± 150	- 17 ± 1
CS–PLGA	580 ± 230	- 6 ± 1
CL113	580 ± 50	- 12 ± 1

Table 6.2. Physical properties of the NCs used in current experiments. KET = ketoprofen, IND = indometacin, CS = chitosan (as CL113), HA = hyaluronate, PLGA = Poly(D,L-lactide-co-glycolide).

Type	Size (nm)	ZP (mV)	PDI	EE (% w/v)	DL (% w/w)
HA:CS Pos	173 ± 2	+ 33 ± 1	0.10 ± 0.02	N/A	N/A
HA:CS Pos KET	187 ± 2	+ 34 ± 1	0.10 ± 0.05	28.0 ± 4.5	5.3 ± 0.8
HA:CS Pos IND	196 ± 1	+ 38 ± 3	0.24 ± 0.05	62.5 ± 2.4	13.1 ± 0.5
HA:CS Neg	192 ± 1	- 32 ± 5	0.12 ± 0.02	N/A	N/A
HA:CS Neg KET	173 ± 1	- 28 ± 1	0.10 ± 0.03	63.1 ± 5.6	11.4 ± 1.1
HA:CS Neg IND	186 ± 1	- 36 ± 1	0.14 ± 0.05	17.6 ± 1.7	3.5 ± 0.3
PLGA	267 ± 3	- 41 ± 1	0.08 ± 0.02	N/A	N/A
PLGA IND	323 ± 8	- 35 ± 1	0.20 ± 0.10	93.2 ± 2.2	19.0 ± 0.4
PLGA KET	316 ± 3	- 33 ± 1	0.31 ± 0.09	41.3 ± 11.1	12.0 ± 3.0

6.3 Aggregometry Studies

6.3.1 Empty Carriers

Three different types of NCs were used for these experiments; PLGA, HA:CS negatively charged and HA:CS positively charged as described in **Chapter 3, 4** and **5**, respectively. It was established in previous work by Li et al. (2009) that non drug loaded PLGA NPs did not induce platelet aggregation when incubated with platelets alone and thus these PLGA NPs were compatible with platelets.

This current work verified the PLGA NPs compatibility with platelets by again testing the platelet response to being incubated with empty PLGA NPs. No aggregation was seen over the entire incubation period (**Figure 6.1**). This was also completed for the HA:CS empty PECNs with both negative and positive charge for the first time. It was essential to be sure that the NCs were platelet compatible before testing them while loaded with KET and IND. For the HA:CS PECNs no platelet aggregation was seen over the duration of the incubation time. This was true for those with a positive charge and those with a negative charge (**Figure 6.1**).

None of the NCs tested prevented platelet aggregation once stimulated with collagen (2 ug/ml) at the highest concentration of empty NCs tested (80 ug/ml). This shows that the NCs did not interfere with the action of collagen on the platelets.

The PLGA, HA:CS positively charged (pos) and HA:CS negatively charged (neg) NCs did not induce platelet aggregation or prevent collagen induced aggregation at their maximum concentration used for these experiments (80 ug/ml). This was assumed to be a high concentration to use as it was double the maximum concentration used for the drug loaded NCs in this chapter. **Figure 6.1** shows that over 25 minutes (min) of incubation post addition of the NCs minimal aggregation was seen.

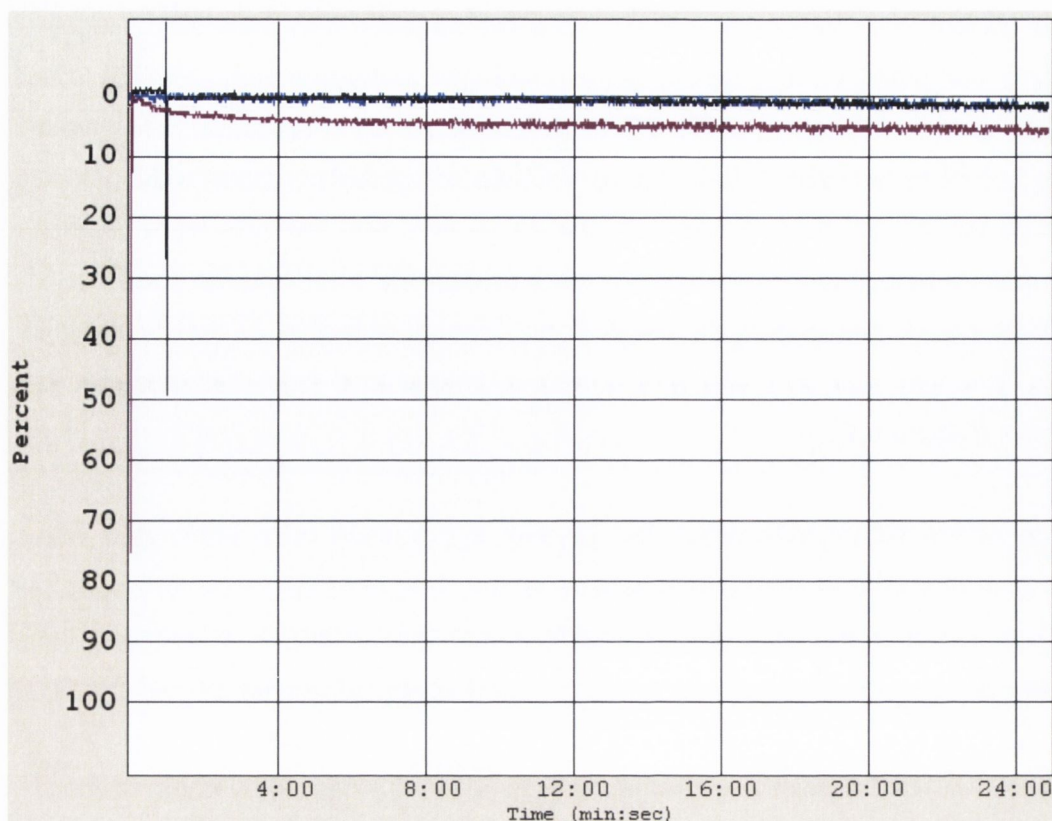


Figure 6.1 Aggregometry traces of empty NCs incubated with platelets for 25 min. The NCs were added after 2 min at a concentration of 80 ug/ml. Blue = PLGA NPs, Red = HA:CS PECNs Neg, Black = HA:CS PECNs Pos.

These NCs were compatible with platelets but it was unknown what effect they would have on drugs that are known to affect platelet aggregation. Once it was known that the NCs were compatible it was decided to load them with KET and IND. Both of these drugs prevent platelet aggregation.

6.3.2 Drug Loaded NCs

As observed in the previous **Chapters 3, 4 and 5**, all of the KET and IND loaded PLGA NPs and HA:CS PECNs prepared had higher DL at 0.2 mg/mg than 0.1 mg/mg. For this reason it was decided to focus on one concentration of DL in this chapter - 0.2 mg/mg.

Free KET had a potent effect on the inhibition of collagen stimulated platelet aggregation. The reversible inhibition of *in vitro* aggregation induced by KET was investigated by research groups including Gandini et al. (1983) who studied the effect of KET on ADP, epinephrine and collagen induced platelet aggregation in

eleven patients with a daily intravenous dose (600 mg) of KET being given on days 4 and 8 during a 7-day treatment (200 mg i.v. every 8 hours). Significant reduction of platelet aggregation was observed for KET with complete or almost complete recovery 36 hours after the last dose. They concluded that KET affects platelets with readily reversible inhibition of *in vitro* aggregation and a slight increase of bleeding time.

Similar reversible inhibition of platelet aggregation has also been reported with IND for example Cheung et al. (1999) examined collagen-induced (5 µg/ml) platelet aggregation in premature infants. IND, given in 3 intravenous doses (0.2 mg/kg followed by 0.1 mg/kg at 12 and 36 hours later) significantly inhibited the *ex-vivo* platelet aggregatory response with a 54% decrease in aggregation. Full normalisation of platelet function occurred 3 to 7 days later.

6.3.2.1 KET Loaded PLGA NPs

While it was decided to use one concentration of DL for these experiments (0.2 mg/mg) two concentrations were tested for one of the NC types to verify that the 0.1mg/mg DL samples were not likely to show advantages. For this reason two concentrations of KET (0.1 mg/mg and 0.2 mg/mg) were loaded initially into PLGA NPs. This resulted in DL for KET 0.1 mg/mg PLGA NPs of 2 ± 1 % and for KET 0.2 mg/mg NPs 12 ± 3 %.

The ability of KET loaded PLGA NPs at 0.1 mg/mg to inhibit aggregation did not show any advantages over PLGA NPs loaded with KET at 0.2 mg/mg. The only significant differences from the collagen treated sample were seen at KET 0.2 mg/mg loading after 10 min of incubation ($p < 0.001$). So it was concluded that further experiments would be narrowed to the 0.2 mg/mg concentration only (Figure 6.2).

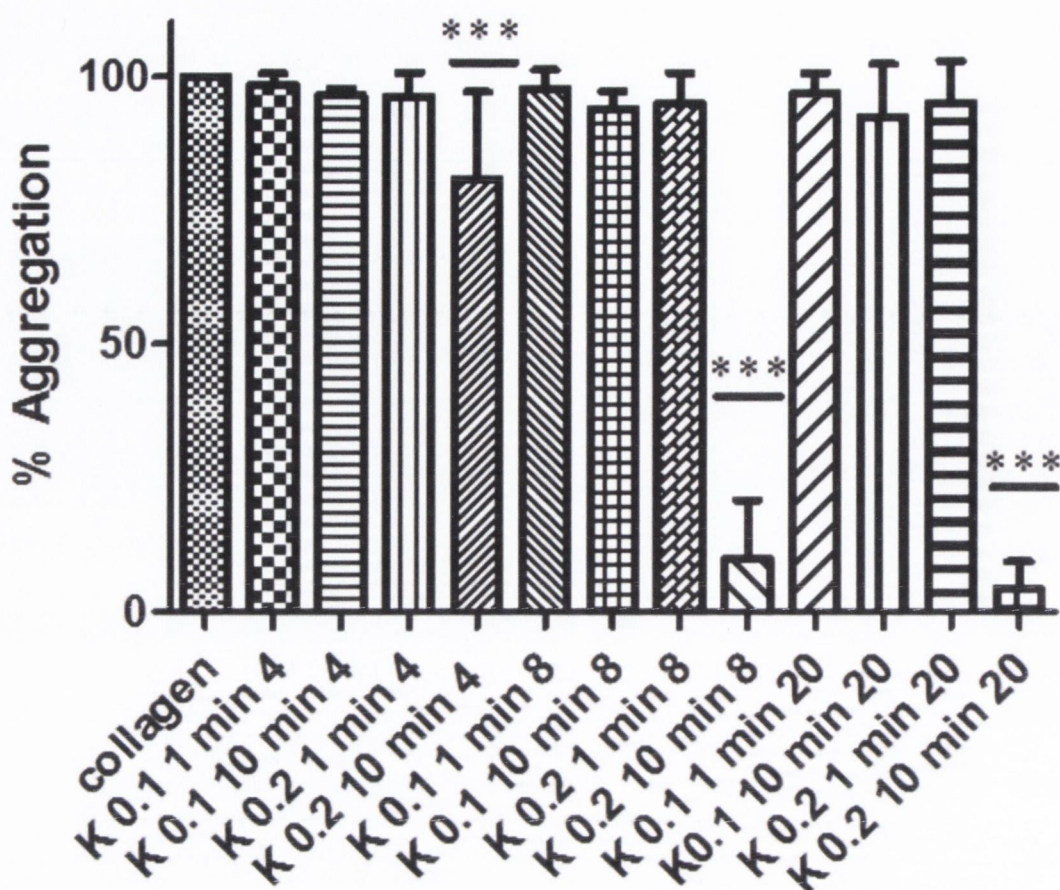


Figure 6.2 A comparison of KET 0.1 mg/mg loaded PLGA NPs vs 0.2 mg/mg PLGA NPs. Samples are shown post 1 minute (min) and 10 min incubation. Only KET 0.2 mg/mg samples after 10min showed significant differences to collagen (2 ug/ml) ($p < 0.001$). K = KET, 1 min and 10 min refer to incubation time and 4, 8, 20 are the concentration of NPs added in ug/ml. (***) = $p < 0.001$).

The % aggregation observed when PLGA KET NPs were added to the platelets prior to collagen (2 ug/ml) addition was significantly different to the effect of collagen alone only when the incubation time of PLGA KET NPs with platelets prior to collagen (2 ug/ml) addition was 10 min (**Figure 6.2**). One min did not show significant effect compared to collagen for KET 0.1 mg/mg or KET 0.2 mg/mg PLGA NPs. This is most likely due to the drug being released over time once the PLGA KET NPs were added to the platelet samples. The DL in the 0.1 mg/mg KET loaded PLGA NPs may not have been high enough to influence the platelet aggregation as at 0.2 mg/mg loading the actual observed loading was 10% higher than at 0.1 mg/mg.

The effect of KET 0.2 mg/mg loaded PLGA NPs alone can be observed in **Figure 6.3**. Significant differences were found between 1 and 10 min incubation time of the KET loaded PLGA NPs with the platelets prior to addition of collagen (2 ug/ml). The p-value for the difference in inhibition at 4 ug/ml of KET PLGA NPs with platelets at 1 min and 10 min was < 0.05 with a decrease in aggregation from $95 \pm 5 \%$ to $76 \pm 17 \%$. At higher PLGA KET NPs concentrations of 8 ug/ml and 20 ug/ml the p value was < 0.001 and thus the difference between 1 and 10 min incubation time, which was highly significant for these PLGA KET NPs. The decrease in aggregation for 8 ug/ml was $92 \pm 4 \%$ to $5 \pm 9 \%$ (1 vs. 10 min incubation) and for 20 ug/ml was $92 \pm 11 \%$ to $3 \pm 3 \%$ (1 vs. 10 min incubation).

At 10 min incubation all concentrations of KET loaded PLGA NPs were significantly different to collagen ($p < 0.001$).

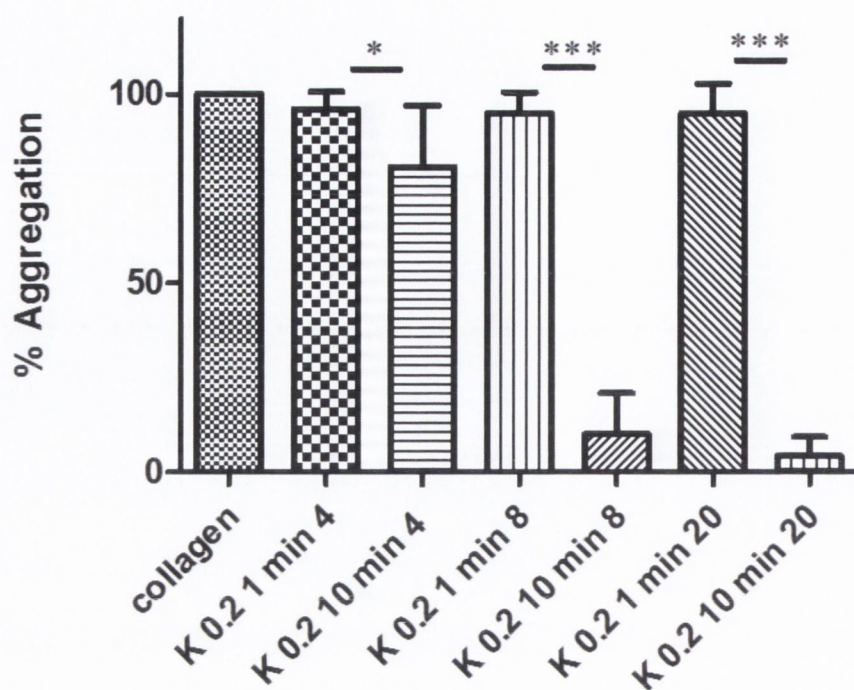


Figure 6.3 PLGA KET loaded NPs. A comparison of the ability of KET to inhibit the aggregation induced by collagen (2 ug/ml). Significant effects were seen between 1 and 10 min for all concentrations with greater differences at higher concentrations. (* = $p < 0.05$, *** = $p < 0.001$) K = KET, 1 min and 10 min refer to incubation time and 4, 8, 20 are the concentrations of NPs added in ug/ml. At 10 min all samples were significantly different to collagen ($p < 0.001$).

If the DL was taking into account for these PLGA KET NPs it was possible to look at the action of the KET PLGA NPs in comparison to a solution of free KET (**Figure 6.4**). The concentration of KET in the PLGA NPs was calculated and compared to samples of free drug but it was not possible to match the concentrations of drug exactly for each sample due to variations in DL. PLGA KET NPs after 1 min of incubation with the platelets showed activity lower than that seen with KET solution at approximately the same drug concentration. Free KET showed maximum inhibition of platelet aggregation at 1 ug/ml and beyond this concentration the effect of KET did not increase the reduction of platelet aggregation. Petruszewicz et al. (1994) stated that the concentration of KET that could inhibit 10 ug/ml of collagen had an IC_{50} at 5.30×10^{-6} M (1.3 ug/ml) and an IC_{25} at 7.20×10^{-7} M (0.2 ug/ml). This collagen concentration was higher than that for the current work (2 ug/ml) but studies by Leach and Thorburn (1982) demonstrated that the difference between human platelet inhibition *in vitro* was

not considerably different at different concentrations of collagen addition (1 ug/ml – 5 ug/ml). Thus KET was expected to have IC_{50} values lower than those found for 10 ug/ml of collagen but the values were not predictable by a directly proportional amount.

At an incubation time of 10 min of incubation a significant increase in the level of inhibition of platelet aggregation was shown. The inhibition of aggregation seen after 10 min of incubation became closer to that observed for the free KET solution than that witnessed for KET PLGA NPs after 1 min of incubation. After 10 min of incubation the inhibition of aggregation rose more slowly than that seen for free KET solution but both reached the same level of inhibition (free KET solution $92 \pm 6 \%$ and PLGA KET $97 \pm 6 \%$). While the inhibitory effect of KET solution levelled off at higher concentrations than 1 ug/ml, further inhibition could be observed for KET above this concentration when loaded into PLGA KET NPs, which had undergone 10 min of incubation. This may be due to drug being continuously released from the PLGA KET NPs and inhibiting aggregation while the effect for free KET may be less due to competition at the receptor sites. It may also be due to steric effects of the PLGA NPs or synergistic effects between the PLGA and the KET.

