

Nanomedicines for the Treatment of Coagulation Disorders

Submitted by

Ciarán Manus Maguire



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Under the supervision and direction of

Adriele Prina-Mello, PhD

and

Professor Yuri Volkov, MD, PhD

and

Professor James O'Donnell, MB, PhD

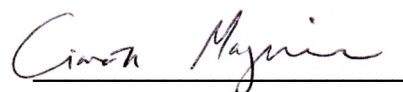
Department of Clinical Medicine
Trinity College, The University of Dublin

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Declaration

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A handwritten signature in black ink, reading 'Ciarán Manus Maguire', written over a horizontal line.

Ciarán Manus Maguire

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Summary

Nanomaterials are becoming increasingly utilised in all areas of science, technology and healthcare. More specifically in terms of nanomedicine, the physical and chemical properties of the materials allow for application in diagnostics, therapeutics, drug delivery and multifunctional theranostic agents. Prior to any potential clinical application, nanomaterials intended for use *in vivo* must be comprehensively examined for toxicity and other bio-incompatibility issues. Following introduction into the human body one can expect a critical interaction will take place between the nanoparticle and the blood components, most notably with the constituents of the coagulation cascade.

One of fundamental challenges in the area of application development is related to generating reliable and reproducible characterisation data for nanomaterials. Characterisation is critical, not only for understanding the mechanism of action of nanotherapeutics, but also to comply with directives, procedures and regulation requested by regulatory agencies. As such, in this presented research, we set out to develop robust, reliable and accurate characterisation protocols for the analysis of nanoparticles in liquid suspension using Nanoparticle Tracking Analysis (NTA) developed by NanoSight Malvern Ltd.

Through the implementation of multiple rounds of interlaboratory comparison (ILC) with 12 pan-national institutions, the percentage coefficients of variation for monodisperse and complex mixtures of nanoparticles were dramatically reduced across the board. The characterisation of nanoparticles in complex dispersal media was also examined. This led to the refinement of standard operating procedures not only for sample characterisation, but also sample shipping, handling and preparation. Software advancements also improved the resolution of size distribution graphs and were instrumental in the analysis of multimodal samples.

The refinement of the NTA technique was critical in the analysis of multiple sample types presented in this thesis. Throughout the course of this study, various nano-objects were characterised, including gold nanoboxes and bismuth ferrite nanoparticles, as well as more biologically and clinically relevant samples including exosomes and virus particles. NTA was utilised to study the stability of particles in cell culture medium, as well as the impact of storage and isolation method on exosome size. It was demonstrated that nanomaterial size and material type are important factors when studying the interaction of nanoparticles with cell lines. Similarly, NTA was used to assess the impact on size

and concentration of Adenovirus particles following incremental heating and exposure to gold nanoparticles. Here it was illustrated that exposure to gold nanoparticles increase the temperature at which the virus capsid ruptures.

NTA was also utilised in the characterisation of functionalised iron oxide nanoparticles for applications in the treatment of coagulation disorders. Here, a selection of particles were screened for their effect on the coagulation cascade and the most appropriate candidate was then selected to be functionalised with the anticoagulant heparin. The nanoparticles were functionalised using numerous approaches and then characterised by NTA. Following characterisation, the functionalised materials were tested for their anticoagulant activity. It was demonstrated that upon conjugation of heparin to the nanoparticle, heparin loses its anticoagulant properties, most likely due to its inability to bind its targets antithrombin and thrombin.

Finally, cadmium telluride quantum dots (QDs) were investigated for their effect on the coagulation cascade. It was demonstrated that 3.2 nm QDs illicit a strong anticoagulant response on the intrinsic coagulation pathway. No anticoagulant response was observed for their relatively larger 3.6 nm counterparts. It was reported that the activity of the intrinsic coagulation factors were within their normal respective ranges. The greatest reduction in activity was observed for Factor VIII, with an activity of 51 % of normal. This reduction in activity would not typically cause the massive prolongation of the clotting times observed in this thesis, but the effect may be additive to alterations in function of other coagulation proteases and cofactors. It was also demonstrated that increases in calcium concentration result in a corresponding increase in clotting times suggesting that the 3.2 nm QDs interact with the calcium ions required for normal haemostasis.

In conclusion, this thesis demonstrates that the development of robust procedures for the characterisation of nanoparticles by NTA reduces the user-to-user variation and increases the reproducibility of the analysis. Further, it was shown that when heparin is conjugated onto the surface of nanoparticles, a corresponding loss in the anticoagulant properties of the drug is observed. Finally, it was illustrated that cadmium telluride quantum dots illicit a size- and concentration-dependent anticoagulant effect on haemostasis. It is anticipated that the results presented in this thesis will add to the expanding knowledge base describing the biological effects of nanoparticles, and provide further opportunities for nanomedicine applications for the treatment of coagulation disorders.

Abbreviations

ADP	Adenosine diphosphate
AgNPs	Silver Nanoparticles
APC	Activated protein C
APTES	(3-Aminopropyl)trimethoxysilane
APTT	Activated Partial Thromboplastin Time
Arg	Arginine
Asn	Asparagine
AuNB	Gold Nanobox
AuNPs	Gold Nanoparticles
BSA	Bovine Serum Albumin
CaCl ₂	Calcium chloride
cDNA	Complementary Deoxyribonucleic acid
CdTe	Cadmium Telluride
CNCs	Carbon nanocapsules
CNTs	Carbon nanotubes
Col	Collagen
DEPC	Diethylpyrocarbonate
DI	Deionised
DIC	Disseminating Intravascular Coagulation
DLS	Dynamic Light Scattering
DMEM	Dulbecco's Modified Eagle Medium
DMSA	Dimercaptosuccinic acid
DNA	Deoxyribonucleic acid
DRVVT	Dilute Russell's Viper Venom time
DVT	Deep Vein Thrombosis
EDC	1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide
EGF1	Epidermal Growth Factor 1
ELISA	Enzyme Linked Immunosorbent Assay
ENP	Engineered nanoparticle
EPCR	Endothelial Protein C Receptor
Epi	Epinephrine
ETP	Endogenous Thrombin Potential
FBS	Fetal Bovine Serum
FITC	Fluorescein Isothiocyanate
FIX	Factor IX
FIX _a	Activated Factor IX
FTLA	Finite Track Length Adjustment
FV	Factor V
FV _a	Activated Factor V
FVII	Factor VII

FVII _a	Activated Factor VII
FVIII	Factor VIII
FVIII _a	Activated Factor VIII
FX	Factor X
FX _a	Activated Factor X
FXI	Factor XI
FXI _a	Activated Factor XI
FXII	Factor XII
FXII _a	Activated Factor XII
FXIII	Factor XIII
FXIII _a	Activated Factor XIII
GAGs	Glycosaminoglycans
Gla	γ-carboxyglutamate
GlcA	D-glucuronic acid
GlcNAc	α-D-N-acetylglucosamine
GlcNS	α-D-N-sulfoglucosamine
Glu	Glutamic acid
Gly	Glycine
GP1b	Glycoprotein 1b
GPIIb-IIIa	Glycoprotein IIb-IIIa
HRP	Horseradish peroxidase
IdoA	L-Iduronic acid
IFS	Intrinsic Factor Screen
IgG	Immunoglobulin G
ILC	Interlaboratory comparison
LMWH	Low Molecular Weight Heparin
LOD	Limit of Detection
Lys	Lysine
MAD	Median absolute deviation
MPTES	Mercaptopropyltriethoxysilane
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NF-κβ	Nuclear Factor κβ
NHS	n-Hydroxysuccinimide
NP	Nanoparticle
NTA	Nanoparticle Tracking Analysis
PBS	Phosphate Buffered Saline
PBS-T	Phosphate Buffered Saline-Tween solution
PEG	Polyethylene glycol
PFA-100	Platelet Function Analyser-100
PFC NPs	Perfluorocarbon Core Nanoparticles
PLGA	Poly(D,L-lactide-coglycolide)
PM	Particulate matter

PPACK	D-phenylalanyl-L-prolyl-L-arginyl-chloromethyl ketone
PPP	Platelet Poor Plasma
PRP	Platelet Rich Plasma
PT	Prothrombin time
QD	Quantum Dot
QDs	Quantum Dots
RCo	Ristocetin Cofactor
RPMI-1640	Roswell Park Memorial Institute 1640
SCT	Silica clotting time
SD	Standard deviation
SiNPs	Silica Nanoparticles
siRNA	Small Inhibitory Ribonucleic Acids
SOP	Standard Operating Procedures
SPION	Superparamagnetic Iron Oxide Nanoparticle
TEM	Transmission electron microscopy
TF-FVII	Tissue Factor-Factor VII complex
TFPI	Tissue Factor pathway inhibitor
TGA	Thioglycolic acid
TMB	3,3',5,5'-Tetramethylbenzidine
TNF- α	Tumour Necrosis Factor- α
TT	Thrombin Time
UFH	Unfractionated Heparin
VWD	Von Willebrand Disease
VWF	Von Willebrand Factor

Units

% CV	Percentage Coefficient of Variation
° C	Degrees Celsius
μ g	Microgram
μ g/mL	Micrograms per Millilitre
μ L	Microliter
μ m	Micrometre
μ M	Micromolar
Å	Angstrom
g	Gram
IU/mL	International Units per Millilitre
KDa	Kilodalton
L	Litre
M	Molar
min	Minutes
mL	Millilitre

mV	Millivolts
nm	Nanometre
nM	Nanomolar
pM	Picomolar
v/v	Volume per Volume
w/v	Weight per Volume

Communications from Thesis

1.1 Peer Reviewed Publications

Patrick Hole, Katherine Sillence, Claire Hannell, **Ciaran Manus Maguire**, Matthias Roesslein, Guillaume Suarez, Sonja Capracotta, Zuzana Magdolenova, Limor Horev-Azaria, Agnieszka Dybowska, Laura Cooke, Andrea Haase, Servane Contal, Stein Manø, Antje Vennemann, Jeans-Jacques Sauvain, Kieran Crosbie Staunton, Sergio Anguissola, Andreas Luch, Maria Dusinska, Rafi Korenstein, Arno C. Gutleb, Martin Wiemann, Adriele Prina-Mello, Michael Riediker, Peter Wick. (2013) **“Interlaboratory comparison of size measurements on nanoparticles using Nanoparticle Tracking Analysis (NTA).”** Journal of Nanoparticle Research 2013, 15:2101 doi:10.1007/s11051-013-2101-8

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Jolanda Spadavecchia, Dania Movia, Caroline Moore, **Ciaran Manus Maguire**, Hanane Moustou, Sandra Casale, Yuri Volkov, Adriele Prina-Mello. (2016). **“Targeted polyethylene glycol gold nanoparticles for the treatment of pancreatic cancer: from synthesis to proof-of-concept in vitro studies.”** International Journal of Nanomedicine 2016, 11:791-822, doi:10.2147/IJN.S97476

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1.2 Oral Presentations

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Ciaran M. Maguire, *Nanoparticle Assessment Case-Studies in Trinity College Dublin*. Celtic Alliance for Nanohealth: Nanoscience Event. Swansea, Wales. April 2014.

Ciaran M. Maguire, *Size-Dependent Anti-Coagulant Properties of Cadmium Telluride Quantum Dots*. 6th International Congress Nanotechnology in Medicine and Biology. Graz, Austria. April 2015.

1.3 Poster Presentations

Ciaran M. Maguire, *Towards non-toxic haemostatically active ENPs for biomedical applications*. European Nanoforum, Convention Centre Dublin, Ireland. June 2013.

Ciaran M. Maguire, *Systematic Approach to the Evaluation of Blood Compatible Nanoparticles for Treatment of Coagulation Disorders*. Haematology Association of Ireland Annual Meeting, Europa Hotel Belfast, Northern Ireland. October 2013.

Ciaran M. Maguire, *Investigating the Anti-Coagulant Properties of Cadmium Telluride Quantum Dots*. Haematology Association of Ireland Annual Meeting, Clarion Hotel Sligo, Ireland. October 2014.

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Chapter 1

Introduction

1 Introduction

1.1 Background

Nanoparticles are typically defined as having one dimension in the size range of 1-100 nm [1] and can be stratified into two main subclasses: environmental nanoparticles and engineered nanoparticles. Environmental nanoparticles are released by natural processes such as volcanic eruptions and have been present in the environment throughout the history of the earth, but also incorporate contemporary combustion by-products such as diesel particulate matter. Engineered nanoparticles (ENPs) are those that have been intentionally produced and have applications in all areas of science, technology and healthcare, diagnostics and medicine.