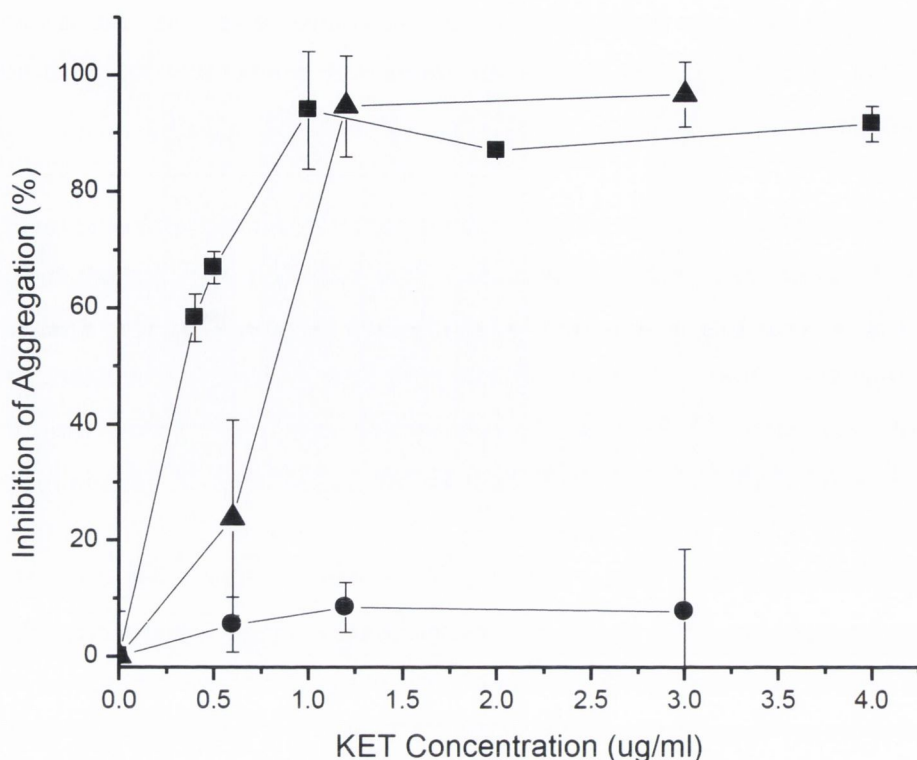


Figure 6.4 Comparison of the ability of KET to inhibit platelet induced aggregation between free KET solution, PLGA KET loaded NPs after 1 and 10 min. ■ = free KET, ● = PLGA KET 1 min, ▲ = PLGA KET 10 min.

6.3.2.2 KET loaded HA:CS Polyelectrolyte Complex NPs (PECNs)

6.3.2.2.1 KET loaded HA:CS PECNs - Positive Charge

Platelets have a negative surface charge and thus it could be assumed that HA:CS KET loaded PECNs with a positive charge would have been more attracted to the platelet surface than the negatively charged HA:CS PECNs or PLGA NPs. Li et al. (2009) have contradicted this idea as they found no relationship between their PLGA, PLGA-CS and CL113 NPs and the platelet surface based on charge. There have also been arguments against the use of positively charged PECNs in the bloodstream. For example Dobrovolskaia et al. (2008) found that the presence of unprotected primary amines (positive charges) on the surface of polyamidoamine (PAMAM), carbosilane, polypropylene imine and polylysine dendrimers was associated with erythrocyte damage in a dose-dependent manner.

Positively charged HA:CS KET loaded PECNs quickly release the cargo with the complete drug load being released rapidly within thirty min to one hour (**Chapter 4**). According to the studies on the isoelectric point determination of these PECNs the positively charged HA:CS PECNs (**Chapter 4**) have an isoelectric point at pH 6.8. Thus, they may be stable at the blood pH value of 7.4 but their stability would be very sensitive to fluctuations in the ionic content of the plasma. The DL for these KET loaded HACS PECNs was 5.3 ± 0.8 % when an initial concentration of 0.2 mg/mg of drug was added. This meant that there was 0.05 mg KET for every 1 mg of HA:CS polymers present.

For the positively charged HA:CS KET loaded PECNs the % reduction in aggregation was significantly different to collagen with a p-value < 0.01 seen at all concentrations of particles added: 4 ug/ml, 8 ug/ml and 40 ug/ml at 1 and 10 min of incubation. There were also significant differences seen for the % reduction in aggregation at all concentrations between 1 and 10 min incubation of the positively charged HA:CS PECNs and platelets. At 4 ug/ml the reduction was from 77 ± 12 % to 8 ± 4 % aggregation, for 8 ug/ml it was from 44 ± 13 % to 6 ± 5 % aggregation and for 40 ug/ml it was lowered from 38 ± 12 % to 6 ± 4 % aggregation after 10min. (**Figure 6.5**).

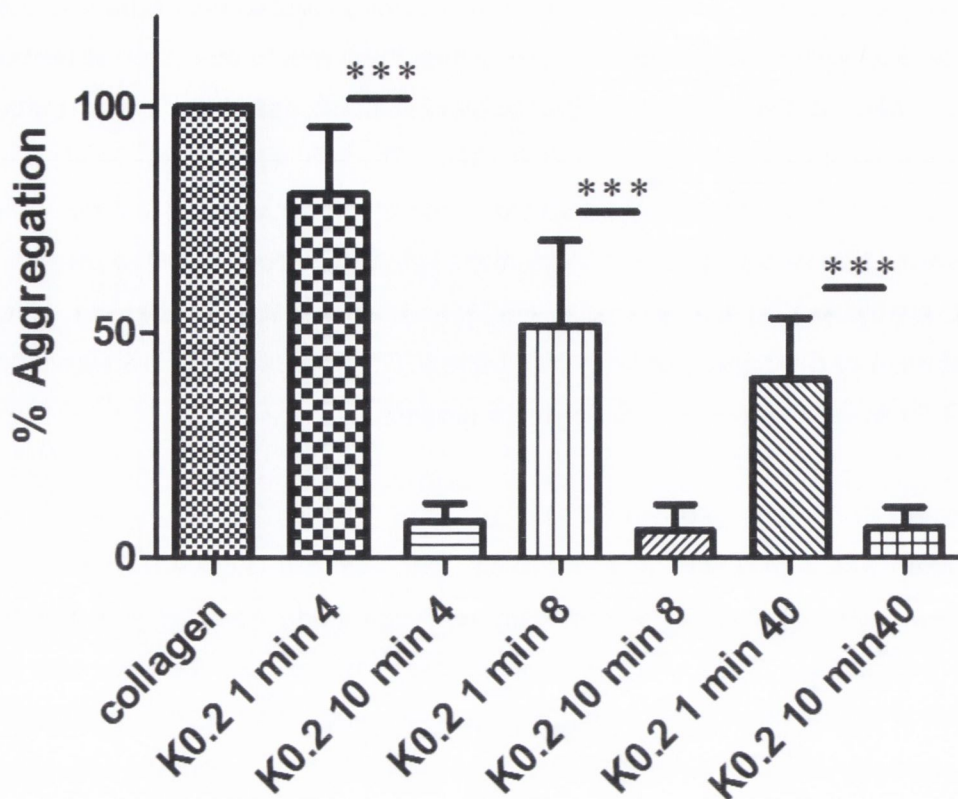


Figure 6.5 HA:CS positively charged KET loaded PECNs. A comparison of the ability of KET to inhibit the aggregation induced by collagen. Significant differences were seen between 1 and 10 min incubation of HA:CS PECNs with the platelets for all concentrations. (***) = $p < 0.001$) K = KET, 1 min and 10 min refer to incubation time and 4, 8, 40 are the concentrations of PECNs added in ug/ml. Collagen showed significant differences to all samples at 1 min or 10 min incubation ($p < 0.01$).

If the drug concentration loaded was investigated in comparison to the sample of free KET solution, the performance of the KET loaded HA:CS positively charged PECNs surpassed that of the free drug after 10 min of incubation with the platelets (**Figure 6.7**). After 1 min of incubation with the platelets the % inhibition of aggregation seen for positively charged KET loaded HA:CS PECNs was close to the inhibition measured for the free KET solution with only approx. 10% difference seen in the inhibition of aggregation at 4 ug/ml of HA:CS KET PECNs (equivalent to 0.3 ug/ml of drug) and 8 ug/ml of HACS KET NPs (equivalent to 0.5 ug/ml of drug). For KET loaded PLGA NPs at 1 min incubation the % reduction of aggregation was much lower.

After 10 min of incubation with the platelets the % inhibition of aggregation for positively charged HA:CS KET loaded PECNs was higher than the free KET. At 4 ug/ml HA:CS KET PECNs (equivalent to 0.3 ug/ml of KET) the inhibition was 92 ± 4 %, at 8 ug/ml of HACS KET PECNs (equivalent to 0.5 ug/ml of KET) the inhibition was 94 ± 6 % and free KET showed 58 ± 4 % at 0.4 ug/ml and 67 ± 3 % at 0.5 ug/ml of free KET. This observed inhibition of aggregation (%) was greater than that predicted by Petrusiewicz et al. (1994). It was calculated that PLGA NPs at 10 min incubation should have released approximately 0.10 ug KET/mg of PLGA NPs which is double that released by HA:CS positively charged PECNs at the same time point.

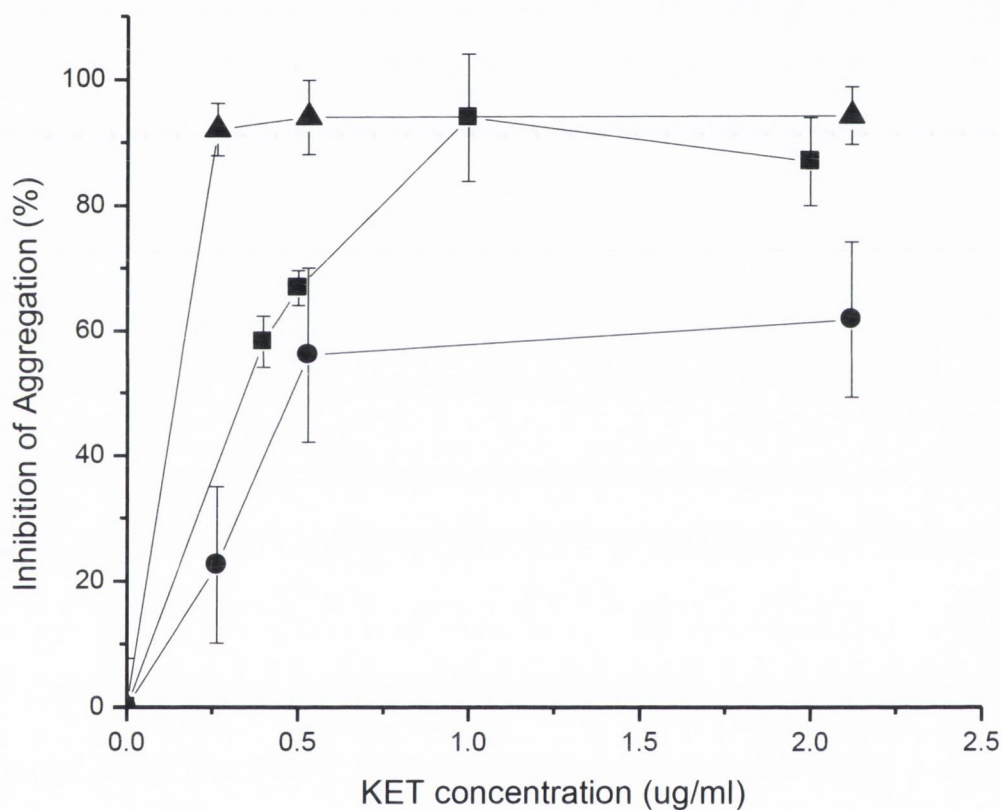


Figure 6.7 Comparison of the ability of KET to inhibit platelet induced aggregation between free KET solution and HA:CS positively KET loaded PECNs after 1 and 10 min incubation with platelets. ■ = free KET, ● = KET loaded HA:CS positively charged PECNs at 1 min incubation, ▲ = KET loaded HA:CS positively charged PECNs at 10 min incubation. Collagen showed significant differences to all samples at 1 min or 10 min incubation ($p<0.01$).

6.3.2.2.2 KET loaded HA:CS PECNs Negative Charge

Negatively charged HA:CS KET loaded PECNs were prepared as a comparison to the positively charged HA:CS PECNs to test if the surface charge would have a major impact on the performance of these HA:CS PECNs when loaded with KET. The KET loading was higher for these negatively charged HA:CS PECNs than for the positively charged HA:CS KET loaded PECNs. When 0.2 mg of KET was added/mg the DL was initially 11.4 ± 1.1 %, which was the equivalent of 0.11 mg drug/mg of HA:CS polymers. These HA:CS KET loaded negatively charged particles had the same quick release profile as the positively charged HA:CS KET loaded PECNs (**Chapter 4**). Despite having a higher DL these negatively charged KET loaded HA:CS PECNs did not show superior efficiency at inhibiting platelet aggregation.

Compared to collagen, significant differences were seen for all of the negatively charged HA:CS PECNs loaded with KET at 1 and 10 min incubation ($p < 0.05$). There was no significant decrease in aggregation between 1 and 10 min incubation time for the 4 ug/ml and 8 ug/ml concentrations. A significant decrease was seen between 1 and 10 min at 40 ug/ml concentration ($p < 0.05$). For 4 ug/ml NPs aggregation changed from 65 ± 3 % to 67 ± 19 %, from 66 ± 10 % to 60 ± 8 % at 8 ug/ml and from 65 ± 20 % to 28 ± 4 % at 40 ug/ml (1 min vs. 10 min of incubation) (**Figure 6.8**). The pI for these negatively charged HA:CS PECNs was 4.5 so they were expected to be stable in the plasma pH (**Chapter 4**).

When the inhibition of aggregation was compared for the free KET solution and the estimated KET released from the negatively charged HA:CS PECNs at each particle concentration it could be seen that the free KET had a greater ability to inhibit platelet aggregation, assuming the estimated amount of KET released is accurate. This may be a result of KET being immediately available and therefore can inhibit aggregation quicker than the KET associated with PECNs. It is also possible that these negatively charged HA:CS PECNs were not as attracted to the platelets due to their negative surface charge (Albanese et al. 2012). They may have repelled the platelets and thus taken longer to release the KET and affect the platelet aggregation. The inhibition of aggregation was not greater than that predicted by Petruszewicz et al. (1994) for these samples. At 1 min and 10 min it could be seen that the negatively charged HA:CS PECNs were releasing

low amounts of KET but once the concentration of negatively charged HA:CS PECNs rose to 40 ug/ml the incubation time had a significant effect on the inhibition of aggregation ($p < 0.05$). At this point sufficient KET must have been released to have a stronger effect on the collagen induced aggregation. Consequently, the incubation allowed optimal release and the inhibition to occur. After 10 min incubation using 40 ug/ml of negatively charged HA:CS PECNs with the platelets the the expected concentration of of KET present with the platelets was 4.6 ug/ml. This result showed no significant differences to the inhibition seen for free KET at 4 ug/ml ($p = 0.091$). It is concluded that once the KET had been released it was having a similar effect to KET at higher concentration and thus the negative surface charge may have decelerated the KET release at lower concentration or may have prevented the KET binding as the negatively charged HA:CS PECNs were not attracted to the platelets (**Figure 6.9**). Albanese et al. (2012) reported in their review paper 'The Effect of Nanoparticle Size, Shape, and Surface Chemistry on Biological Systems' that the surface charge of the nanoparticle plays a pivotal role in downstream biological responses to NPs. It has also been concluded that compared with NPs with a neutral or negative charge, positively charged NPs are taken up at a faster rate (Jin et al. 2009, Wang et al. 2010). It has been suggested that the cell membrane possesses a slight negative charge and cell uptake is driven by electrostatic attractions (Slowing et al. 2006 and Mukherjee et al. 2010).

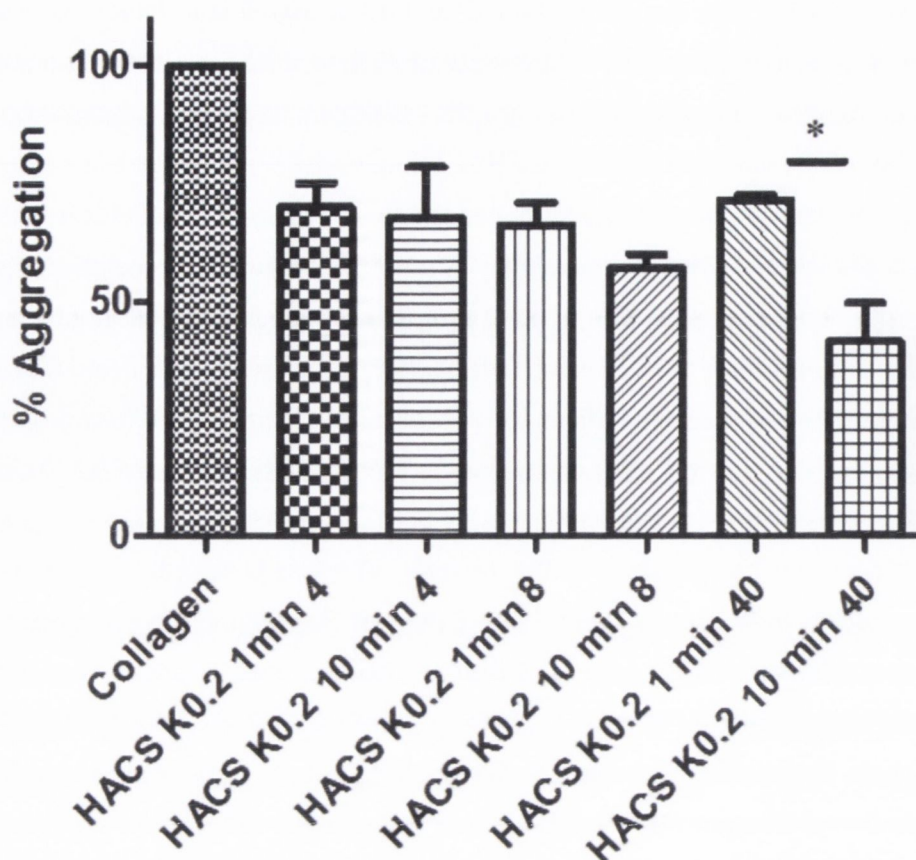


Figure 6.8 A comparison of the ability of KET to inhibit the aggregation induced by collagen compared to KET loaded negatively charged HA:CS PECNs. Significant effects were seen only between 1 and 10 min incubation with platelets. (* = $p < 0.05$) at 40 $\mu\text{g/ml}$ of PECNs. K = KET, 1 min and 10 min refer to incubation time and 4, 8, 40 are the concentrations of NPs added in $\mu\text{g/ml}$. Collagen showed significant differences to all samples at 1 min or 10 min incubation ($p < 0.05$).

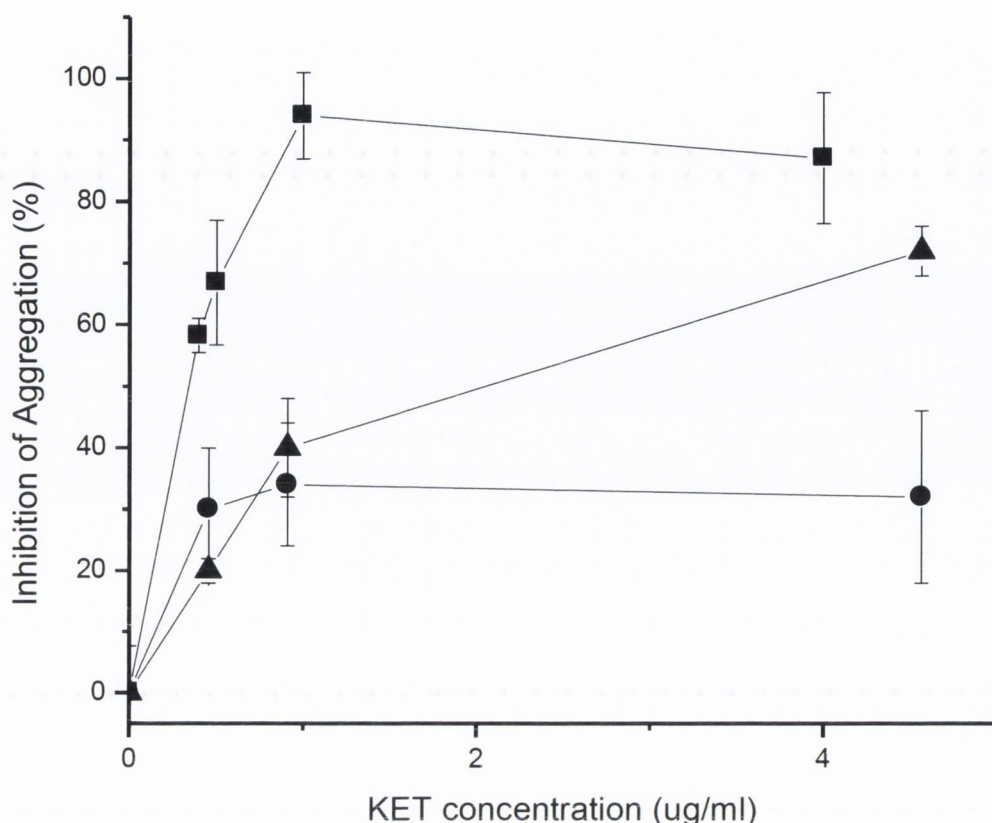


Figure 6.9 Comparison of the ability of KET to inhibit platelet induced aggregation between free KET solution and negatively charged KET loaded HA:CS PECNS after 1 and 10 min incubation time. ■ = free KET, ● = KET loaded negatively charged HA:CS PECNs at 1 min incubation, ▲ = KET loaded negatively charged HA:CS PECNs at 10 min incubation.

6.3.2.3 IND loaded PLGA NPs

To look at the effect of more than one anti-platelet drug in these NPs IND was chosen. It has different physical properties to KET as described previously (**Chapter 4 and 5**), and is effective at higher concentrations than KET. The IC_{50} for KET was reported by Petrusiewicz et al. (1994) to be at 5.30×10^{-6} M (1.3 ug/ml) and the IC_{25} at 7.20×10^{-7} M (0.2 ug/ml) when platelets were stimulated by using collagen (10 ug/ml). Leach and Thorburn (1982) found that the therapeutic range for IND to inhibit collagen 5 ug/ml induced aggregation was 43 umol (0.016 ug/ml) to 43 mmol (15.8 ug/ml). The concentration of collagen used in the studies described in this chapter was lower (2 ug/ml). PLGA IND loaded NPs

had a higher DL than PLGA KET NPs with 19 ± 0.4 % w/v loaded. This meant that 0.19 mg IND/mg of PLGA was present.

Significant differences were observed at all concentrations for the % aggregation between 1 and 10 min for IND loaded PLGA NPs incubation with the platelets (**Figure 6.10**). The p value for all samples was highly significant with $p < 0.001$ in all cases. At 4 ug/ml of PLGA IND NPs the difference in % aggregation was 89 ± 5 % at 1 min compared to 31 ± 28 % at 10 min, at 8 ug/ml of PLGA IND NPs the difference in % aggregation was 86 ± 10 % at 1 min compared to 13 ± 9 % at 10 min, at 20 ug/ml of PLGA IND NPs the difference in % aggregation was 85 ± 12 % at 1 min compared to 10 ± 4 % at 10 min. This was most likely due to the slow release of IND over 10 min increasing the amount of drug present in the platelet samples. At 10 min it was estimated that 0.13 ug IND/ml of PLGA NPs would be released.

Moreover, all PLGA NP concentrations showed significant differences to collagen ($p < 0.001$) at 10 minutes, whereas no significant differences were seen at 1 min incubation.

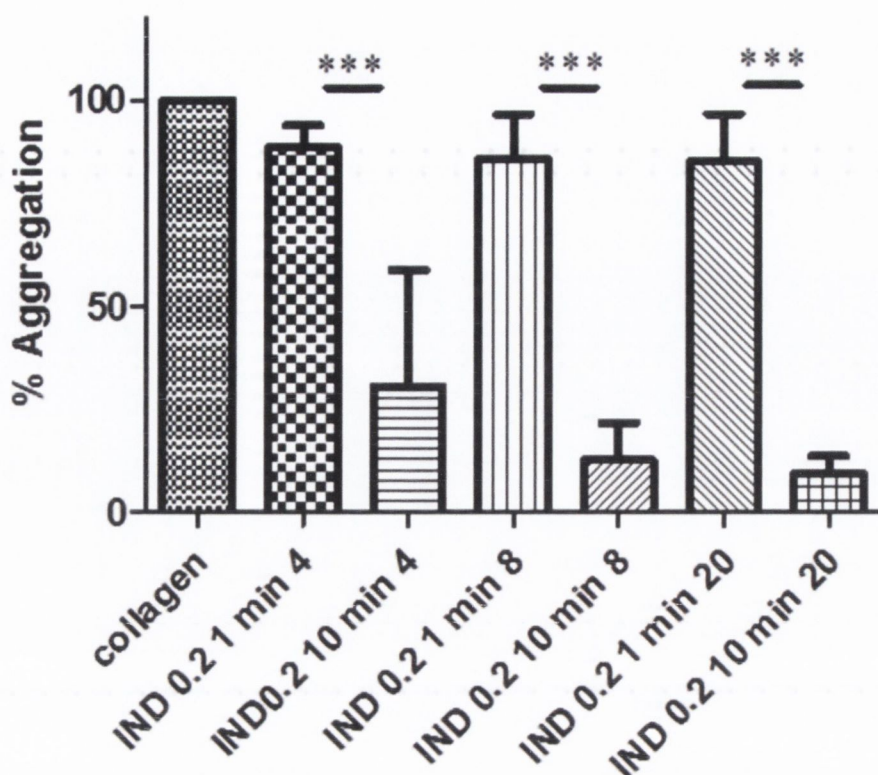


Figure 6.10 PLGA IND loaded NPs. A comparison of the ability of IND to inhibit the aggregation induced by collagen. Significant effects were seen for all concentrations between 1 and 10 min incubation. (***) = $p < 0.001$. 1 min and 10 min refer to incubation time, 4, 8, 20 are the concentration of NPs added in ug/ml. At 10 min incubation all samples were significantly different to collagen ($p < 0.001$).

By converting the concentration of particles added to the concentration of encapsulated drug in these particles it was possible to compare the drug loaded NPs to the free IND solution (**Figure 6.11**). It was observed that after 1 minute of incubation, the level of inhibition was low, In contrast, after 10 minutes of incubation with the platelets a large increase in the % inhibition of aggregation was found with the results being higher than the free IND solution alone at all concentrations tested. All concentrations were within the 0.0158 ug/ml to 15.8 ug/ml range reported by Leach and Thorburn (1982).

Free IND solution had lower activity than free KET solution at inhibiting platelet aggregation (**Figures 6.9 and 6.11**).

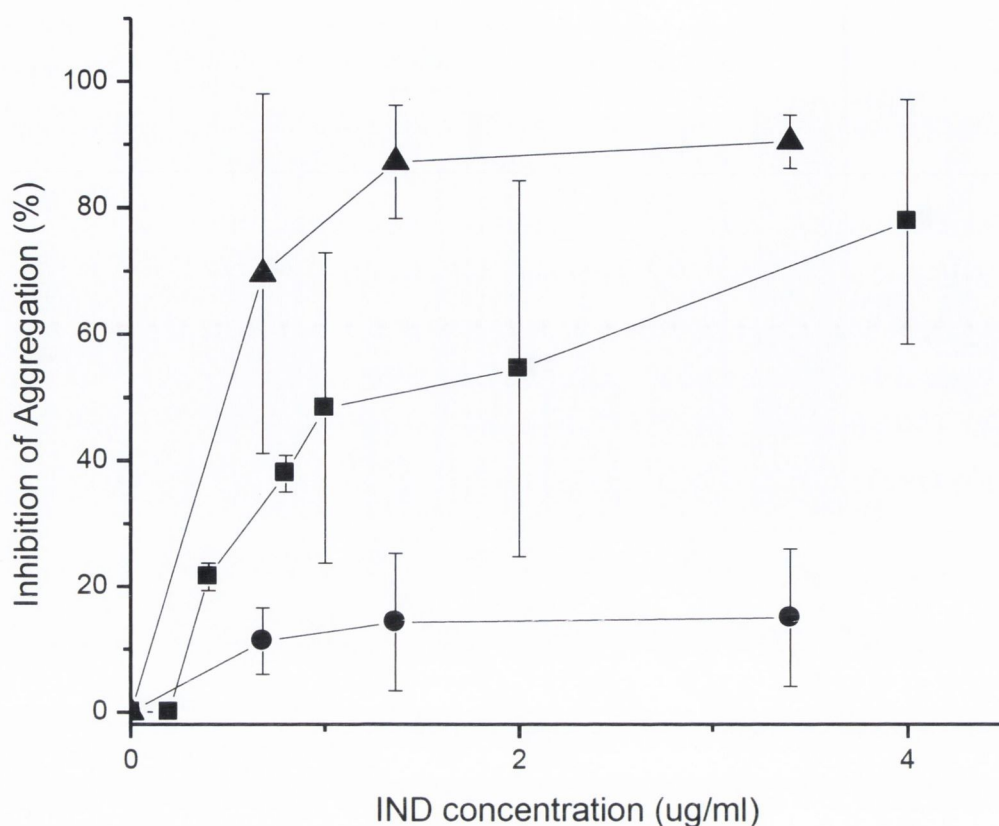


Figure 6.11 Comparison of the ability of IND to inhibit platelet induced aggregation between free IND solution and PLGA IND loaded NPs after 1 and 10 min incubation. ■ = free IND, ● = PLGA IND NPs at 1 min incubation, ▲ = PLGA IND NPs at 10 min incubation.

6.3.2.4 IND loaded HA:CS Polyelectrolyte Complex PECNs

6.3.2.4.1 IND loaded HA:CS PECNs with Positive Charge

Positively charged HA:CS PECNs loaded with IND had a lower DL than the PLGA IND PECNs with DL at 13.1 ± 0.05 % w/v which is 0.13 mg IND/mg of HA:CS. This is approximately half the DL of PLGA IND NPs DL values. The positively charged HA:CS PECNs were a quick release formulation with the majority of IND expected to be released within the first 30 min of addition to the platelets (**Chapter 5**).

As shown in **Figure 6.12**, significant differences were seen between collagen and positively charged HA:CS PECNs loaded with IND for 8 ug/ml and 40 ug/ml of the NCs at 10 minutes incubation only ($p < 0.01$). Moreover, for these concentrations (8 ug/ml and 40 ug/ml/min of HA:CS IND loaded PECNs) significant differences were seen between the particles at 1 min and 10 mins of incubation with the platelets. After 10 minutes of incubation, the % of aggregation decreased from $100 \pm 1\%$ to $61 \pm 32\%$, from $96 \pm 6\%$ to $51 \pm 29\%$ and from $90 \pm 5\%$ to $46 \pm 26\%$ at 4, 8 and 40 ug/ml HA:CS IND PECNs, respectively.

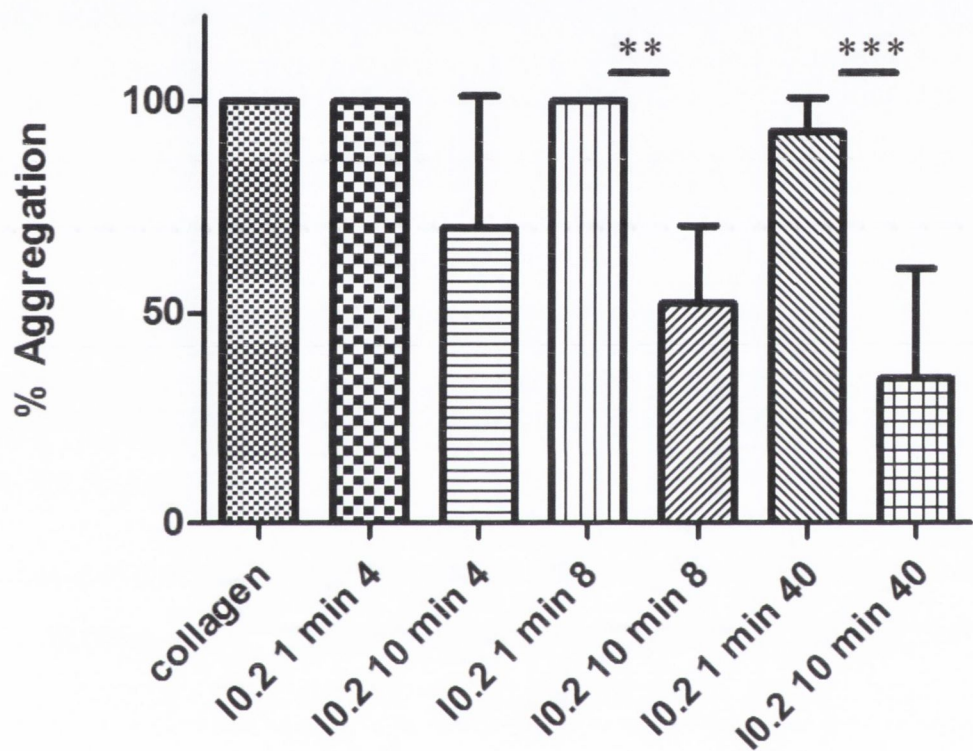


Figure 6.12 A comparison of the ability of IND to inhibit the aggregation induced by collagen compared to HA:CS positively charged IND loaded PECNs. Significant effects were seen for 8 ug/ml and 40 ug/ml concentrations between 1 and 10 min with greater differences being seen at higher concentrations. (** = $p < 0.01$, *** = $p < 0.001$) I = IND, 1 min and 10 min refer to incubation time and 4, 8, 40 are the concentrations of PECNs added in ug/ml. Collagen showed significant differences to the 8 ug/ml and 40 ug/ml samples at 10 min incubation ($p < 0.01$).

When drug concentration of free IND and positively charged HA:CS IND loaded PECNs was compared, as displayed in the profile of inhibition of aggregation (%) to IND concentration (**Figure 6.13**), it was seen that the effectiveness of positively charged HA:CS IND loaded PECNs increased from 1 minute to 10 minutes incubation. After 1 minute, a low level of % inhibition of aggregation was observed for the loaded PECNs, which rose to the same level of inhibition as that of free IND after 10 minutes incubation.

The inhibition was not as high as that for the PLGA IND PECNs and is assumed to be a result of the lower DL observed for these HA:CS IND loaded PECNs. At 10 minutes it was estimated that 0.10 ug of IND/mg was released from the positively charged HA:CS PECNs and thus showed a lower response than expected.

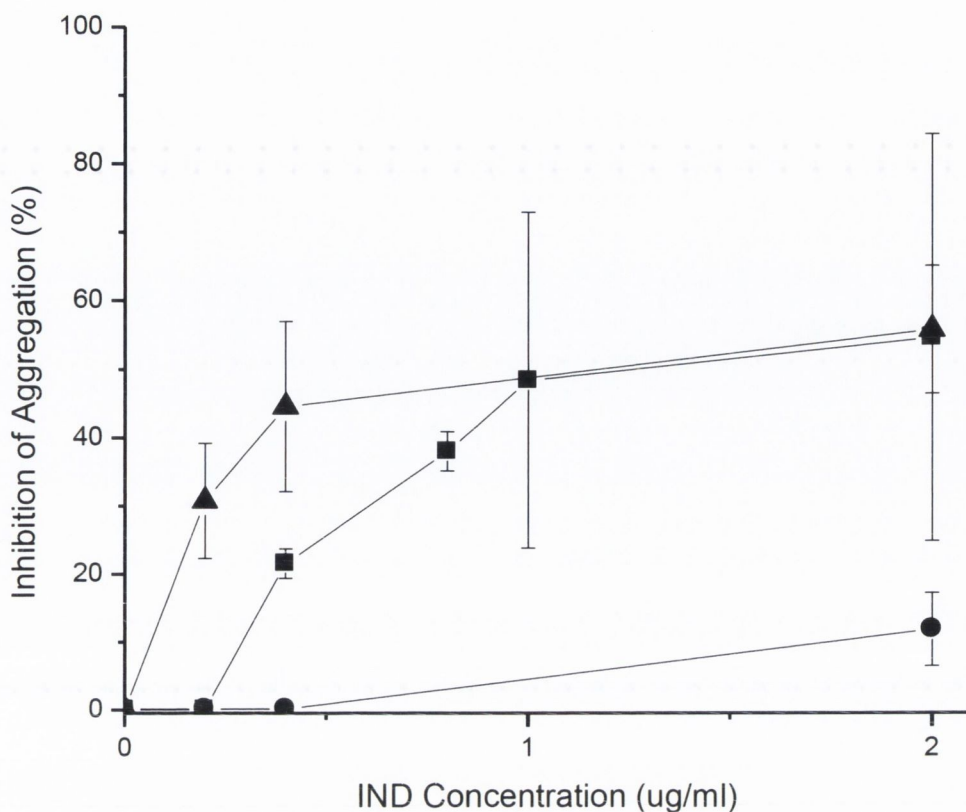


Figure 6.13 Comparison of the ability of IND to inhibit platelet induced aggregation between free IND solution and positively charged IND loaded HA:CS PECNs after 1 and 10 min incubation time. ■ = free IND, ● = positively charged IND loaded HA:CS PECNs at 1 min incubation, ▲ = positively charged IND loaded HA:CS PECNs at 10 min incubation.