In nanomedicine, nanotechnology is being utilised to deliver drugs and imaging agents, to change pharmacokinetic profiles and to target particular sites of interest [2, 3]. Examples of currently available nanotechnology-enabled medicinal drugs are the paclitaxel-albumen nanoparticle Abraxane®, first licenced by the Food and Drug Administration (FDA) in 2005 for the treatment of breast cancer [4], and the liposomal formulation of doxorubicin, DOXIL®, approved in 1995 for the treatment of ovarian cancer [5]. Such nanomedicines can be used to circumvent some drug mediated side-effects by improving targeting and maintaining bioavailability.

However, prior to any potential clinical application, nanomaterials intended for use *in vivo* must be compressively examined for toxicity and other bio-incompatibility issues. One critical interaction will be between the nanoparticle and blood components, most notably interactions with the coagulation cascade.

1.2 The Burden of Haemostatic Disorders

Disorders of haemostasis and coagulation can have a large burden on society and healthcare, with 1,900 deaths in 2010, in the United States alone, being attributed to coagulation defects, as shown in Figure 1.1 [6]. The bleeding disorder Haemophilia A is associated with a deficiency in coagulation Factor VIII, and has an incidence of 1 in every 10,000 male births. Patients generally require replacement therapy throughout their life on an ongoing, or as-needed basis following injury or bleeding. Likewise, Von Willebrand Disease (VWD) is the most common hereditary bleeding disorder [7].

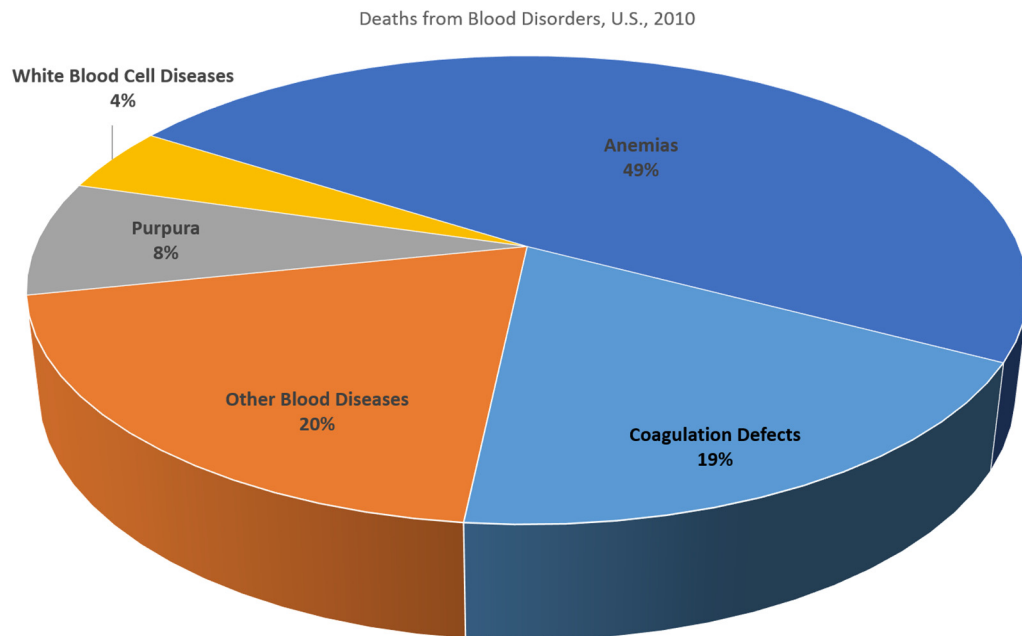


Figure 1.1: Deaths related to blood disorders in the US, modified from [6]. 1,900 deaths were attributed to blood disorders in the United States for the year 2010. 19 % of these deaths were due to coagulation defects.

It has been noted that 1 % of the general population experience reduced or altered VWF concentrations and activities [7, 8], with clinically significant bleeding occurring 1 per 10,000 [9]. It is associated with deficiencies of Von Willebrand Factor (VWF), a protein required for platelet adhesion. Patients generally require the synthetic hormone, desmopressin (DDAVP) to promote the release of more VWF and subsequent increase in circulation of Factor VIII.

In general, treatments for coagulation disorders require the replacement of the absent, mutated or deficient factor or protein. In severe cases this can require frequent infusions, reducing the quality of life of the patient, and provides an opportunity for the use of nanomaterials which may act to increase the circulation and retention times of coagulation factors and haemostatic proteins.

1.3 Haemostasis

1.3.1 The Coagulation Cascade

The coagulation cascade is the biochemical pathway responsible for maintaining haemostasis (Figure 1.2). It maintains the balance between the pro- and anti-thrombotic pathways. Coagulation is the mechanism by which the blood loss is halted following vascular injury [10]. Typically, injury to the blood vessels causes the exposure of endothelial cells to blood proteins such as tissue factor, thereby initiating a cascade of zymogens which become activated, ultimately leading to platelet activation, thrombin generation and the conversion of soluble fibrinogen to an insoluble fibrin clot [11, 12].

Traditionally viewed as two separate pathways, the intrinsic pathway and extrinsic pathway converge to form the common pathway [13]. Although these pathways are not so straight forward *in vivo*, they are still commonly referred to as they form the basis of important tests for coagulation disorders [14, 15] (Table 1.1).

1.3.1.1 *The Extrinsic Pathway*

In this pathway, tissue factor released due to vascular damage binds to Factor VII, which initiates a cascade whereby Factor X is activated, leading to the production of thrombin from prothrombin. The activity of this pathway is measured through the prothrombin time (PT) test, and measures prothrombin. It is also sensitive to changes in factors VII, X, V and thrombin and fibrinogen. The PT test is based on the time required for a fibrin clot to form following the addition of tissue factor, phospholipids and calcium to decalcified platelet poor plasma. The normal time for clot formation using this test is between 10 and 14 seconds [15].

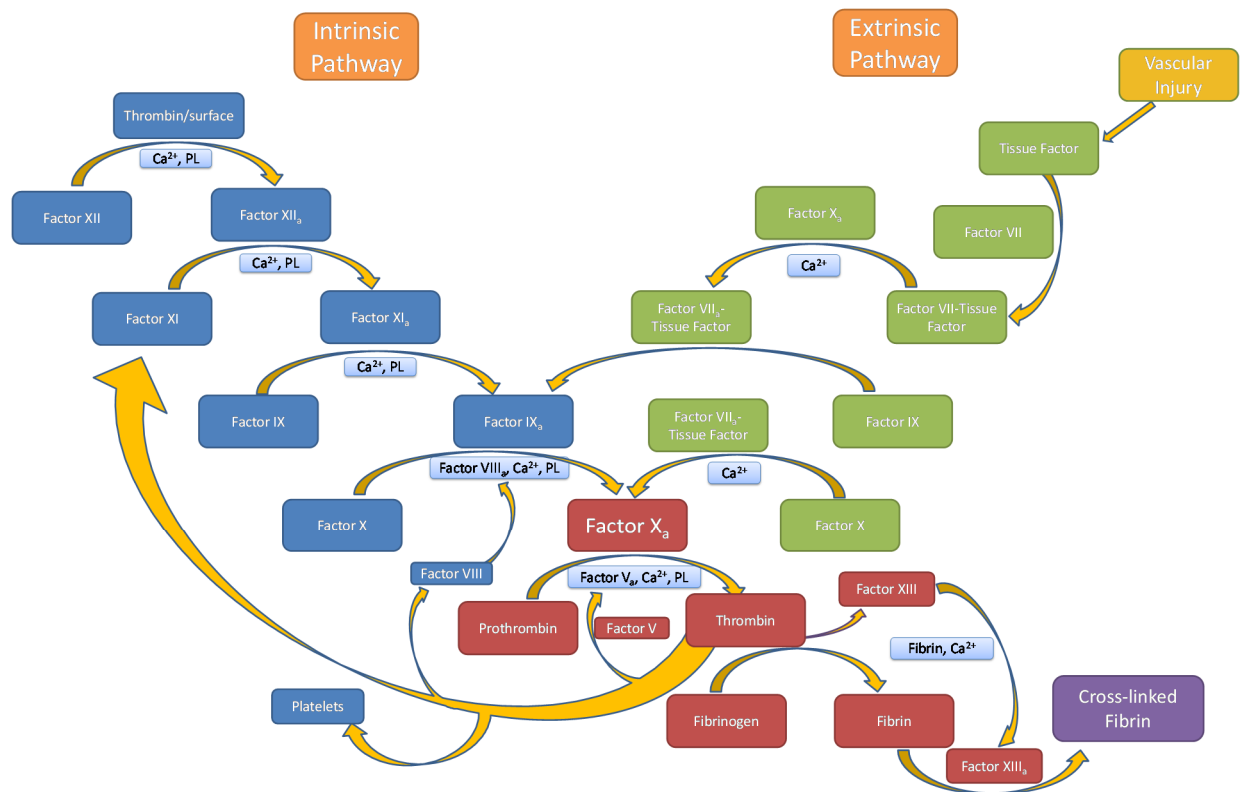


Figure 1.2: The coagulation cascade. This pathway is responsible for maintaining the balance between a pro-thrombotic and anti-thrombotic state. Contact with a negatively charged surface or following vascular injury results in the release of tissue factor, platelet activation and activation of Factor X, followed by the conversion of fibrinogen to fibrin and the formation of a cross-linked fibrin clot.

Table 1.1: Screening tests used in the diagnosis of coagulation disorders [15].

Screening Test	Abnormalities Indicated by prolongation	Most common cause of coagulation disorder
APTT	Deficiency/inhibition of factors XII, XI, IX, VIII, X, V, II, fibrinogen	Haemophilia A, Haemophilia B, heparin or warfarin therapy, disseminated intravascular coagulation (DIC)
PT	Deficiency/inhibition of factors VII, X, V, II, fibrinogen	Liver disease, warfarin therapy, DIC
TT	Deficiency/abnormality of fibrinogen, inhibition of thrombin by heparin	DIC, heparin therapy



Figure 1.3: Tissue Factor-Factor VII_a complex modified from [16]. This complex is required to activate Factor IX to Factor IX_a and Factor X to Factor X_a. Deficiency in Factor VII is known as Alexander's Disease, and is considered to be one of the most frequently occurring rare bleeding disorders with an incidence of 1 per 300,000-500,000 [17].

The predominant function of the extrinsic pathway is to produce a burst of thrombin following the release of tissue factor. Circulating Factor VII (FVII) comes into contact with exposed tissue factor leading to the formation of an activated tissue factor-FVII complex (TF-FVII_a) (Figure 1.3).

This complex then acts to activate Factor IX (FIX) to FIX_a and Factor X (FX) to FX_a. FX_a and its cofactor Factor V_a (FV_a) form the prothrombinase complex which cleaves prothrombin into thrombin. The prothrombinase complex assembles on the negatively charged phospholipid membrane of platelets in the presence of calcium ions [18], with cleavage of prothrombin to thrombin occurring at Arg271 initially, followed by Arg320 [19] as shown below in Figure 1.4.

1.3.1.2 *The Intrinsic Pathway*

The intrinsic pathway can also be known as the contact activation pathway, as a contact activator such as kaolin is required to initiate clotting [20]. This pathway is measured by the activated partial thromboplastin time (APTT) test, and is sensitive to changes in levels of fibrinogen, thrombin and factors V, VIII, IX, X, XI and XII. The normal time to clot formation is typically between 25 and 46 sec [21, 22].

In this pathway, Factor XII (FXII) becomes auto-activated to FXII_a when bound to a negatively charged surface, such as a phospholipid membrane. FXII_a is then involved in the activation of Factor XI (FXI) and Factor IX. Deficiencies of these factors can cause prolongation of the APTT, but do not lead to bleeding disorders *in vivo* [13], with the exception of Factor XI [23]. Thrombin is also involved in the activation of FXI suggesting that FXI has a secondary role in the production of more thrombin following vascular injury [24].

1.3.1.3 *The Intrinsic and Extrinsic Pathways Combine*

Following activation of FIX through the intrinsic or extrinsic pathway, FIX forms a complex with Factor VIII_a (FVIII_a), calcium and phospholipids to activate Factor X [25]. Under normal conditions, FVIII circulates as a complex with von Willebrand Factor (VWF), which protects it from proteolytic activation to FVIII_a [13]. When VWF binds to platelets attached to damaged endothelium, the VWF dissociates from FVIII causing it to become activated to FVIII_a. As mentioned earlier, FX_a then forms the prothrombinase complex with FV_a to facilitate the cleavage of prothrombin to thrombin.