6.3.2.4.2 IND loaded HA:CS PECNs with Negative Charge

Negatively charged HA:CS PECNs loaded with IND had a low DL at 3.5 ± 0.3 % w/v which is equivalent to 0.035 mg IND/mg of HA:CS. The HA:CS PECNs were a quick release formulation with the majority of drug expected to be released within the first 30 min of addition to the platelets (**Chapter 5**). It had the lowest DL of all the PECNs investigated in this chapter. The DL was approximately one quarter that of the positively charged IND loaded HA:CS PECNs (13.1 ± 0.5 %). This indicates that negatively charged HA:CS PECNs were not the most suitable NCs for IND. IND may have required the excess CS in the positively charged HA:CS PECNs to stabilise it.

No significant differences were seen between the particles at 1 min and 10 min of incubation time with the platelets for the 4 ug/ml and 8 ug/ml particle concentrations, only at 40 ug/ml. At 4 ug/ml of HA:CS Neg IND loaded PECNs the % of aggregation decreased from 93 ± 6 % to 93 ± 1 % after 10 min of incubation, at 8 ug/ml the % aggregation dropped from 92 ± 6 % to 82 ± 4 % after 10 min of incubation at 8 ug/ml/min PECNs and from 76 ± 9 % to 50 ± 6 % at 40 ug/ml of HA:CS Neg IND loaded PECNs (**Figure 6.14**). Because the IND loading was low for these samples it is likely that the concentration of IND for 4 and 8 ug/ml of HA:CS PECNs was too low to overcome the strong aggregating power of collagen. When the concentration of particles reached 40 ug/ml, a significant decrease was seen in aggregation once the PECNs had been incubated for 10 min releasing the drug.

Collagen showed significant differences to IND loaded HA:CS PECNs at 1 min and 10 min of incubation for 40 ug/ml ($p < 0.01$) and at 10 min of incubation only for 8 ug/ml ($p < 0.05$) .

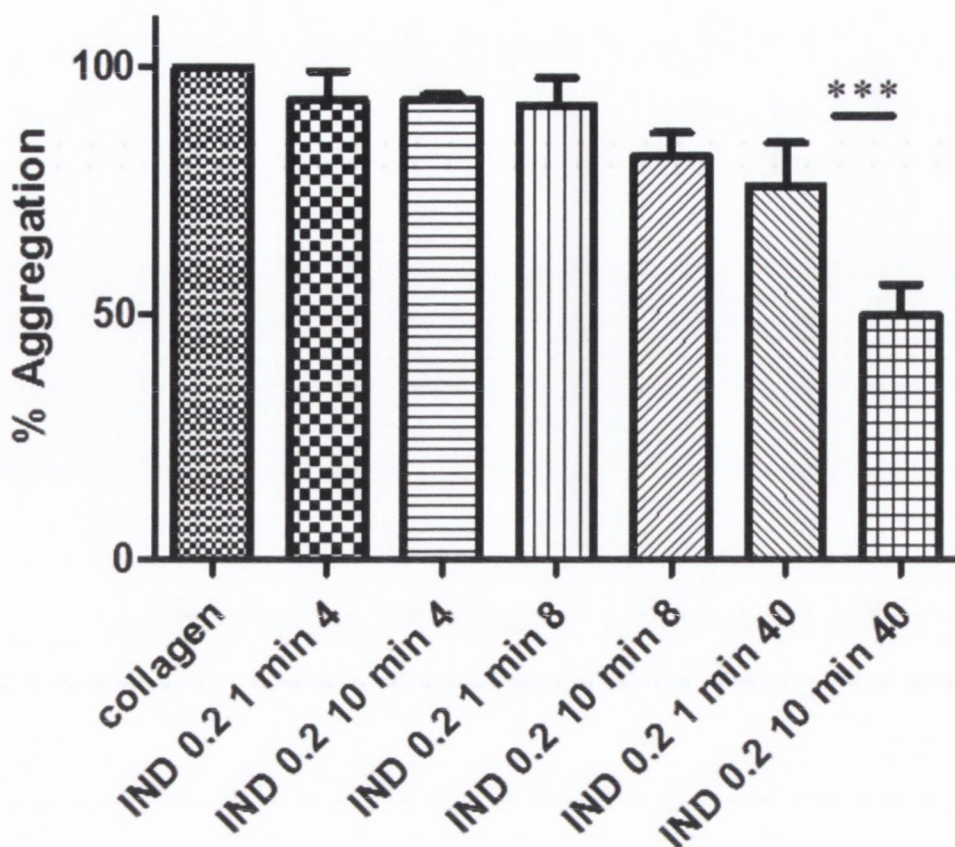


Figure 6.14 A comparison of the ability of IND to inhibit the aggregation induced by collagen for negatively charged IND loaded HA:CS PECNs. Significant effects were seen between 1 and 10 min incubation time at 40 ug/ml only. (***) = $p < 0.001$) I = IND, 1 min and 10 min refer to incubation time with platelets and 4, 8, 40 are the concentrations of PECNs added in ug/ml. Collagen showed significant differences to IND loaded HA:CS PECNs at 1 min for 40 ug/ml ($p < 0.01$) and at 10 min for 8 ug/ml ($p < 0.05$) and 40 ug/ml ($p < 0.001$).

By comparing the estimated actual drug released from the IND loaded negatively charged HA:CS PECNs with the free IND concentration (**Figure 6.15**) it appeared that when the drug was at higher concentrations in the 40 ug/ml sample (estimated 1.4 ug/ml IND released after 10 min) the inhibition of aggregation was similar to that seen with free IND. At 1 min incubation the effect of the negatively charged HA:CS PECNs was not as strong as from the equivalent KET HA:CS PECNs. At 10 min incubation the lower concentrations of negatively charged HA:CS PECNs at 4 ug/ml (estimated IND released after 10 min of 0.1 ug/ml) and 8 ug/ml (estimated IND released after 10 min of 0.3 ug/ml) were compared to free IND. At 0.1 ug/ml IND (4 ug/ml HA:CS Neg PECNs) the

inhibition of aggregation was 7 ± 1 % and was 14 ± 8 % at 0.3 ug/ml (8 ug/ml HA:CS Neg PECNs). In comparison to this for free IND at 0.2 ug/ml the inhibition of platelet aggregation was 1 ± 0.3 %. It was assumed that the additional inhibition seen for IND loaded HA:CS was not related to the solid state of IND in the negatively charged HA:CS PECNs because it was being compared to free IND which was in solution already. In the case that free IND precipitated out of solution due to poor solubility it is possible that IND associated with the negatively charged HA:CS PECNs may have dissolved quicker if it was in the amorphous state. The alternative explanation is that the negatively charged HA:CS PECNs acted synergistically with IND to reduce platelet aggregation. HA has been reported by Verheye et al. (2000) to have reduced platelet aggregation when used to coat endovascular devices. It was calculated that the IND loaded negatively charged HA:CS PECNs were releasing 0.03 ug of IND/mg. This value was lower than that for IND loaded PLGA NPs and for IND loaded positively charged HA:CS PECNs. Thus the response was expected to be less to that experimentally observed.

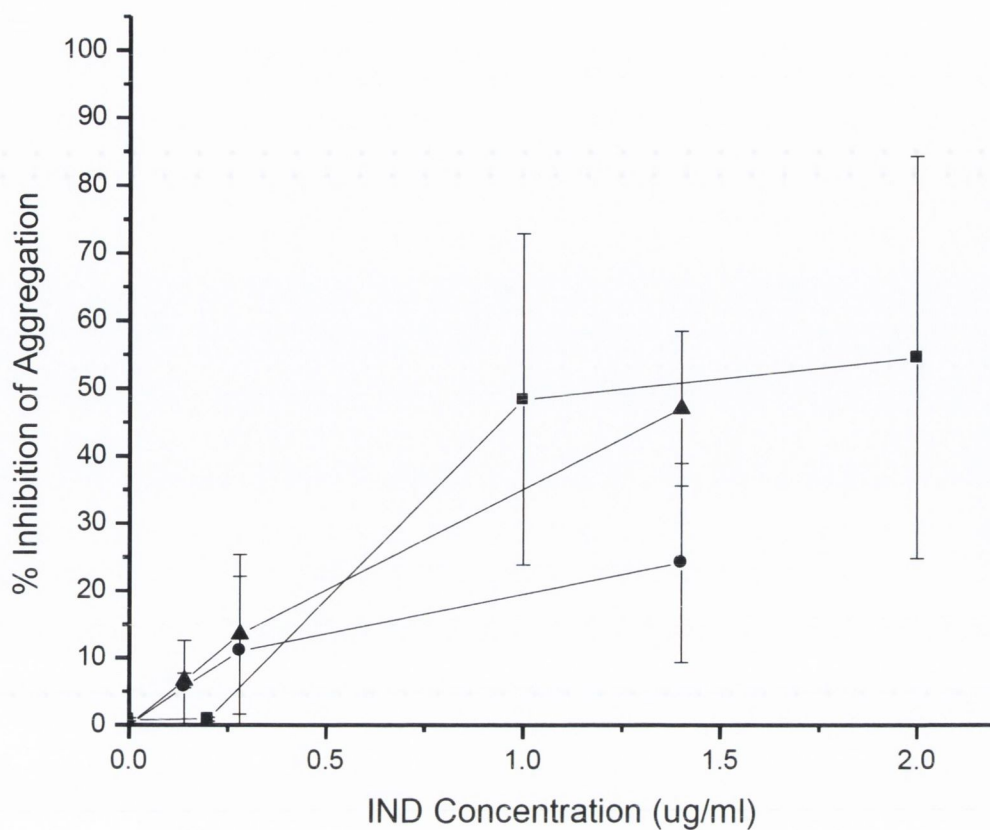


Figure 6.15 Comparison of the ability of IND to inhibit platelet induced aggregation between free IND solution, negatively charged IND loaded HA:CS PECNs after 1 and 10 min incubation. ■ = free IND, ● = negatively charged IND loaded HA:CS PECNs at 1 min incubation, ▲ = negatively charged IND loaded HA:CS PECNs at 10 min incubation.

6.4 Flow Cytometry

Flow cytometry was carried out to support the data obtained using aggregometry. It was decided to test the effect of the IND and KET loaded NCs on the P-selectin expression on platelets as was used by Li et al. (2009) in order to link her work with the current research in this chapter.

The second to minimum concentrations at which each type of NCs significantly inhibited platelet aggregation are shown in **Table 6.3**. It was decided to choose the second to minimum inhibitory concentration for these experiments to assure that the effect of inhibition on P-selectin receptors could be measured. Samples were tested for 10 min of incubation time as more significant effects were seen for these samples due to greater drug release. Negatively and positively charged KET loaded HA:CS PECNs were incubated for 1 minute, randomly, to check if any major differences would be seen between 1 minute and 10 minutes incubation time.

Table 6.3 The minimum concentration of NCs inducing significant inhibition of collagen-induced platelet aggregation (n = 3; p < 0.05).

Sample Type	1 min incubation (µg/ml)	10 min incubation (µg/ml)	second to min concentration at 10 min incubation (ug/ml)
PLGA KET	-	8	20
HA:CS KET Pos	4	4	8
HA:CS KET Neg	8	4	8
PLGA IND	-	4	8
HA:CS IND Pos	-	40	40
HA:CS IND Neg	-	8	40

6.4.1 PLGA NPs

The empty PLGA NPs were tested by adding them to resting platelets without the addition of collagen to ensure that they themselves would not induce p-selectin expression themselves. No significant differences were observed between the empty PLGA NPs (80 ug/ml) and resting platelets (**Table 6.4**, **Figure 6.16**).

PLGA NPs loaded with KET and IND were tested post-addition of collagen (2 ug/ml) to see the effect of the NPs on the collagen induced P-selectin expression. The result was compared to the effect of collagen alone and to the results for resting platelets without collagen and for empty PLGA NP addition without collagen addition. **Figure 6.16** shows that the agonist collagen significantly increased the expression of P-selectin on the surface of the platelets ($p < 0.001$). For both the KET and the IND loaded PLGA NPs the number of P-selectin receptor copies on the surface of the platelets following incubation were significantly reduced ($p < 0.001$) at 10 min of incubation. PLGA KET loaded NPs were not significantly different to PLGA IND loaded NPs in terms of P-selectin expression. There were no significant differences observed between these drug loaded PLGA NPs and the resting platelets which were measured without collagen addition (**Table 6.4**).

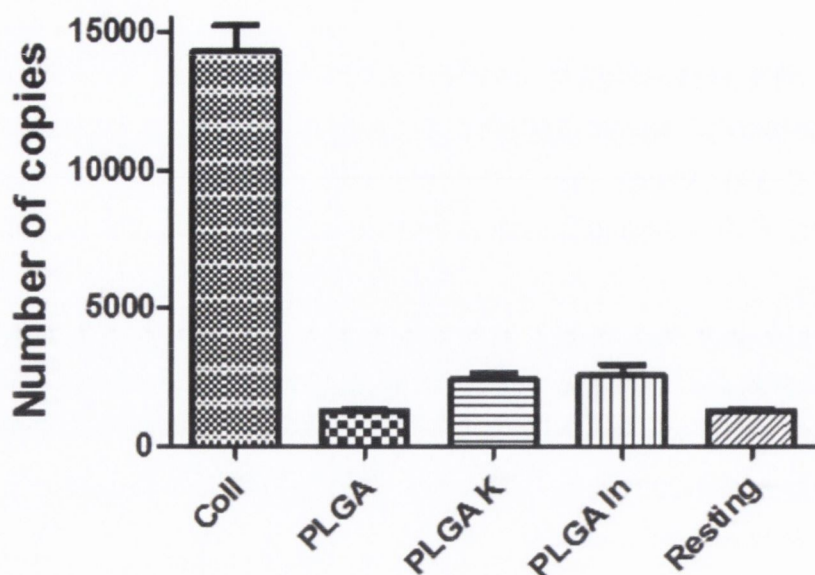


Figure 6.16 Flow cytometry analysis of collagen induced P-selectin expression on platelets in the presence of KET loaded PLGA NPs, IND loaded PLGA NPs and empty PLGA NPs and resting platelets (both without collagen stimulation) following 10 min of incubation. Collagen was used as positive control, resting platelets used as negative control.(n = 3; ***p < 0.001 collagen vs. all)

Table 6.4 The number of receptor copies expressed (mean $\pm$ SD) on the surface of the resting platelets, on the platelets pre-incubated for 10 min with KET and IND loaded PLGA NP pre-collagen addition and on platelets pre-incubated for 10 min with empty PLGA NPs without collagen addition (n= 3).

Sample Type	Number of receptor copies
Resting platelets	1231 $\pm$ 122
0.2 mg/mg PLGA KET	2405 $\pm$ 418
0.2 mg/mg PLGA IND	2545 $\pm$ 689
Empty PLGA	1237 $\pm$ 101
Collagen	14287 $\pm$ 1667

6.4.2 HA:CS PECNs

6.4.2.1 Positively Charged HA:CS PECNs

The positively charged empty HA:CS PECNs were measured without collagen addition as a control to see if the positively charged HA:CS PECNs were

affecting the p-selectin expression on resting platelets. They did not show significant differences to the resting platelets indicating that the positively charged HA:CS PECNs were not inducing p-selectin expression (**Figure 6.17**). For both the KET and the IND loaded positively charged HA:CS PECNs the number of P-selectin receptor copies on the surface of the platelets induced by collagen (2 ug/ml) following incubation were significantly reduced ($p < 0.001$) at 10 min of incubation. There were no significant differences observed between these drug loaded positively charged HA:CS PECNs and the resting platelets which were measured without collagen addition (**Figure 6.17**). There were also no significant differences observed between IND loaded and KET loaded positively charged HA:CS PECNs (**Table 6.5**). As was seen for PLGA NPs the agonist collagen significantly increased the expression of P-selectin on the surface of the platelets ($p < 0.001$) without the presence of NCs or drugs.

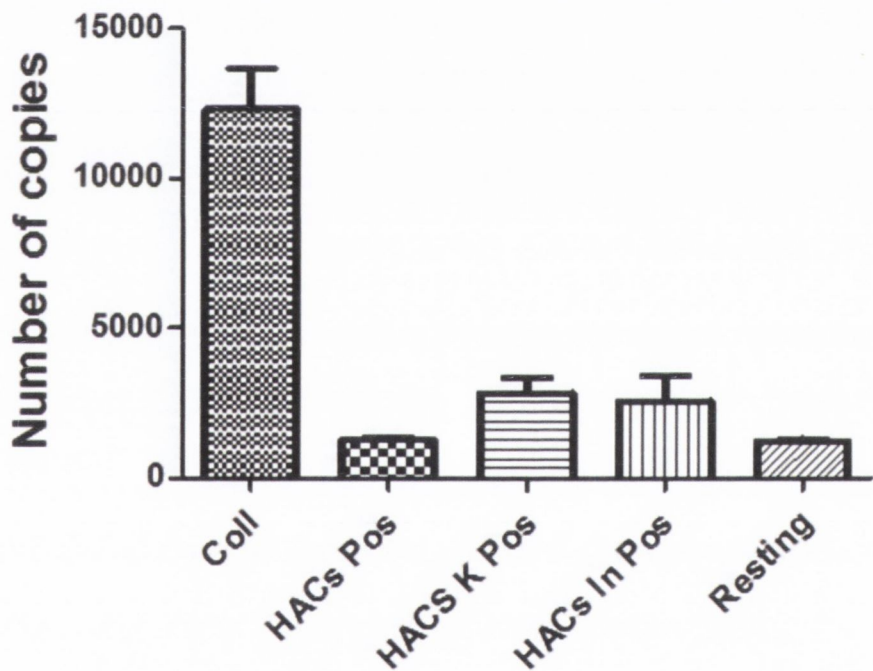


Figure 6.17 Flow cytometry analysis of P-selectin expression on platelets following collagen (2ug/ml) addition in the presence of positively charged KET and IND loaded positively charged HA:CS PECNs, as well as positively charged empty HA:CS PECNs without collagen addition following 10 min of incubation with the platelets. Collagen was used as positive control, resting platelets were used as negative control. (n=3; *** $p < 0.001$ collagen vs. all).

Table 6.5 The number of receptor copies expressed (mean $\pm$ SD) on the surface of the resting platelets, on the platelets incubated for 10 min with KET and IND loaded positively charged HA:CS PECNs and on platelets incubated for 10 min with empty positively charged HA:CS PECNs (n= 3).

Sample Type	Number of receptor copies
Resting platelets	1231 $\pm$ 122
0.2 mg/mg HA:CS KET Pos	2834 $\pm$ 830
0.2 mg/mg HA:CS IND Pos	2567 $\pm$ 1464
Empty HA:CS Pos	1271 $\pm$ 144
Collagen	12310 $\pm$ 2300

6.4.2.2 Negatively Charged HA:CS PECNs

Figure 6.18 shows that the negatively charged empty HA:CS PECNs without the addition of collagen (2 ug/ml) did not induce P-selectin expression. The IND and KET loaded HA:CS PECNs significantly decreased the abundance of P-selectin on the platelet surface as evident by the number of receptor copies expressed in comparison to that expression induced by collagen (2 ug/ml) without NCs, indicating that these drug loaded PECNs inhibited collagen-induced platelet aggregation. There was no significant difference between the effect of KET and IND for these samples. The number of receptor copies expressed on the surface of the platelets following incubation with the IND and KET loaded HA:CS PECNs post –collagen (2 ug/ml) addition was not significantly different to that of the resting platelets without collagen addition (**Table 6.6**).

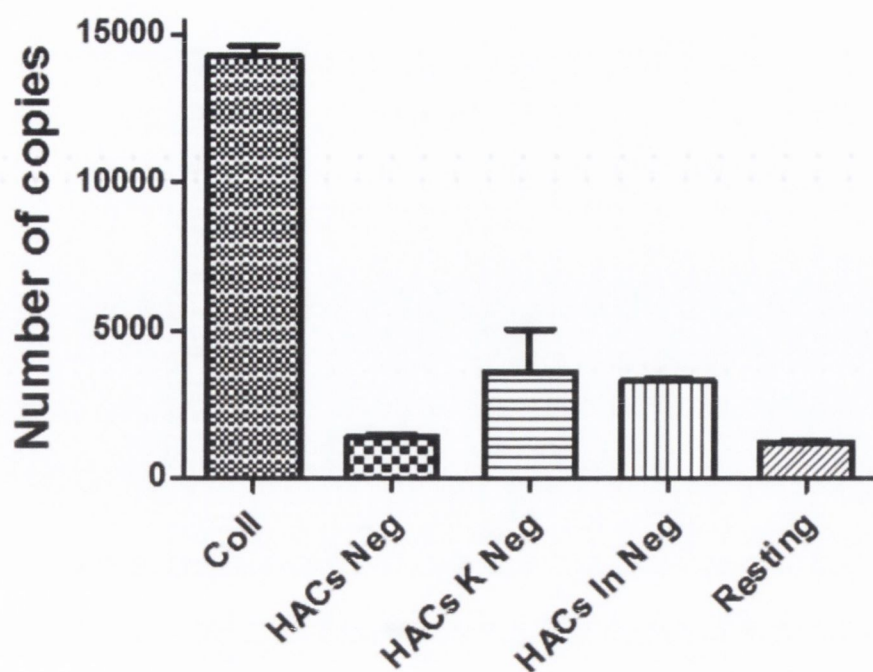


Figure 6.18 Flow cytometry analysis of P-selectin on platelets in the presence of negatively charged KET and IND loaded HA:CS PECNs post-collagen addition, and negatively charged empty HA:CS PECNs without collagen addition, following 10 min of incubation. Collagen was used as positive control, resting platelets used as negative control. (n = 3; ***p < 0.001 collagen vs. all)

Table 6.6 The number of receptor copies expressed (mean $\pm$ SD) on the surface of the resting platelets, on the platelets pre-incubated for 10 min with negatively charged KET and IND loaded HA:CS PECNs prior to collagen addition and on platelets pre-incubated for 10 min with negatively charged empty HA:CS PECNs without collagen addition (n= 3).

Sample Type	Number of receptor copies
Resting platelets	1231 $\pm$ 122
0.2 mg/mg HA:CS KET Neg	2955 $\pm$ 1330
0.2 mg/mg HA:CS IND Neg	3311 $\pm$ 101
Empty HA:CS Neg	1393 $\pm$ 193
Collagen	14268 $\pm$ 646

6.4.2.3 Comparison of Free KET or Free IND with the drug loaded NCs

Direct comparison between the concentration of free drug and the concentration of drug loaded into the NCs could not be made due to differences in the exact amount of drug released at any given time. The value taken for free drug was an average value for the amount of free drug released from the NPs (PLGA, positively charged HA:CS PECNs and negatively charged HA:CS PECNs) at 10 min. A comparison was made to see if there were any major differences when using the different NCs. **Figure 6.19 and Table 6.7** showed no significant differences between the different types of NCs at 10 min incubation for the 40 ug/ml concentration of particles (20 ug/ml PLGA NPs) and free KET at 0.69 ug/ml. The predicted KET release for KET loaded NCs at 10 min incubation was 0.78 ug/ml for PLGA NPs, 0.41 ug/ml for positively charged HA:CS PECNs and 0.91 ug/ml for negatively charged HA:CS PECNs. The number of P-selectin copies expressed verified that the NCs were not enhancing the effect of KET on p-selectin expression but at the same time they were not lessening the effect of KET as there was no significant differences.

Table 6.7 The number of receptor copies expressed (mean $\pm$ SD) on the surface of the resting platelets without collagen, on the platelets incubated with free KET and free IND with collagen addition (n= 3).

Sample Type	Number of receptor copies
Resting platelets	1231 $\pm$ 122
Free KET	2002 $\pm$ 521
Free IND	2092 $\pm$ 187
Collagen	10556 $\pm$ 2539

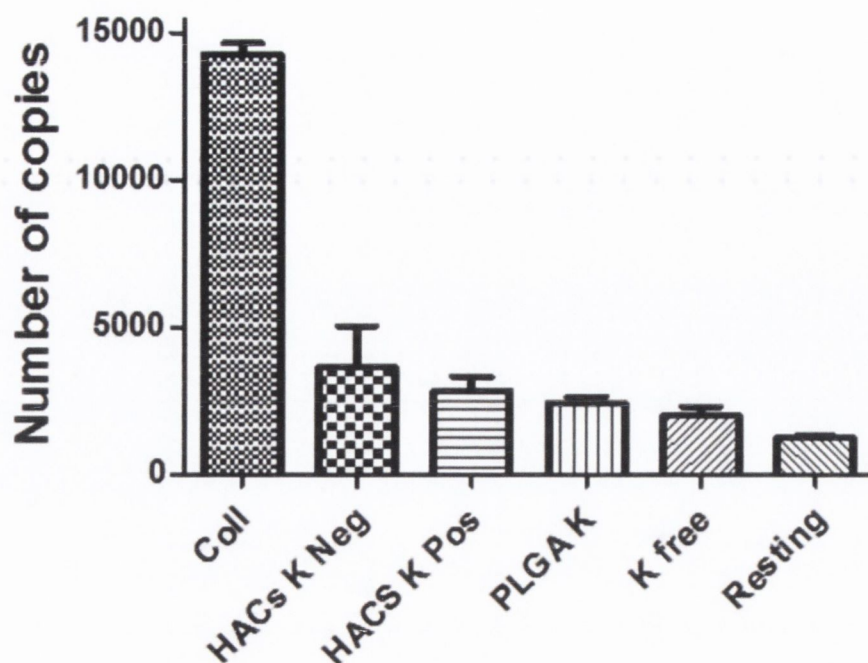


Figure 6.19 Flow cytometry analysis of P-selectin on platelets in the presence of KET loaded NPs and free KET. Negatively charged KET loaded HA:CS PECNs, positively charged KET loaded HA:CS PECNs, PLGA NPs and free KET are shown following 10 min of incubation. Collagen used as positive control, resting platelets used as negative control. (n=3; ***p < 0.001 collagen vs. all).

Again for IND loaded NCs direct comparison could not be made to free IND due to differences in the exact amount of drug released at that time. **Figure 6.20** shows no significant differences between the different types of NCs at 10 min incubation for the 40 ug/ml concentration of particles (20 ug/ml for PLGA NPs) and free IND at 1.05 ug/ml. The predicted IND release for these NCs at 1.40 ug/ml for 10 min incubation was 1.02 ug/ml for PLGA NPs, 1.22 ug/ml for positively charged HA:CS PECNs, and 0.91 ug/ml for negatively charged HA:CS PECNs. The number of receptor copies expressed indicated that IND loaded NPs did not enhance the effect of IND on inhibition of p-selectin expression but they also did not prevent IND from being effective.

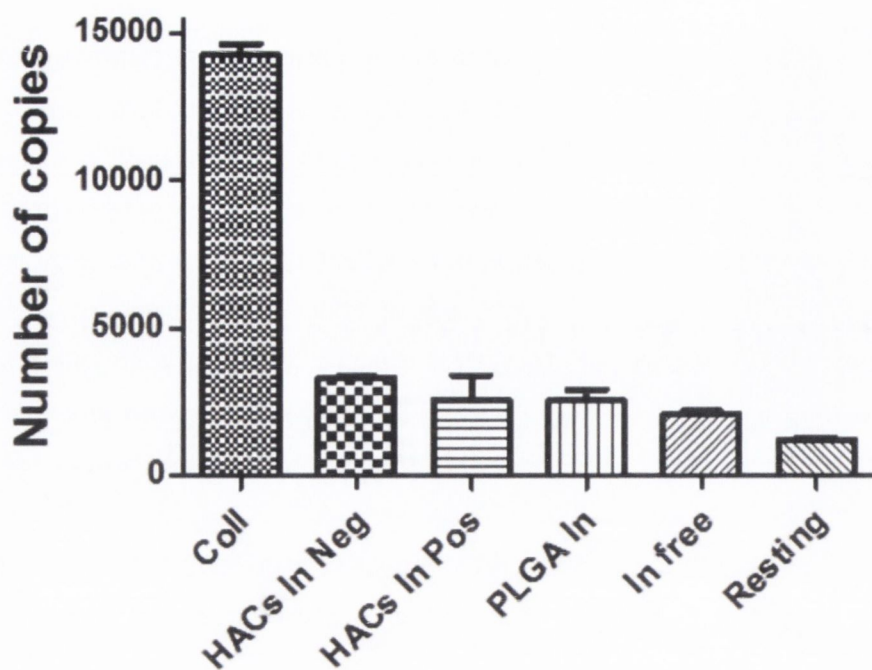


Figure 6.20 Flow cytometry analysis of P-selectin on platelets in the presence of IND loaded NCs and free IND. Negatively charged IND loaded HA:CS PECNs, positively charged IND loaded HA:CS PECNs, PLGA NPs and free IND are shown following 10 min of incubation. Collagen used as positive control, resting platelets used as negative control. (n=3; ***p < 0.001 collagen vs. all).

It was interesting to note that both IND and KET NCs showed the same pattern for the average number of copies of P-selectin expressed. Although no significant difference was seen, the average was highest for the negatively charged HA:CS PECNs, followed by the positively charged HA:CS PECNs, the PLGA NPs and finally the free drug. This pattern was the same for both drugs regardless of DL. To illustrate the fact that this may have been related to the NC type, as opposed to the DL, the results for the positively charged HA:CS PECNs can be considered. Positively charged KET loaded HA:CS PECNs released approximately 0.41 ug/ml of KET after 10 min of incubation which was lower than that released for the other KET loaded NCs. Conversely, positively charged IND loaded HA:CS PECNs released 1.22 ug/ml IND after 10 min incubation which was higher than the amount of IND released from the other IND loaded NCs. Regardless of the amount of IND or KET released from the positively charged HA:CS PECNs at 10 min incubation the sequence of average number of copies of P-selectin was still the same. This may suggest that the NC itself has some impact on the number of copies of P-selectin expressed.

Figure 6.21 (a-d) gives an example of how the flow cytometry is graphically represented resting platelet, platelets activated with collagen (2 ug/ml) and platelets activated with collagen and then incubated drug loaded NCs. **Figure 6.21 (c and d)** gives an example of drug loaded NC: negatively charged KET loaded HA:CS PECNs at 1 min **(c)** and 10 min **(d)** of incubation. These images show that the KET loaded HA:CS PECNs were capable of reducing platelet activation in the presence of collagen. The obvious spread of the scatterplot was reduced in the presence of the KET loaded negatively charged HA:CS PECNs to resemble that of resting platelets.

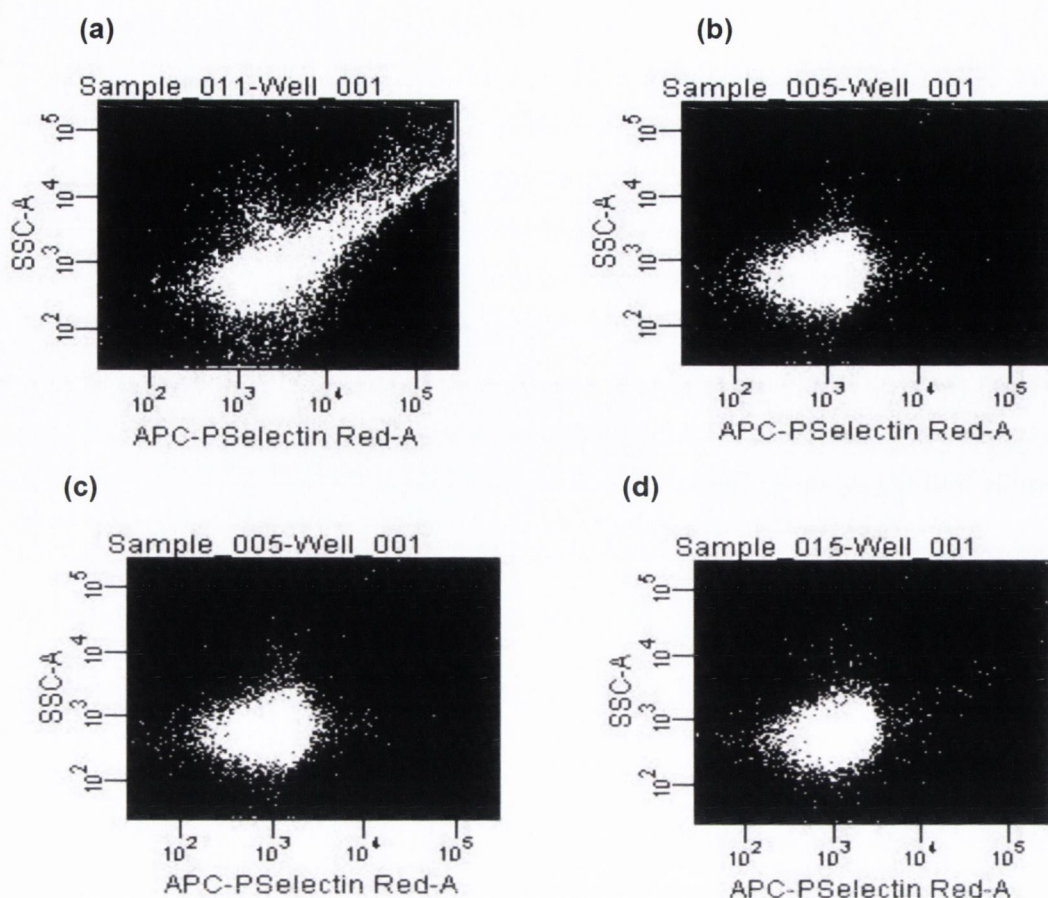


Figure 6.21 Flow cytometric analysis of platelet activation: scatter plots illustrating the extent of platelet activation of (a) platelets activated with collagen, (b) resting platelets, and of platelets activated with collagen in the presence of (c) positively charged KET loaded HA:CS nanoparticle following 10 min of incubation and (d) negatively charged KET loaded HA:CS PECN following 1 min of incubation. (n=3).

6.4.2.4 Comparison between 0.1 mg/mg KET loading and 0.2 mg/mg KET loading

A comparison was made between the negatively charged HA:CS PECNs loaded with 0.1 mg/mg and 0.2 mg/mg of KET to confirm, as was done for aggregometry (**Section 6.3.2.1**), that there was no advantage in using the lower concentration of KET in these experiments. There was no difference observed in the number of receptor copies measured for KET 0.1 mg/mg and KET 0.2 mg/mg loaded HA:CS PECNs (**Table 6.8**).

Table 6.8 The number of receptor copies expressed (mean $\pm$ SD) on the surface of the resting platelets, on the platelets incubated with negatively charged KET loaded HA:CS PECNs at 0.1 mg/mg and 0.2 mg/mg, negatively charged empty HA:CS PECNs and collagen stimulated platelets (n= 3)

Sample Type	Number of receptor copies
Resting platelets	1231 $\pm$ 122
0.1 mg/mg KET neg HA:CS PECNs	2955 $\pm$ 1330
0.2 mg/mg KET neg HA:CS PECNs	2209 $\pm$ 1514
Empty HA:CS neg	1393 $\pm$ 193
Collagen	14268 $\pm$ 646

6.5 Incubation Time Effect

Incubation for 10 min had a significant effect on the percent inhibition of aggregation for all types of NCs prepared with either KET or IND with the exception of negatively charged KET and IND loaded HA:CS PECNs (**Table 6.9 and 6.10**). There was a higher percent increase in inhibition of aggregation for PLGA NPs as opposed the HA:CS PECNs, which would be expected as PLGA NPs are a slow release formulation. Thus the drug would release more slowly with time into the surrounding medium, while the HA:CS PECNs would start releasing their drug more quickly once added and so would be expected to have more of an immediate effect at 1 min incubation as was observed, except for HA:CS IND.

While for the free drug solution the amount of drug added was instantly available to the platelets to impact upon their response to collagen induced aggregation, this was not the case for the drug loaded into NCs. For the PLGA KET and IND loaded NPs the drug was released slowly, so the nominal drug content was not the amount of drug immediately available in solution with the platelets. The percent increase in inhibition with incubation time shows that there was increasing inhibitory activity and thus the drug was being continuously released over the incubation period.

For the HA:CS PECNs loaded with KET or IND the DL was lower but the drug release was expected to be quicker based on the measured drug release profiles

(Chapter 4 and 5). For this reason the percent inhibition of aggregation did not increase as much for HA:CS PECNs as for the PLGA NPs between 1 and 10 min incubation time. IND showed a larger increase when loaded into the HA:CS PECNs from 1 to 10 min of incubation than KET loaded HA:CS PECNs most likely due to the drug content being so low that the concentration of drug present immediately did not have pharmacological activity to inhibit platelet aggregation. The increase observed may also be related to IND having a lower solubility than KET.