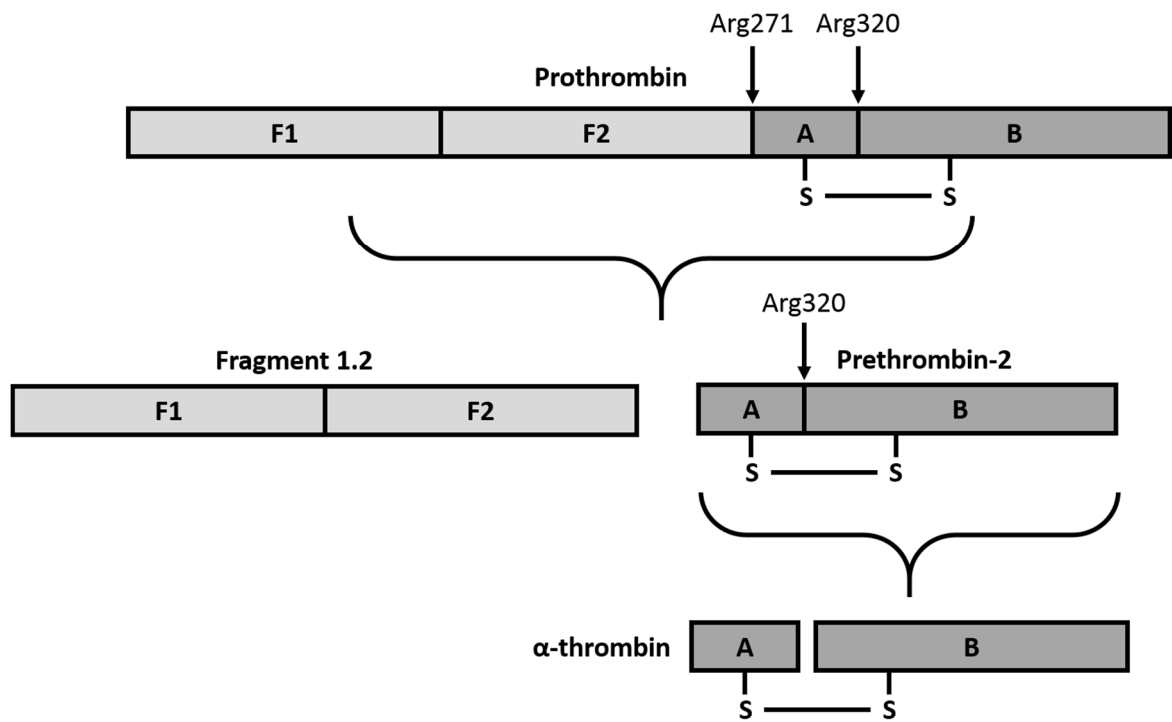


Figure 1.4: Cleavage of prothrombin into α -thrombin by prothrombinase occurs at residues Arginine271 and Arginine320, modified from [19]. Fragment 1.2 can be useful in the diagnosis of a hypercoagulable state [26], while α -thrombin is involved in the cleavage of fibrinogen to fibrin.

1.3.1.4 Fibrinogen Cleavage

The final stages of the coagulation cascade involves the conversion of soluble fibrinogen to insoluble fibrin. This conversion of fibrinogen to fibrin can be measured using the thrombin time (TT) test, with a normal reference range for this test lying between 12 and 14 seconds [15]. The test involves the addition of an excess of bovine or human thrombin to plasma, which results in the cleavage of fibrinogen to fibrin.

Fibrinogen exists as two sets of disulphide-linked peptide chains: A α , B β , and γ , which are cleaved by thrombin to produce fibrinopeptide A and B, with cleavage occurring at A α Arg16-Gly17 and B β Arg14-Gly15 [27]. The release of fibrinopeptide A from fibrinogen appears to be of greater importance for fibrin formation, and occurs faster than the B peptide [28]. These cleavage products are then cross-linked by FXIII_a, by first polymerising end to end to form protofibrils and then laterally to form fibrin fibres [29, 30].

1.3.2 Platelets, Von Willebrand Factor and the Endothelium

Platelets are anucleate cells, 2-3 μ m in diameter [31] derived from megakaryocytes [32], with a primary function in haemostasis. Under normal non-activating conditions, platelets circulate in quiescent discoid shape. Following damage to the endothelium of blood vessels, sub-endothelial proteins such as collagen and tissue factor initiate the activation of platelets and the formation of a platelet plug. Following stimulation and activation, actin becomes polymerised into filaments which aid in the formation of the platelet network [33].

One of the critical stages in primary haemostasis is the interaction of platelets with collagen and von Willebrand Factor (VWF). VWF is a large glycoprotein that plays a role in primary haemostasis by first binding to collagen and then activated platelets [34]. It is synthesised and stored in Weibel-Palade bodies in endothelial cells and also in α -granules in platelets [35]. Circulating VWF binds to exposed collagen following damage to the endothelium and is stretched by shear stress in the blood vessel. This leads to the exposure of the binding sites in the A1 domain for platelet GP1b (Figure 1.5) and ultimately platelet aggregation (Figure 1.6).

Similarly, platelet adhesion glycoprotein GPIIb-IIIa mediates the binding of platelets to fibrinogen and VWF and aids in platelet activation and is critical in thrombus formation. Although GPIIb-IIIa is present on the surface of unstimulated platelets, it only binds fibrinogen after activation [36].

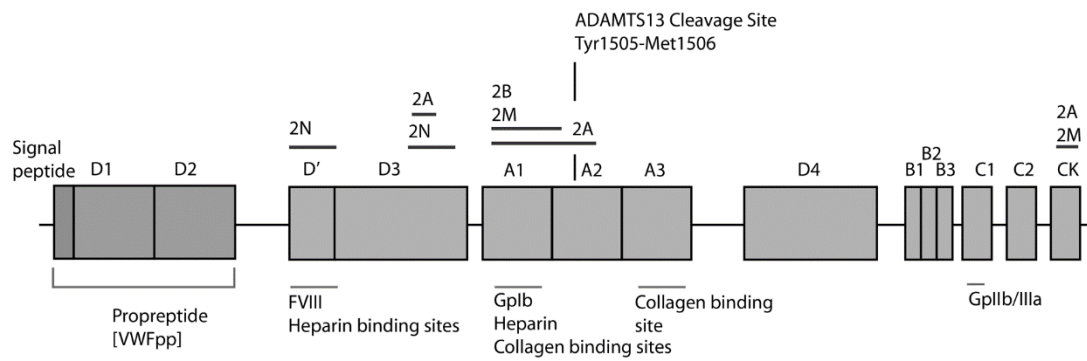


Figure 1.5: The domains and binding sites of von Willebrand Factor [37]. Areas of note include the FVIII binding site in the D' domain, as well as the collagen binding sites in A1 and A3. The propeptide region is essential for multimer formation, while the C1 domain is involved in platelet binding.

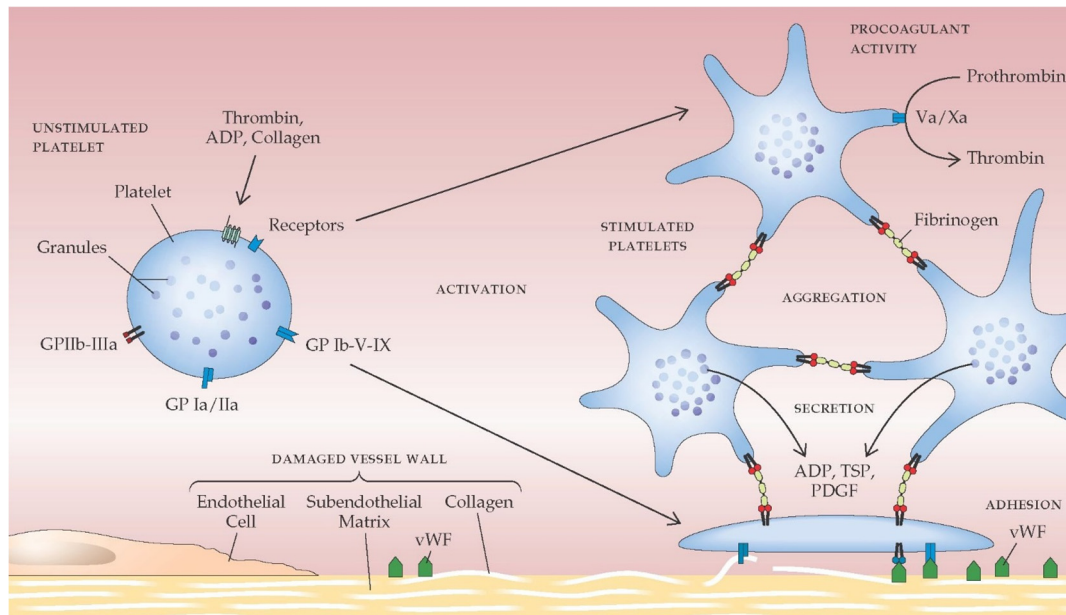


Figure 1.6: Activation of platelets following damage to blood vessel wall from [38]. Platelet glycoproteins bind to collagen bound VWF leading to the formation of a platelet plug.

1.4 Controlling Coagulation

Maintaining the balance between a pro- and anti-coagulative states is essential for haemostasis, as alterations can lead to serious bleeding or thrombotic consequences (Figure 1.7).

1.4.1 Activated Protein C

Under normal conditions, activated Protein C (APC) and Protein S function as essential anti-coagulants. Protein C is a vitamin K-dependent serine protease containing a γ -carboxyglutamate (Gla) domain which is crucial for binding to negatively charged phospholipid membranes [39]. It becomes activated upon binding of Protein C and thrombin to a cell surface protein, thrombomodulin.

APC acts by irreversibly proteolytically inactivating Factor V_a and Factor $VIII_a$. APC inactivates Factor V_a by cleaving it at residues arginine (Arg) 306, Arg506 and Arg679 diminishing its affinity for Factor X_a [39], and total loss of FV_a activity is caused by cleavage at Arg306 [40].

Regulation of the $FVIII_a$ complex by APC requires the cofactor Protein S. This protein is also a vitamin K-dependent plasma protein, where only the free form has cofactor activity [41]. Protein S has been suggested to decrease the distance between the active site of APC and FX_a , located on the phospholipid membrane [39].

The activity of protein C can be down regulated by reducing the levels of thrombomodulin and the endothelial protein C receptor (EPCR) by inflammatory cytokines such as interleukin-1 β and tumour necrosis factor- α (TNF- α) [42].

1.4.2 Tissue Factor Pathway Inhibitor

Tissue Factor pathway inhibitor (TFPI) is a single-chain polypeptide that can reversibly inhibit Factor X_a . It is the primary regulator of the Factor VII_a -Tissue Factor complex ($FVII_a$ -TF) and inhibits its activity towards Factor IX and X in a Factor X_a dependent manner [43].

TFPI is a Kunitz-type inhibitor containing three Kunitz-type domains. These are domains that inhibit the function of protein degrading enzymes, i.e. they act as protease inhibitors [44]. The first Kunitz-domain is known to bind Factor VII_a , with the second binding factor X_a [43]. Protein S also acts as a cofactor for TFPI by reducing the inhibition constant for FX_a , and the third TFPI Kunitz-domain is required for this enhancement [45].