Table 6.9 Increase in inhibition of platelet aggregation seen after 10 min incubation for IND 0.2mg/mg NCs compared to 1 min incubation with platelets (based on aggregometry studies).

IND HA:CS Pos Conc (ug/ml)	% Increase of Inhibition	IND HA:CS Neg Conc (ug/ml)	% Increase of Inhibition	IND PLGA Conc (ug/ml)	% Increase of Inhibition
0	39	0	0	0	0
4	48	4	1	4	18
8	44	8	3	8	73
40	40	40	23	20	75

Table 6.10 Increase in inhibition of platelet aggregation seen after 10 min incubation for KET 0.2 mg/mg NCs compared to 1 min incubation with platelets (based on aggregometry studies).

HA:CS KET Pos Conc (ug/ml)	% Increase of Inhibition	HA:CS KET Neg Conc (ug/ml)	% Increase of Inhibition	KET PLGA Conc (ug/ml)	% Increase of Inhibition
0	0	0	0	0	0
4	15	4	70	4	18
8	16	8	38	8	86
40	3	40	33	20	89

A dose-response sigmoidal curve was fitted to the free KET (**Figure 6.22**) and free IND (**Figure 6.23**) data to compare the response of the KET and IND loaded NCs to that of free drug. **Table 6.11** compares the amount of KET released, as predicted by the curve for KET based on the % inhibition of aggregation, as a percentage of the estimated actual amount of KET loaded into each NC type.

The table showed that for KET loaded PLGA NPs at 1 min incubation the inhibitory response at 1 min confirmed that there release of KET was not complete while at 10 min incubation for PLGA KET loaded NPs the amount of drug predicted to be present by the curve was 100 % that predicted as loaded at 8 ug/ml of NPs and 98 % at 20 ug/ml of NPs. The positively charged HA:CS PECNs showed excellent responses compared to the predicted responses at the concentration of KET loaded. The responses observed after 10 min incubation surpassed the predicted response for the concentration of KET loaded (467 % at 4 ug/ml and 200 % at 8 ug/ml) while at 40 ug/ml the response was 100 % of the predicted. This means that for the positively charged HA:CS PECNs the response was amplified which may be worth further investigation. For negatively charged HA:CS PECNs the increase in correlation between drug loaded and effect was not observed from 1 min incubation to 10 min incubation. In fact, for these negatively charged KET loaded PECNs the response was less than 50% the predicted response for the concentration of KET loaded. This may mean that the KET did not completely release from the negatively charged HA:CS PECNs as predicted.

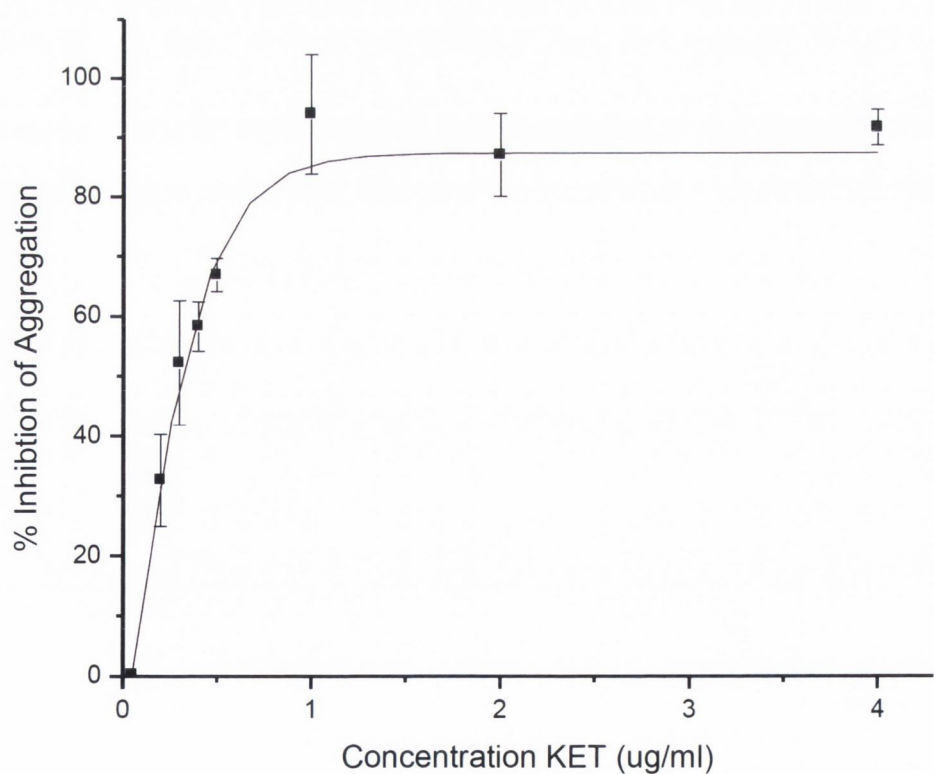


Figure 6.22 Dose-response curve for free KET.

Table 6.11 The % KET release for each NC after 1 min and 10 min incubation with the platelets was estimated from a sigmoidal dose-response curve fitted to free KET against % inhibition of aggregation. This table shows the effect of the NCs as a % of the expected effect for each NC based on the actual KET loaded.

Conc of NC (ug/ml)	PLGA		HA:CS		HA:CS	
	Pos	Neg	Pos	Neg	Pos	Neg
	1m	10 m	1m	10m	1m	10m
0	0	0	0	0	0	0
4	11	25	57	467	41	41
8	5	100	67	200	23	27
20	3	98	N/A	N.A	N/A	N/A
40	N/A	N/A	19	100	4	12

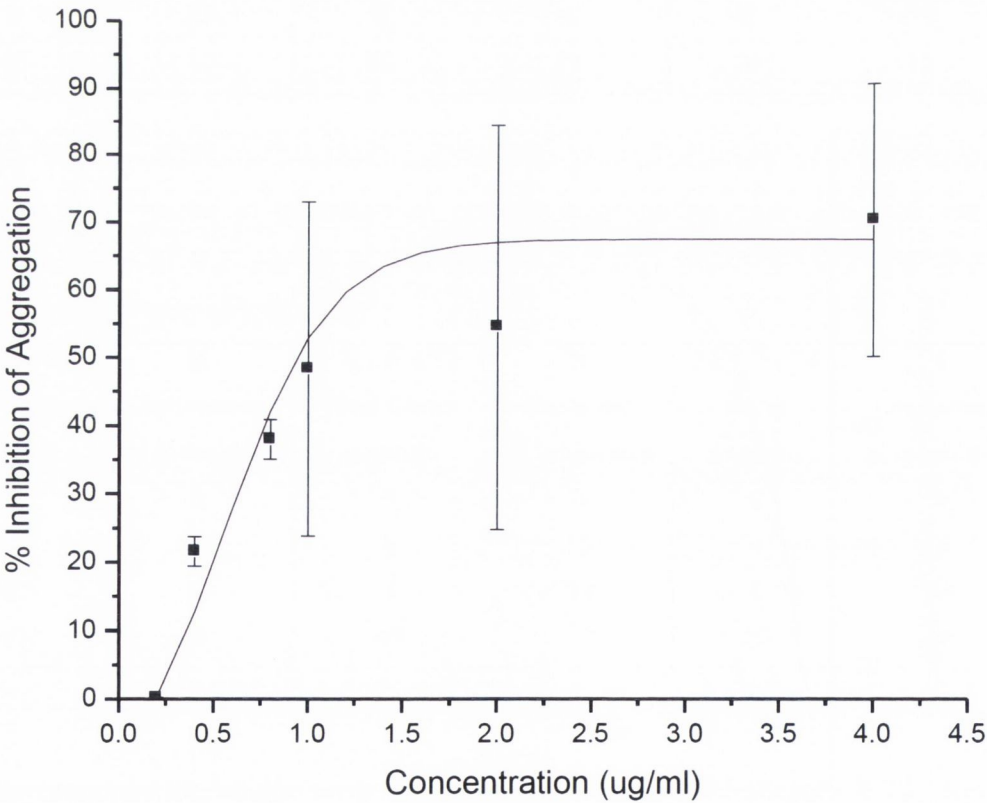


Figure 6.23 Dose-response curve for free IND.

Table 6.12 compares the amount of IND released, as predicted by the sigmoidal dose-response curve for IND based on the % inhibition of aggregation, as a percentage of the estimated actual amount of IND released at the 1 min or 10 min incubation. This table shows that for many of the IND loaded NCs the observed responses were greater than the responses predicted for the concentration of IND loaded. To be more specific this table shows that IND loaded PLGA NPs at 1 min incubation showed a response below that expected confirming that there was not complete release of IND at this point. After 10 min incubation for the PLGA IND loaded NPs the amount of drug predicted to be present by the curve was 455 % of the response predicted as loaded at 4 ug/ml, 228 % at 8 ug/ml and 91 % at 20 ug/ml. This showed that the response observed was beyond that seen with free IND at the concentrations loaded.

The positively charged HA:CS PECNs showed a similar pattern with no response seen at 1 min, followed by a rapid increase in response at 10 min to above that expected for this concentration. The actual responses observed compared to the predicted responses after 10 min was again way above that predicted. (379 % at 4 ug/ml, 224 % at 8 ug/ml) while at 40 ug/ml the response was 81 % of the predicted response.

Finally for negatively charged IND loaded HA:CS PECNs the increase in correlation between drug loaded and effect was not observed from 1 min to 10 min incubation. For these negatively charged HA:CS PECNs the response at 1 min was similar to that at 10 min and both exceeded the predicted response again at the lower concentrations of PECNs added: 4 ug/ml and 8 ug/ml. This showed that at lower concentrations of IND the inclusion of IND in the NCs was enhancing the response of free IND.

It should be noted that the IND dose-response curve constructed in **Figure 6.23** was not as close a fit as that for KET (**Figure 6.22**) due to large variations in the donor responses to free IND.

Table 6.12 The % IND release for each NC after 1 min and 10 min incubation with the platelets was estimated from a sigmoidal curve fitted to free IND against % inhibition of aggregation. This table shows the effect of the NCs as a % of the expected effect for each NC based on the actual IND loaded.

Conc of NC (ug/ml)	PLGA		HACS Pos		HACS Neg	
	1m	10m	1m	10m	1m	10m
0	0	0	0	0	0	0
4	56	455	0	379	211	222
8	31	228	0	224	134	147
20	13	91	NA	NA	NA	NA
40	NA	NA	19	81	40	63

6.6 Conclusions

All of the empty NCs used; PLGA NPs and HA:CS PECNs with a positive or a negative charge did not induce platelet aggregation within a concentration up to 80 ug/ml when incubated with platelets. This supports the findings of Li et al. (2009) who studied the effect of PLGA, PLGA-CS and CS NPs on platelet function. The current work supports this study to suggest that these NCs are compatible with platelets. This is an important statement as many other research groups have found that NPs induce platelet aggregation. Radomski et al. (2005) found that platelet aggregation was induced by carbon nanotubes, Corbalan et al. (2012) tested the reaction of platelets to amorphous silica NPs and found that they induced severe platelet aggregation. Jun et al. (2011) found that silver NPs also induced platelet aggregation and Miller et al. (2009) even suggested a use for NPs in the formation of more effective blood clots.

The NCs tested in the current work were prepared using either PLGA or a HA:CS combination. These two types of NCs have very different physical properties as outlined in **Chapters 3-5**. HA:CS PECNs could be further divided based on their surface charge.

KET loaded PLGA NPs showed an increase in the % inhibition of aggregation between 1 and 10 min which was not observed for the negatively charged KET loaded HA:CS PECNs. The results observed in this current chapter support the

idea that PLGA KET release is slower than negatively charged HA:CS KET release. Interestingly, the observed effectiveness of the KET loaded PLGA NPs was greater than that for negatively charged KET loaded HA:CS PECNs despite them having the same KET loading. When compared to the inhibition effect of free KET, the KET loaded PLGA NPs seemed to match the effect of free KET while this was not observed for the negatively charged KET loaded HA:CS PECNs. This may indicate that KET is still associated with the HA:CS polymers and thus cannot interact with the platelets or is not being fully released in the platelet sample.

It was more difficult to compare PLGA and HA:CS NCs when loaded with IND due to greater differences in DL values. Both formulation types showed an increase in % inhibition from 1 to 10 min of incubation but the increase was much more pronounced for the IND loaded PLGA NPs. As the IND loaded PLGA NPs showed a far higher DL capacity than the HA:CS PECNs, it would appear that PLGA NPs seem to be more suitable NPs to target platelets. The highest concentration of PLGA NPs used was 20 ug/ml while 40 ug/ml of the HA:CS PECNs positively charged or negatively charged had to be used to get the same effect.

There were noticeable differences between the negatively and positively charged HA:CS PECNs (KET and IND loaded) observed. For the negatively charged HA:CS PECNs loaded with either IND or KET there was less of an increase in the effect of the drug after 10 min of incubation while the positively charged HA:CS PECNs with KET or IND showed a marked increase in the inhibition in platelet aggregation after 10 min of incubation. Regardless of the DL, for KET the positively charged HA:CS PECNs were more effective. This could be related to attraction between the positively charged HA:CS PECNs and the platelets or it could relate to the stability of the HA:CS PECNs. As was explained in **Chapter 4** for KET and **Chapter 5** for IND the pI for negatively charged HA:CS PECNs was 4.5 for KET and 3.8 for IND, while the pI for positively charged HA:CS PECNs was 6.8 for KET and 6.4 for IND.

The final comparison that can be made from the data in this chapter is between IND and KET. KET has been proven to have a strong anti-platelet action relative to IND (Petrusewicz et al. 1994 and Leach and Thorburn 1982). In this chapter the KET loaded NCs showed stronger anti-platelet action at low concentrations

regardless of the type of NC used. The performance of KET was superior to that of IND in these NCs with greater inhibitory effects being observed. Despite KET having stronger effects, the effect of IND loaded NCs was seen to surpass the response expected from free IND while the response for KET loaded NCs was in most cases in line with the response for free KET. At the higher concentrations of NCs (20 ug/ml for PLGA and 40 ug/ml for HA:CS) the concentration of IND in contact with blood platelets was similar to that which is bioavailable post intramuscular injection (2.7 ug/ml) as reported by Jensen and Grenabo (1985). For KET Ishizaki et al. (1980) reported plasma concentrations of 0.43 to 5.62 ug/ml after the final dose of a 50 mg q.i.d. intramuscular injection regimen. Due to the requirement to reach these plasma concentrations it is necessary to use the most efficient NCs with the highest DL and efficacy. For this reason it would be recommended not to further investigate the negatively charged HA:CS PECNs. PLGA NPs and positively charged HA:CS PECNs showed more promise for future experimentation. It may be worth further investigation into whether the NCs were helping the less soluble drug IND to exert their anti-platelet action. It could also be of interest to examine whether the NCs were having any effect on the half-life of the drugs.

General Discussion

Chapter 7

7.1 Introduction

This thesis focused on the development of polymeric NCs, PLGA NPs and HA:CS PECNs loaded with the same drugs, IND and KET, to study their effects on platelet aggregation. Two types of NCs were chosen: PLGA NPs were a hydrophobic system formed by an emulsion/solvent-diffusion technique and HA:CS PECNs, which were hydrophilic systems formed based on electrostatic interactions between two polymers. The drugs IND and KET were chosen to represent the growing category of hydrophobic, organic, small drug molecules and also because they inhibit platelet aggregation. Previous studies by Li et al. (2010) demonstrated that PLGA, CS and PLGA-CS NPs did not induce platelet aggregation. In fact, they showed some inhibition of platelet aggregation at high concentrations (100 ug/ml). Therefore, it was seen as an interesting progression to load NCs with KET and IND to see how the NCs would behave in the presence of platelet active drugs. This current research expanded the work on PLGA NPs to include HA:CS PECNs. For this reason the research in this thesis started with optimising and characterising the NCs with and without KET and IND before conducting the studies on platelet compatibility.

7.2 KET Loaded NPs

Chapter 3 and **Chapter 4** proved that KET could be successfully loaded into all three NC types: PLGA NPs, positively charged HA:CS and negatively charged HA:CS PECNs. KET represents a hydrophobic drug, which has good aqueous solubility within certain pH ranges. Sheng et al. (2006) in their research into the effect of pH and solubilisation on the solubility of KET called for a waver of *in vivo* bioequivalence for KET given that found its solubility in McIlvaine buffer (0.1 M citric acid and 0.2 M disodium phosphate solutions) prepared at different pH values to be 0.28 ± 0.01 mg/ml at pH 4 increasing at pH 6.8 to 40.76 ± 0.01 mg/ml. The addition of sodium lauryl sulphate (SLS) at 1% w/v further increased the solubility at pH 4 to 4.70 ± 0.04 mg/ml and at pH 6.8 to 49.63 ± 0.01 mg/ml. This meant that KET appeared to be soluble at higher pH values.

The size of KET loaded NPs was greatest for PLGA NPs at 344 ± 2 nm at KET 0.1 mg/mg and 316 ± 3 nm at KET 0.2 mg/mg. These NPs were significantly

larger ($p < 0.001$) than the HA:CS PECNs which ranged in size from 173 nm – 187 nm depending on the loading and charge. This means that the PLGA NPs were larger and expected to have a lower surface area as microscopy revealed them to be spherical, non-porous NPs in comparison to the more disordered particles observed for HA:CS PECNs. The larger average size was accompanied by a larger PDI suggesting that there is wider size distribution within the PLGA samples (**Table 7.1**).

The ZP for the NCs loaded with KET was negative for PLGA NPs and the ZP of HA:CS PECNs was dependent on the HA:CS composition.. There was no significant difference between the ZP of PLGA NPs and of negatively charged HA:CS PECNs (**Table 7.1**).