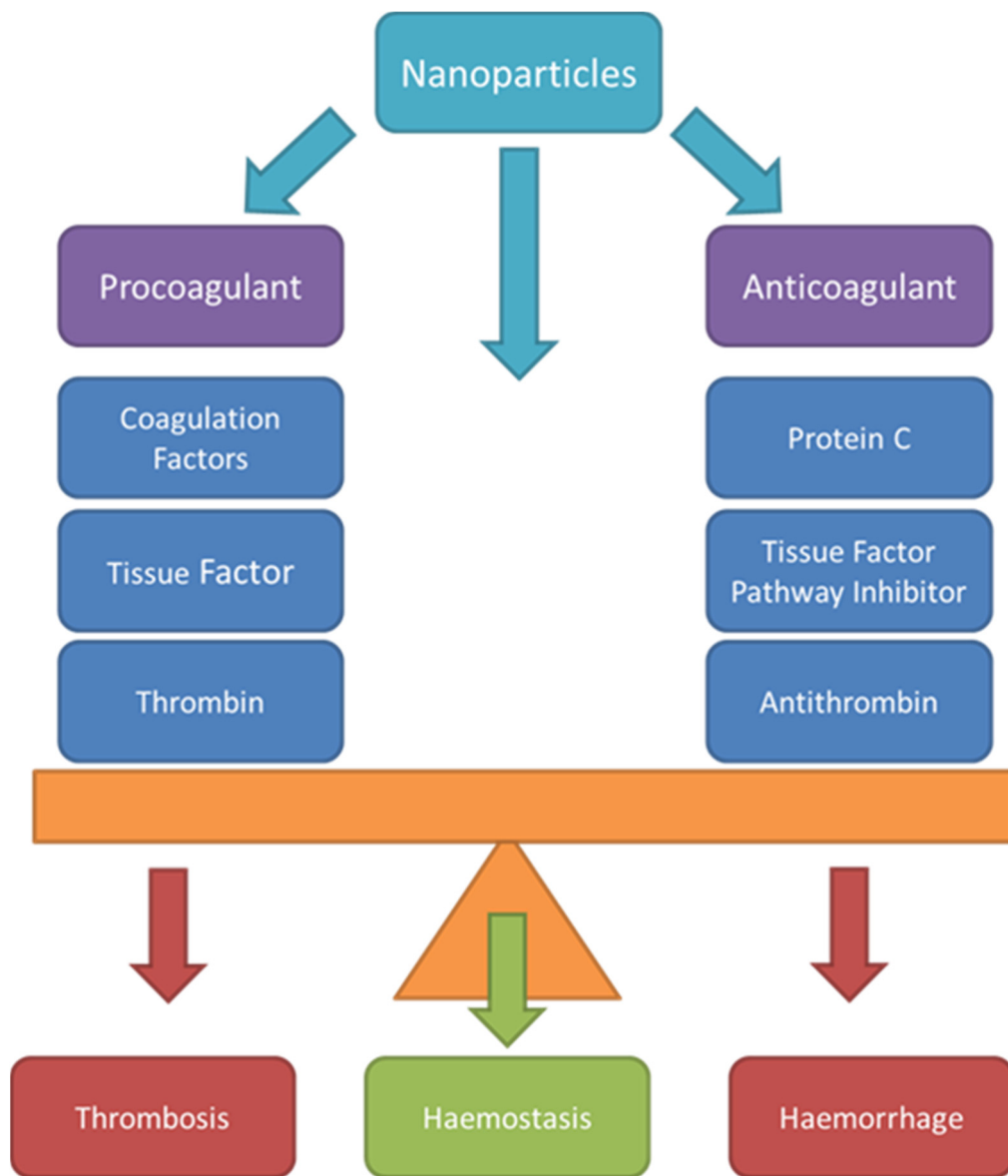


Figure 1.7: Maintaining haemostasis. The balance between pro- and anti-coagulant factors is crucial to prevent pathological haemostatic processes. The absence of inhibitors of coagulation can result in an increased tendency towards thrombosis, while over active anti-coagulant factors can result in an increased risk of haemorrhage. Nanoparticles can interact with components of the coagulation cascade, leading to desirable or unintended consequences, or have no effect.

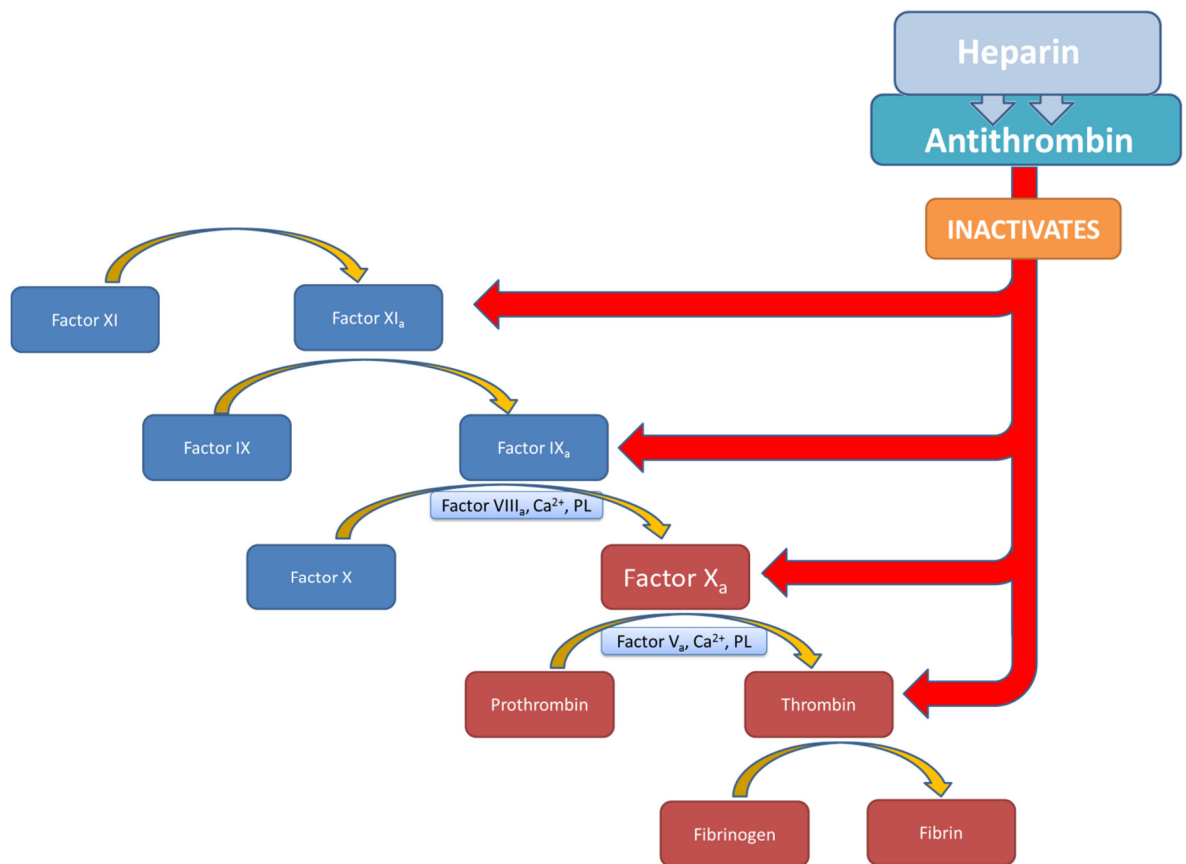


Figure 1.8: Heparin mechanism of action. Heparin activates antithrombin which in turn forms complexes with and inactivates activated coagulation factors.

It has been postulated that the inhibitory effect of TFPI occurs in a twostep process, where TFPI binds to Factor X_a, followed by the X_a-TFPI complex binding the FVII_a-TF complex, blocking its activity [43].

1.4.3 Heparin

Heparin is one of the most commonly used anticoagulants used in the treatment of prothrombotic complications [46], particularly in the treatment of deep vein thrombosis (DVT). It is a polymer with a size range from 3-30 kDa, approximately 9 nm in length [47, 48], with average chains in the 15-18 kDa size range [15]. The anticoagulant nature of heparin is due to its ability to bind to the inhibitory enzyme, antithrombin. Antithrombin inhibits procoagulant enzymes by forming a kinetically stable irreversible complex with them [49]. Heparin markedly accelerates this inhibitory effect of antithrombin.