Despite the surface charges being close, there was a considerable difference in the KET loading for these NCs. KET loading varied for each NC. The highest KET loading values were for HA:CS negatively charged PECNs, followed by PLGA NPs and lastly HA:CS positively charged PECNs. The KET loading was highest in negatively charged HA:CS PECNs with EE of $63.1 \pm 5.7\%$ w/w, DL $11.4 \pm 1.1\%$ w/w for an initial KET loading of 0.2 mg/mg and $94.1 \pm 1\%$ w/w, DL $8.6 \pm 0.1\%$ w/w for an initial KET loading of 0.1 mg/mg. This was much greater than that seen for PLGA NPs or positively charged HA:CS PECNs (**Table 7.1**). All of the KET loaded NCs showed a higher DL for an initial loading of 0.2 mg/mg than an initial loading of 0.1 mg/mg but this difference for HA:CS positively charged PECNs was not significant. Given the difference in results for HA:CS PECNs loading of a higher amount of KET 0.4mg/mg was attempted. Negatively charged KET loaded HA:CS PECNs showed that the capacity of these PECNs to load KET was high as the EE was $51.0 \pm 3.6\%$ w/w which resulted in a DL of $18.7 \pm 1.0\%$ w/w when the initial KET added was 0.4 mg/mg. For positively charged HA:CS PECNs increasing the KET initial loading to 0.4 mg/mg did not result in increased DL with an EE of $14.9 \pm 1.7\%$ w/w and DL of $5.9 \pm 0.4\%$ w/w. This shows that there may have been a limit to the capacity of HA:CS positively charged PECNs to load KET that was not present for negatively charged HA:CS PECNs. Given that the negative charge did not seem to be the reason as negatively charged PLGA NPs had DL values similar to positively charged HA:CS PECNs it was assumed that this is due to the solubility of KET at pH of the HA:CS PECNs. The other reason for the high DL may have been due to KET

interactions with HA as HA was in excess according to the weight ratio and charge ratio for the negatively charged HA:CS PECNs.

Thermal analysis for KET loaded PLGA NPs showed that in all cases KET was present in the amorphous form, as no crystalline peaks were seen using PXRD analysis and no melting points for KET were observed using DSC analysis. Peaks on PXRD diffractograms were observed pre-washing for some higher concentration HA:CS PECNs but once the washing steps were completed this crystalline content had been removed. This indicates that KET was miscible with the polymers so that on precipitation and washing it did not separate and crystallise. This was the case for the hydrophilic HA:CS PECNs and the hydrophobic PLGA NPs showing the KET is versatile and can mix well with either system.

The results of the KET release studies showed that the PLGA NPs gave the slowest KET release of all of the KET loaded NCs. Release experiments into PBS showed an initial burst release of 61.1 ± 3.2 % for KET 0.1 mg/mg and 86.1 ± 1.4 % for KET 0.2 mg/mg loaded PLGA NPs followed by a gradual release of the remaining drug over a 14 day period to a final release of 92.3 ± 4.2 % for KET 0.1 mg/mg and 96.0 ± 5.7 % for KET 0.2 mg/mg. HA:CS PECNs showed a very rapid release of the KET content. For HA:CS PECNs given that their structure was stabilised by electrostatic interactions their release was investigated in three different release media. For PBS and water HA:CS PECNs released their drug content almost completely within 10 minutes. When the release medium was 0.01 M HCl the full amount of KET was not released over 14 days for negatively charged KET 0.2 mg/mg HA:CS PECNs, which could be due the drug having reduced solubility at this low acidic pH compared to pH 7.4 for PBS.

KET loaded NCs showed good physical stability over a 14-day period with no visible precipitation obvious and little changes in the ZP being observed. PLGA NPs showed the least change in the NC structure over the 14-day period as observed using DLS but positively charged HA:CS PECNs showed the greatest reduction in size over the 14 day period.

It was difficult to know exactly where the KET was located in these NCs. Corrigan et al. (2009) stated that the drug present on the surface of the PLGA NPs is generally released during the burst release, which would allow an

estimation of the % of KET loaded into these PLGA NPs on the surface compared to the fraction present deeper inside the PLGA NPs. For the HA:CS PECNs it is more difficult to say as these structures released amorphous KET straight away and their structure was less solid, as they appear to be held together by entangled polymer chains interacting based on charge. It could be assumed that the KET was interacting with the polymers in HA:CS PECNs based on charge but given its amorphous nature and its higher DL in negatively charged HA:CS PECNs it is possible that KET had dissolved or mixed with HA and was reliant on HA dissolution or disassociation from CS for release.

Both of these NC types appeared suitable for KET loading. The main deciding factor in choosing between formulations would be the duration of KET release required and pH of the biological fluid for KET release.

Table 7.1 Comparison of the physical properties of KET loaded NCs as investigated in **Chapter 3** and **Chapter 4**. PDI = polydispersity index, ZP = ZP, EE = encapsulation efficiency, DL = drug loading, pI = isoelectric point, N/A = not analysed.

Type of KET NC	Size (nm)	PDI	ZP (mV)	EE (%)	DL (%)	pI	Solid-State
PLGA NP 0.1 mg/mg	344 ± 2	0.22 ± 0.07	- 29. ± 1	19.1 ± 4.0	2.0 ± 0.4	N/A	amorphous
PLGA NP 0.2 mg/mg	316 ± 3	0.31 ± 0.09	- 33. ± 1	41.4 ± 11.3	8.2 ± 3.4	N/A	amorphous
HA:CS Neg PECN 0.1 mg/mg	187 ± 1	0.15 ± 0.01	- 31 ± 1	94.0 ± 0.9	8.6 ± 0.1	N/A	amorphous
HA:CS Neg PECN 0.2 mg/mg	173 ± 1	0.10 ± 0.04	- 28 ± 1	63.1 ± 5.6	11.4 ± 1.1	4.5	amorphous
HA:CS Neg PECN 0.4 mg/mg	170 ± 2	0.09 ± 0.02	- 27 ± 1	51.0 ± 3.6	18.7 ± 1.0	N/A	amorphous
HA:CS Pos PECN 0.1 mg/mg	182 ± 1	0.11 ± 0.02	+ 37 ± 1	38.4 ± 7.3	3.7 ± 0.1	N/A	amorphous
HA:CS Pos PECN 0.2 mg/mg	187 ± 2	0.10 ± 0.03	+ 34 ± 1	28.0 ± 4.5	5.3 ± 0.8	6.8	amorphous
HA:CS Pos PECN 0.4 mg/mg	282 ± 3	0.10 ± 0.05	+ 41 ± 1	14.9 ± 1.7	5.9 ± 0.4	N/A	amorphous

7.3 IND Loaded NCs

Chapter 3 and **Chapter 5** discussed the loading of IND into all three NC types: PLGA NPs, HA:CS positively charged and HA:CS negatively charged PECNs. IND represented a hydrophobic drug, which had very poor aqueous solubility within certain pH ranges.

The size of IND loaded NPs was greatest for PLGA NPs at 291 ± 3 nm at IND 0.1 mg/m and 323 ± 8 nm at IND 0.2 mg/mg. These NPs were significantly larger ($p < 0.001$) than the HA:CS PECNs which ranged in size from 184 – 200 nm depending on the loading and charge. This means that the PLGA NPs were larger and were expected to have a lower surface area as microscopy revealed them to be spherical, non-porous NPs in comparison to the more disordered particles observed for HA:CS PECNs. This size behaviour and morphology were the same as that observed for KET loaded NCs. The difference between KET and IND was that the IND loaded PLGA NPs were not as large as the KET loaded PLGA NPs and the PDI for IND loaded PLGA NPs was lower than that for KET loaded PLGA NPs and not significantly larger than that seen for IND loaded HA:CS PECNs. The IND loaded HA:CS PECNs also had a slightly larger size range and higher PDI values than was observed for KET loaded HA:CS PECNs (**Table 7.2**).

As was the case for KET loaded NCs, the ZP for the NCs loaded with IND was negative for PLGA NPs and the ZP of HA:CS PECNs was dependent on the HA:CS composition. There was no significant difference between the ZP of PLGA NPs and of negatively charged HA:CS PECNs. While the positively charged HA:CS PECNs loaded with IND had ZP values close to those for KET loaded positively charged HA:CS PECNs, the values observed for negatively charged HA:CS PECNs and PLGA NPs loaded with IND were significantly more negative than the KET loaded equivalent NCs ($p < 0.01$).

Despite the surface charges being close, there was a considerable difference in IND loading for these NCs. IND loading varied for each NC, having its highest loading values for PLGA NPs, then HA:CS positively charged PECNs and lastly HA:CS negatively charged PECNs. The IND loading was highest in PLGA NPs with EE of 91.2 ± 4.0 % w/w, DL 9.0 ± 0.8 % w/w for an initial IND loading of 0.1 mg/mg and EE of 93.2 ± 2.0 % w/w, DL 19.1 ± 0.4 % w/w for an initial IND loading of 0.2 mg/mg. This was much greater than that seen for positively charged HA:CS PECNs or negatively charged HA:CS PECNs (**Table 7.2**). All of the IND loaded NCs showed a higher DL for an initial loading of 0.2 mg/mg than for an initial loading of 0.1 mg/mg but the increase for HA:CS negatively charged from 0.1 mg/mg to 0.2 mg/mg was not significant. This pattern was different to that observed for KET loaded NCs. PLGA NPs loaded with IND were smaller,

less polydisperse and had higher DL than KET loaded PLGA NPs, which was mostly likely directly related to the hydrophobicity IND in comparison to KET. In contrast to KET loaded HA:CS PECNs, IND loaded HA:CS PECNs had higher DL for positively charged HA:CS PECNs than negatively charged HA:CS PECNs. This may be due to the solubility of IND. As IND has very low solubility it may not have mixed as well with the HA and CS polymers and thus its loading may have relied on the electrostatic interactions with CS, the positively charged polymer. This could be supported by the fact the IND loaded HA:CS PECNs were larger in size than KET loaded HA:CS PECNs.

In the same test as was used for KET, it was attempted to load a higher amount of IND (0.4 mg/mg) into the HA:CS PECNs. For negatively charged HA:CS PECNs 0.4 mg/mg IND loaded HA:CS PECNs would not form, and for positively charged HA:CS PECNs they did form but they were very physically unstable with a large particle size and high polydispersity (397 ± 3 nm with PDI 0.3 ± 0.071). This showed that the capacity of these PECNs to load IND had been reached at this concentration and the drug could not mix with the polymer solution to form particles. This was in contrast to KET where DL and low particle size and PDI were still observed at 0.4 mg/mg for positively and negatively charged HA:CS PECNs.

Thermal analysis for IND loaded NCs was not as straightforward as for KET loaded NCs. PLGA NPs showed that in all cases IND was present in the amorphous form, as no crystalline peaks were seen using PXRD analysis and no melting points for IND were observed using DSC analysis. This was the same as has been seen for KET loaded PLGA NPs and is most likely related to IND solubility in the non-aqueous environment allowing it to mix with the PLGA polymer in ethyl acetate. For HA:CS PECNs Bragg peaks were observed pre-washing for positive and negatively charged HA:CS PECNs. Post-washing these crystalline peaks actually became more prominent for positively charged HA:CS PECNs but not for negatively charged HA:CS PECNs. This could be because the unbound and loosely bound IND was removed from negatively charged HA:CS PECNs on washing but for positively charged HA:CS PECNs the washing stimulated IND crystallisation. These crystals may have remained associated, based on electrostatic interactions, with the excess presence of CS. The loading remained higher for positively charged HA:CS PECNs than negatively charged post-washing. This was different to what was seen for KET

loaded HA:CS PECNs, where once the washing steps were completed the initial crystalline content had been removed. This showed that bound KET was more miscible with the polymers in solution than IND so that on washing it did not crystallise. The drug content for KET was higher for negatively charged and positively charged HA:CS PECNs than when these NCs were loaded with IND, with the exception of positively charged IND loaded HA:CS PECNs at 0.2 mg/mg. IND showed excellent compatibility with the hydrophobic PLGA NPs but this was not as certain for HA:CS PECNs, where the loading was lower and the association was weaker than for PLGA NPs.

Upon examination of IND release studies, as seen with KET, it was observed that the PLGA NPs gave the slowest IND release of all of the IND loaded NCs. The release into PBS resulted in an initial burst release of 61.1 ± 3.2 % for IND 0.1 mg/mg and 86.1 ± 1.4 % for IND 0.2 mg/mg loaded PLGA NPs followed by a gradual release of the remaining drug over a 14 day period to a final release of 92.3 ± 4.2 % for IND 0.1 mg/mg and 96.0 ± 5.7 % for IND 0.2 mg/mg. HA:CS PECNs showed very rapid release of the IND content, as was seen with KET loaded PECNs, but the behaviour of release was not the same as that observed for KET. In PBS the majority of IND was released within 10 minutes, with the highest release concentrations observed for IND 0.2 mg/mg positively charged HA:CS PECNs. When the release medium was 0.01M HCl the release from all samples was about half the concentration of that released for PBS and IND 0.2 mg/mg positively charged no longer showed the highest release values. This is most likely linked to the low solubility of IND at low pH values. The IND released in this 0.01M HCl is more likely to be the amorphous fraction of loaded IND as the crystalline IND present would not have been expected to dissolve at this pH. When deionised water was the release medium the IND release was in the same concentration range as in 0.01M HCl, which again could be related to pH of the medium. Also, the low release could have been related to the low ionic content of deionised water as compared to PBS. The ionic content of PBS could have disrupted the HA:CS PECNs by changing the electrostatic interactions inducing faster release of all the IND content (Dautzenberg 1997). For PBS and 0.01M HCl all HA:CS PECNs released their drug content almost completely within 10 minutes. In deionised water the greatest amount of IND was not released until 60 minutes.

IND loaded NCs showed good physical stability over a 14-day period with minimal change in the ZP being observed for most of the NCs. HA:CS negatively charged IND 0.2 mg/mg NPs started to show IND precipitation at day 10 and positively charged HA:CS PECNs showed precipitation on day. The particle size of negatively charged IND loaded HA:CS PECNs varied very little but there was a drop in size of approx. 20 % observed for positively charged IND loaded HA:CS PECNs. IND loaded PLGA NPs showed the least change in the NC structure over the 14-day period as observed using DLS, the same observation as was made for KET loaded NCs.

Just like with KET loaded NCs, it was difficult to know exactly where the IND was located in these NCs. The burst release allowed an estimation of the percentage of IND loaded into these NPs on the surface and IND slow release was assumed to be associated with IND located deeper inside the PLGA NPs. For the HA:CS PECNs it is more difficult to say as these structures released IND straight away, their structure was less solid and IND appeared to be present in different solid-state forms. In this case not only was the IND location important but also the solid state as both crystalline IND of different polymorphic forms and amorphous IND were associated. CS is expected to interact with acidic drugs such as IND but this interaction type may be dependent on pH of CS (Mi et al. 2001). As IND had higher DL in positively charged HA:CS PECNs it is possible that most of the IND crystals were associated with CS in the HA:CS PECNs.

Both of these NC types appeared suitable for IND loading but the PLGA NPs had higher DL and were more consistent in the solid-state of IND present. This would make PLGA IND loaded NPs a more reliable formulation as release and stability could be better predicted. For HA:CS PECNs loaded with IND there maybe some use given that they are fast release but overall IND may have been too hydrophobic to make a NC that could be consistent enough for future use. That said, it was very interesting to see that IND, a practically insoluble drug, could be loaded into these hydrophilic systems and to see the solid-state forms and manner of incorporation.

Table 7.2 Comparison of the physical properties of IND loaded NCs as investigated in **Chapter 3 and Chapter 5**. PDI = polydispersity index, ZP = ZP, EE = encapsulation efficiency, DL = drug loading, pl = isoelectric point, N/A = not analysed.

Type of IND NC	Size (nm)	PDI	ZP (mV)	EE (%)	DL (%)	pl	Solid-State
PLGA NP 0.1 mg/mg	291 ± 3	0.160 ± 0.140	- 32 ± 1	91.2 ± 4.0	9.0 ± 0.8	N/A	amorphous
PLGA NP 0.2 mg/mg	323 ± 8	0.200 ± 0.100	- 35 ± 1	93.2 ± 2.0	19.1 ± 0.4	N/A	amorphous
HA:CS Neg PECN 0.1 mg/mg	200 ± 1	0.100 ± 0.020	- 32 ± 1	26.4±5.7	2.6±0.6	N/A	Crystalline and amorphous
HA:CS Neg PECN 0.2 mg/mg	186 ± 1	0.142 ± 0.050	- 36 ± 1	17.6±1.7	3.5±0.3	3	Crystalline and amorphous
HA:CS Pos PECN 0.1 mg/mg	184 ± 3	0.120 ± 0.030	+ 34 ± 1	32.8±6.8	3.3±0.7	N/A	Crystalline and amorphous
HA:CS Pos PECN 0.2 mg/mg	196 ± 1	0.242 ± 0.054	+38 ± 3	50.3±2.4	10.1± 0.5	6.4	Crystalline and amorphous
HA:CS Pos PECN 0.4 mg/mg	397 ± 3	0.300 ± 0.071	+ 46 ± 1	N/A	N/A	N/A	N/A

7.4 PECN Compatibility with Hydrophobic Drugs

Hydrophobic, small drug molecules are being revealed in abundance as potential new drug entities by computational modelling (Jorgensen 2004). A great challenge for these drugs is finding a compatible drug delivery system. Previously, hydrophobic drugs have been loaded with success into polymeric NPs which are hydrophobic in nature, for example PLGA NPs. PECNs are hydrophilic systems formed based on electrostatic interactions between two polymers or a polymer:drug system. Previous studies combining hydrophobic drugs with polymers have been successful. Cheow and Hadinoto (2012) prepared an amorphous drug-polyelectrolyte NP complex, where the nanoplex

was held together by the drug-polyelectrolyte electrostatic interactions coupled with an interdrug hydrophobic interaction. The drugs successfully incorporated were poorly soluble acidic (i.e. ibuprofen and curcumin) and basic (i.e. ciprofloxacin) and the polymers were biocompatible poly(allylamine hydrochloride) for acidic drugs and dextran sulphate for basic drugs. The drug content was 80-85%. These nanoplexes were larger in size (300-500 nm - depending on the drug hydrophobicity) than those HA:CS PECNs used in the current studies, and only used one polymer. This essentially means that the particle consists of mostly drug with polymer acting more like a stabiliser for the drug. The ability of hydrophobic drugs to associate with two polymers has only been tested using cross linkers such as TPP or calcium chloride (Jingou et al. 2011, Jingou et al. 2012).

Umerska et al. (2012) developed PECNs using CS and HA, both of which have interesting properties of being biocompatible and biodegradable (Liu et al., 2008), without the use of cross-linkers. CS influences drug reactivity with negatively charged surfaces such as cell membranes and may enhance permeability (Agnihotri et al. 2004) while HA has been reported to act synergistically with CS to enhance mucoadhesion (Wadhwa et al. 2009).

In the current studies HA:CS PECNs were successfully prepared with either a positive or negative charge so given this flexibility it was thought that it would be an interesting experiment to investigate if they were capable of combining with hydrophobic, small drug molecules. Using KET and IND gave useful, new insights into the possible mechanisms for association of hydrophobic drugs in the HA:CS PECN system. KET and IND showed different association behaviour, which was assumed to be related to the solubility of these drugs as they were both NSAIDs with close pKa values (IND = 4.5 and KET = 4.23) but their solubility was notably different (IND in deionised water was 0.031 ± 0.001 mg/ml, KET in deionised water was 0.12 ± 0.04 mg/ml). It was seen when loading KET that it loaded with higher DL values for negatively charged HA:CS PECNs which can be seen **Table 7.3** at 0.2 mg/mg concentration. This was assumed to be related to the solubility of KET in HA solution, which was the excess polymer in the weight and charge ratio for the negatively charged HA:CS PECNs and due to its predicted miscibility with the polymers. The KET loaded was amorphous which showed that good mixing was possible with the HA and CS polymers. IND on the other hand was able to associate with positively charged HA:CS PECNs to give higher DL values. IND associated with these PECNs as crystalline and

amorphous drug, which showed that while some IND may have mixed the rest was most likely associating based on electrostatic interactions with CS. The IND crystals were present in different polymorphic forms highlighting a risk with these IND loaded HA:CS PECNs that the drug release may be difficult to predict due to the fact that IND had loaded in different solid-state forms. The DL values for IND and KET with their most suitably charged NC were close. For example IND in positively charged HA:CS PECNs had a DL at 0.2 mg/mg of $10.1 \pm 0.5\%$ w/w compared to KET negatively charged HA:CS PECNs which had a DL of $11.0 \pm 1.0\%$ w/w. Given that KET could further increase its DL when an initial loading of 0.4 mg/mg KET was tried while IND could not form physically stable PECNs if the drug content was increased this suggests that KET's solubility gave it an advantage. The drug did not greatly affect the charge ratios for IND and KET (**Chapters 4** and **Chapter 5**) and so these drugs are not likely to highly affect the overall charge ratios for these HA:CS PECNs.

It was recognised that it would be a challenge to load hydrophobic drugs into PECNs due to the difference in solubility between the drugs and polymers. These current studies met their aims by successfully loading these hydrophobic drugs (IND and KET) into the HA:CS PECNs. KET was considered more compatible with these carriers than IND because IND precipitated in multiple solid-state forms. This meant it would be difficult in the case of IND loaded HA:CS PECNs to reliably predict the ratio of solid-state forms present, the storage stability and the release pattern for these IND loaded PECNs. This crystallisation could prove troublesome as the IND crystallising was precipitating in more than one polymorphic form. There was evidence of the α , γ and ζ crystalline forms of IND precipitated (**Chapter 5**). Further PXRD studies could be conducted running the test over a longer period of time facilitating better resolution and less noise. This may give more clarity as to the solid state of the drugs in the HA:CS PECNs (Klung and Alexander 1974). While these studies showed that their formation is possible there is definitely room for further development as the EE values observed were low thus there was some drug wastage. Further studies using other hydrophobic drugs would be necessary to build up more data on the association between these HA:CS PECNs and hydrophobic drugs.

Table 7.3 HA:CS PECNs of positive or negative charged loaded with IND or KET. This table compares the properties of HA:CS PECNs with KET and IND as investigated in **Chapter 4 and Chapter 5**. PDI = polydispersity index, ZP = ZP, EE = encapsulation efficiency, DL = drug loading, pI = isoelectric point.

Type of HA:CS PECNs	Size (nm)	PDI	ZP (mV)	EE (%)	DL (%)	pI	Solid-State
KET Pos	187 ± 2	0.10 ± 0.03	+ 34 ± 1	38.4 ± 7.3	3.7 ± 0.1	6.8	amorphous
KET Neg	173 ± 1	0.10 ± 0.04	- 28 ± 1	63.1 ± 5.6	11.4 ± 1.1	4.5	amorphous
IND Pos	196 ± 1	0.2 ± 0.05	+ 38 ± 3	50.3 ± 2.4	10.1 ± 0.5	6.4	Crystalline and amorphous
IND Neg	186 ± 1	0.14 ± 0.05	- 36 ± 1	17.6 ± 1.7	3.5 ± 0.3	3.0	Crystalline and amorphous

7.5 The Effect of Surface Charge on HA:CS PECNs Properties

The effect of NP charge on their compatibility with the human cells is being widely researched with most agreeing that positively charged NPs show better association and uptake into human cells (Arvizo et al. 2010). CS itself has been found to interact with cells for example it is capable of aiding membrane adhesion and lysosomal escape of encapsulated DNA (Mislick and Baldeschwieler, 1996, Panyam and Labhasetwar, 2003). Verma and Stellacci (2010) reported in their review “Effect of Surface Properties on NP–Cell Interactions” that NPs with neutral surface coatings resist interaction with cells and that some studies have observed the uptake of negatively charged NP into cells despite their lack of interaction with the negatively charged membrane but the reviews agreed that based on the evidence, positively charged NPs are most effective in crossing cell-membrane barriers and localising in the cytosol or nucleus and they penetrate cell membranes best. They also pointed out that due to penetration there has been observed cytotoxicity with cationic particles. When Umerska et al. (2012) investigated the cytotoxicity of HA:CS PECNs using Caco-2 cells she observed some cytotoxicity which was CS concentration-dependent.. Umerska’s work concluded that positively HA:CS PECNs increased the IC₅₀ for cell viability by over 3-fold compared to CS alone suggesting that positively

charged HA:CS PENCs could be used as a less toxic alternative to CS. Umerska's work declared negatively charged HA:CS PECNs to be non-toxic in the whole range of concentration studied. Li et al. (2009) did not find that surface charge influenced the induction of platelet aggregation by PLGA, CS and PLGA NPs. This was supported by studies by Ramtoola et al. (2011).

From a physiochemical point of view the charge of the HA:CS PECNs influenced their compatibility with the drugs. IND appeared to have more affinity for the positively charged HA:CS PECNs due to electrostatic interactions with CS, while KET seemed to preferentially load better into negatively charged HA:CS PECNs assumedly as it was soluble in HA solution. The ZP for all HA:CS PECNs formed was stable empty and once loaded with drug for 14 days. It was noted that once drug loaded, the charge of HA:CS PECN seemed to become more positive. Given that the drugs are negatively charged but do not reduce the ZP it may be assumed that the drug could have been sterically blocking the surface charge. The size and PDI were not considerably different for positive or negatively charged HA:CS PECNs and the release profiles showed instant drug release for both types of charge.

When looking at interactions with platelets as discussed in **Chapter 6**, there were noticeable differences between the negative and positive charged HA:CS PECNs observed. Incubation of empty HA:CS PECNs with blood platelets for 14 minutes at 80 ug/ml showed that neither empty HA:CS positively charged PECNs nor negatively charged HA:CS PECNs were seen to induce platelet aggregation. Once loaded with drug it could be seen that there were differences in the behaviour between positively charged and negatively charged HA:CS PECNs. It should be noted that it was difficult to make direct comparison as different amounts of drug were loaded into the HA:CS PECNs system of different types. The positively charged HA:CS PECNs with KET or IND showed a marked increase in the inhibition in platelet aggregation after 10 minutes of pre-incubation, while this was not the case for the negatively charged systems. Regardless of the DL the positively charged HA:CS PECNs were more effective for each drug (IND and KET) at inhibiting platelet aggregation. For KET loaded HA:CS PECNs positively charged the DL was $5.3 \pm 0.8\%$ w/w but for negatively charged it was $11.4 \pm 1.1\%$ w/w and it was observed that the final percentage inhibition after 10 mins was lower for negatively charged KET loaded HA:CS PECNs than positively charged KET loaded HA:CS PECNs. Similarly, although

for IND loaded HA:CS PECNs the DL was higher for positively charged HA:CS PECNs ($13.1 \pm 0.5\%$ w/w) than negatively charged charged HA:CS PECNs ($3.5 \pm 0.3\%$ w/w), the percentage inhibition of aggregation after 10 minutes was still lower for the positively charged IND loaded HA:CS PECNs than the negatively charged HA:CS PECNs. This is very important to note as it shows that the charge of the PECNs had an influence on the platelet compatibility. This could be related to attraction between the positively charged HA:CS PECNs and the platelets or it could relate to the physical stability of the HA:CS PECNs. The positively charged HA:CS PECNs had a pl of 6.8 for KET loaded HA:CS PECNs and pl of 6.4 for IND loaded HA:CS PECNs, while the negatively charged HA:CS PECNs had a pl of 4.5 for KET loaded HA:CS PECNs and pl of 3 for IND loaded HA:CS PECNs. Given that blood pH is closer to pH 6.8 the positively charged HA:CS PECNs we likely to be less stable with the platelets than the negatively charged HA:CS PECNs so stability is most likely not the influencing factor causing greater inhibition of aggregation for positively charged HA:CS PECNs. If the positively charged HA:CS PECNs are neutralised in the blood this could have stabilised the particles by making them less likely to interact with other blood components.

7.6 Compatibility of the IND and KET loaded PLGA NPs and HA:CS PECNs with Platelets

Pharmaceutical anti-coagulation therapy is currently evolving (Lhermusier et al. 2011, Desai and Bhatt 2010). The first-choice combination of aspirin and clopidogrel is not universally efficient for patients and so there is interest in more efficient drugs or new methods of choosing treatments such a platelet function testing (Sinhal and Aylward 2013). Nanotechnology has become popular in medical research and NCs are seen as a possible progression for pharmaceutical drug delivery (Emerich and Thanos 2003). It was considered relevant to investigate how polymeric NCs would behave with blood platelets to expand current knowledge in both NC research and the study of platelet interactions. There has been success for certain types of NCs when tested with platelets including the work of Koziara et al. (2005), which found that PEGylated and non-PEGylated cetyl alcohol/polysorbate NPs (E78 NPs) did not activate platelets. In fact, both NP formulations very rapidly inhibited agonist-induced platelet activation and aggregation in a dose-dependent manner. Additionally,

E78 NPs significantly prolonged *in vitro* whole blood clotting time at a concentration of 500 µg/ml or greater. Cenni et al (2008) tested the effect of NPs made of a conjugate of poly(d,l-lactide-co-glycolide) with alendronate (PLGA–ALE NPs), prepared by emulsion/solvent evaporation technique on platelet activation. The PLGA–ALE NPs size was 198.7 ± 0.2 nm. No platelet activation was observed in the tested concentration range up to 560 µg/ml of PLGA–ALE NPs. Li et al. (2009) which gave an insight into the compatibility of polymeric NPs made from PLGA, CS, and PLGA-CS with platelets, proving them be platelet compatible up to 500 µg/ml and showing that at high concentrations they had some inhibitory effects (10 - 40 %) on platelet aggregation. Most recently, Ramtooia et al. (2011) also had a positive outcome when they reported that NPs based on polymers of PLGA, PLGA-Macrogol and CS had no affect on platelet aggregation and did not inhibit platelet aggregation at the particle concentrations tested (0.01 - 500 mg/ml). The research by Ramtooia et al. did not find that the NPs had an inhibitory effect on platelets, as was observed in the current research, but they used ADP instead of collagen to stimulate platelet aggregation.

In light of the aforementioned studies investigating the compatibility of empty polymeric NCs with platelets, the current studies looking at the platelet compatibility of polymeric NCs loaded with drugs, which is the first time this has been studied to the best of my knowledge, show a salient progression. Firstly the current work showed compatibility between all three NC types and platelets with no aggregation being stimulated by any of the NCs (80 µg/ml over 25 mins). **Chapter 6** details the success of the IND and KET loaded HA:CS PECNs and PLGA NPs at inhibition of collagen (2 µg/ml) induced platelet aggregation. It was confirmed that IND and KET loaded HA:CS PECNs did not affect the anti-coagulation effect of IND and KET. There is a possibility that any of these three NCs - PLGA, positively charged HA:CS PECNs or negatively HA:CS PECNs - could now be further investigated for their compatibility with platelets to make platelet targeting possible. This work also is also a preliminary step in demonstrating that it is likely that there would not be platelet toxicity associated with these NCs. As there was no significant difference between the action of IND and KET when loaded into the different NCs and the effect of free IND and KET it can be said these NCs do not considerably affect the efficacy of KET and IND.

Once loaded with drug, it became apparent that the positively charged HA:CS PECNs were more successfully inhibiting collagen induced aggregation when loaded with IND and KET as discussed in **Chapter 6** and in **Section 7.5**. This is likely to be connected to the interaction with platelets based on the surface charge. Lee et al. (2008) prepared functional group gradients on low-density polyethylene sheets. They found that the N,N-dimethyl aminopropyl acrylamide grafted surface showed a large amount of platelet adhesion, which they attributed to its positive charge character, while the negatively charged acrylic acid grafted surface showed poor platelet adhesion.

The data generated in this research could be used with two purposes. Firstly it could be used to develop platelet-targeted NCs to allow these drugs (IND and KET), or new anti-coagulation drugs, to be delivered more efficiently. The second possibility is that the data could be used to further toxicology studies into the toxicity of NCs. Any drug delivery system that would be delivered parenterally would essentially have to be compatible with blood platelets. The research aims were achieved to the extent that PLGA and HA:CS PECNs of positive and negative charge were all successfully loaded with IND and KET and were tested for their platelet compatibility. They were also successful at delivering KET and IND to the platelets and were no different in effectiveness to the free IND or KET.

7.7 Limitations

The main limitation of this work was that the particles being investigated were in the nanoscale size range. This meant that deep analysis was always challenged by the limitations of the analytical techniques.

When trying to analyse the solid state of the drug by PXRD analysis it could not be determined resolutely whether the drug at the surface was amorphous or whether nanocrystals were present that could not be resolved by PXRD analysis (Ungár 2006, Pinna 2005). Microscopy also proved to be challenging as the NCs the polymers used did not show good resolution and had low T_g values. It was thus difficult to take clear images of the NCs without melting the polymers.

Given the fragile nature of the NCs and their small particle size centrifugation was a challenge as high speeds were required for complete NC separation and yet high speeds were likely to damage the NCs, especially the more fragile HA:CS NCs, and induce aggregation in the process.

Another point to note is that since the NCs were not visible to the naked eye, all conclusions were based solely on the interpretation of data from analytical equipment.

The difference in solubility between IND and KET provided some limitations when preparing HA:CS PECNs. This was because KET is freely soluble in ethanol, which was used to dissolve the drug while IND is only sparingly soluble in ethanol according to the BP 2014. There was also an upper limit on the volume of ethanol that could be used to add the drug to the HA:CS PECNs during preparation (**Chapter 2**). If more than 700 μ l of ethanol was added to the HA:CS systems the PECNs did not form. Thus the solubility saturation of KET in ethanol was not the same as IND. IND dissolved in the volume of ethanol used but it was closer to its saturation solubility. If IND was prepared using a higher volume of ethanol the HA:CS PECNs did not form due to the excess ethanol content. Interestingly, when the volume of KET solution used was lowered to increase the drug saturation solubility, the HA:CS PECNs formed did not have solely amorphous peaks, some crystallinity was observed. For this reason some of the crystallinity observed for IND loaded HA:CS PECNs may have been due to its saturation solubility in the added ethanol. If KET precipitated too quickly from the ethanol solution on addition to the aqueous polymer solutions crystallisation may have been stimulated prior to complete mixing with the polymer solutions. In fact the α polymorph of IND is known to precipitate from ethanol solution. Alhnan et al. (2011) found that the ethanol solubility for KET was $> 600.06 \pm 5.05$ mg/ml while for IND it was only 19.65 ± 0.47 mg/ml.

7.8 Main Findings of this Thesis

- The production of PLGA NPs loaded with IND and KET at 0.1 mg/mg and 0.2 mg/mg by a single emulsion-solvent diffusion method was successfully optimised by investigating each of the important parameters involved in their formation. Previous studies by Corrigan et al. (2009) did

not look at optimisation of PLGA NPs loaded with IND and KET and had lower DL. IND was more hydrophobic than KET which was probably why they optimisation showed higher IND loading.

- The production of HA:CS PECNs loaded with IND and KET at 0.1 mg/mg and 0.2 mg/mg was proven possible. These HA:CS PECNs were physically characterised and observed to be a quick release formulation. Similar HA:CS PECNs were previously prepared by Umerska et al. (2012) but had not been loaded with hydrophobic drugs. Loading was higher for KET than IND showing a preference for hydrophilic drugs.
- HA:CS PECNs were loaded with IND and KET with a positive or a negative ZP but higher DL was obtained for negatively charged HA:CS PECNs for KET and positively charged HA:CS PECNs for IND.
- HA:CS PECNs were interacting with IND and KET by different mechanisms as for KET the drug was loaded in the amorphous form but for IND it was loaded as a combination of amorphous and crystalline drug of different polymorphic forms.
- HA:CS PECNs released the drug load instantly while PLGA NPs showed release over a longer period of time.
- HA:CS PECNs negatively or positively charged and PLGA NPs did not induce platelet aggregation at a concentration of 80 $\mu\text{g/ml}$ over 25 minutes. No inhibition of aggregation was seen at this concentration for any of the empty NCs.
- KET and IND effectively inhibited collagen (2 $\mu\text{g/ml}$) stimulated platelet aggregation when loaded into PLGA NPs, and HA:CS PECNs of a positive or negative charge. The inhibition was not significantly different to the free IND and KET not associated with NCs.
- KET or IND loaded NCs incubated with platelets resulted in platelets with similar P-selectin expression to resting platelets showing that the up regulation of P-selectin induced by collagen was being prevented. The number of P-selectin copies on platelets observed for KET and IND loaded NCs was not significantly different to that observed for free IND and KET.

7.9 Future Work

- Studies on the compatibility of HA:CS NCs with other small, hydrophobic, organic drugs to see if a correlation can be found for the association behaviour of these drugs with hydrophilic NCs.
- Load NCs with other therapeutically active anti-platelet drugs to investigate if these NCs are capable of delivering other currently used anti-platelet drugs. Consider examining the side effect profiles in comparison to the free drugs and the successful response rate.
- Modify NCs to specifically target platelets by altering the surface properties.
- Decide on the most suitable route of administration for platelet targeted drug-loaded NCs and test the NCs using systematic experiments to assess suitability for this route.
- Further microscopy studies would be beneficial to learn more about the interaction of these NCs with platelets. TEM could be used to locate where the particles are interacting with the platelets.
- *In vivo* experiments using mouse models could show the efficacy of these loaded NCs *in vivo* compared to *in vitro* results.
- Further investigation into synergistic effects between the polymers and drugs on platelet aggregation.
- Given that the primary therapeutic use of the NSAID group (IND and KET) is for treating inflammation, it may be useful to test the performance of these loaded NPs on inflammatory models given that they are now known to be compatible with platelets.

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Chapter 8

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