Most notably, the alteration in the antithrombin active site on heparin binding increases its affinity for the procoagulant enzyme, Factor X_a [49], but also thrombin, Factor IX_a and XI_a [15](Figure 1.8). Low molecular weight heparin (LMWH) is produced by enzymatic or chemical depolymerisation of unfractionated heparin into chains of of molecular weight 2,000-10,000. LMWH has a greater ability to inhibit Factor X_a than standard heparin variants. This coupled with its ability to interact less with platelets can result in a lower tendency to cause bleeding, providing a more feasible option for prophylaxis [15].

Antithrombin has been shown to bind heparin with enhanced specificity for a pentasaccharide sequence, GlcNAc/NS(6S)-GlcA-GlcNS(3S,6S)-IdoA(2S)-GlcNS(6S) [50], found in one third of heparin chains [51], and binds this sequence with a 1,000 fold higher affinity than sequences of heparin devoid of this pentasaccharide. Heparin causes the inhibition of Factor X_a (FX_a) by allosteric modification of antithrombin and by acting as a template for antithrombin to inhibit FX_a [52]. In the case of thrombin, inhibition is independent of antithrombin conformation and relies solely on the template effect of heparin [53]. Thrombin binds non-specifically to heparin chains that are at least six saccharides in length [54]. This is demonstrated below in Figure 1.9.

In various studies [55-57] it was shown that the amino acid residues arginine (Arg) 233, Arg101, Arg93 and lysine (Lys) 236 and Lys240 in thrombin were involved heavily in binding heparin. Arg129, asparagine (Asn) 45, Lys125, Arg46, Arg47, Lys11, Lys114, Arg13 and glutamic acid (Glu) 113 are noted as being the critical amino acid residues in antithrombin involved in heparin binding [58]. These residues form a 50 Å long channel in antithrombin that encompasses the above heparin pentasaccharide.

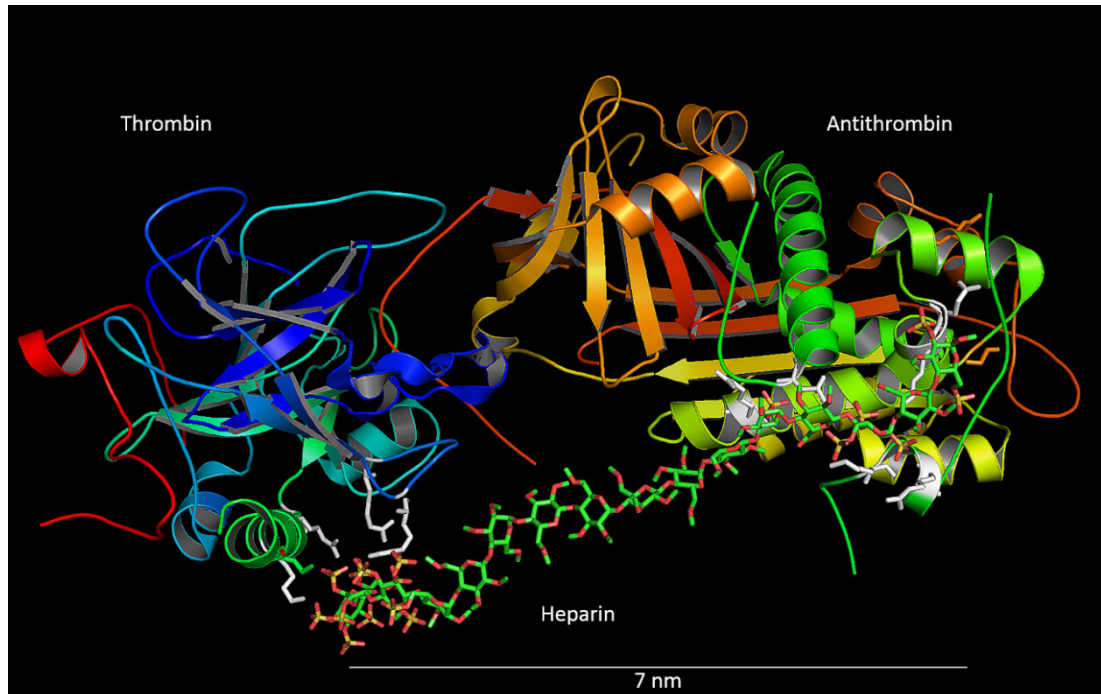


Figure 1.9: The antithrombin-thrombin-heparin ternary complex modified from [55]. The residues Arg233, Arg101, Arg93 and Lys236 and Lys240 in thrombin, and Arg129, Asn45, Lys125, Arg46, Arg47, Lys11, Lys114, Arg13 and Glu113 in antithrombin are heavily involved in heparin binding.

1.5 Role of Calcium and Vitamin K in Haemostasis

Calcium plays a critical role in haemostasis and is required at virtually all stages of zymogen activation [59, 60]. Chelating agents such as EDTA and citrate are frequently used to prevent the clotting of blood samples by binding and eliminating calcium ions. Calcium ion binding is a hallmark of the vitamin K-dependent proteins prothrombin, FVII, FIX, X, protein C, protein S, with vitamin K being essential for the formation of calcium binding sites in these factors [60]. Calcium is crucial for the coordinating the vitamin k-dependent factors to the phospholipid layer of activated platelets to initiate coagulation. This coordination must take place for FIX_a and FVIII to activate FX, and for FX_a to cleave prothrombin to thrombin.

The net negative charge on the phosphatidylserine groups in the activated platelet membrane coordinates calcium ions, with the GLA domain of the coagulation factors then coordinating to these ions to form the coagulation complexes required for the formation of a cross-linked fibrin clot.

The GLA domains are the regions in coagulation factors that contain the vitamin k-dependent carboxylation/gamma-carboxyglutamic (GLA) domain. This GLA domain binds calcium ions by chelating them between two carboxylic acid residues, with the proteins containing a Gla-x(3)-Gla-x-Cys motif [61]. During coagulation, in the presence of calcium ions, the GLA domain of FIX_a undergoes a structural change to become membrane bound [25]. FIX_a and FVIII_a then assemble on the phospholipid membrane to form the tenase complex [62] as demonstrated below in Figure 1.10.

As demonstrated above, the inhibition of calcium binding or diminishing of the calcium ion concentration in the blood would result in a loss of the ability of the body to maintain normal haemostasis. However, this can be used in medicine to prevent a hypercoagulable state. For example, the anticoagulant warfarin inhibits the N-terminal post-translational carboxylation of some residues in prothrombin and Factors VII, IX and X, and diminishes their ability to bind calcium [59], thereby preventing thrombosis.

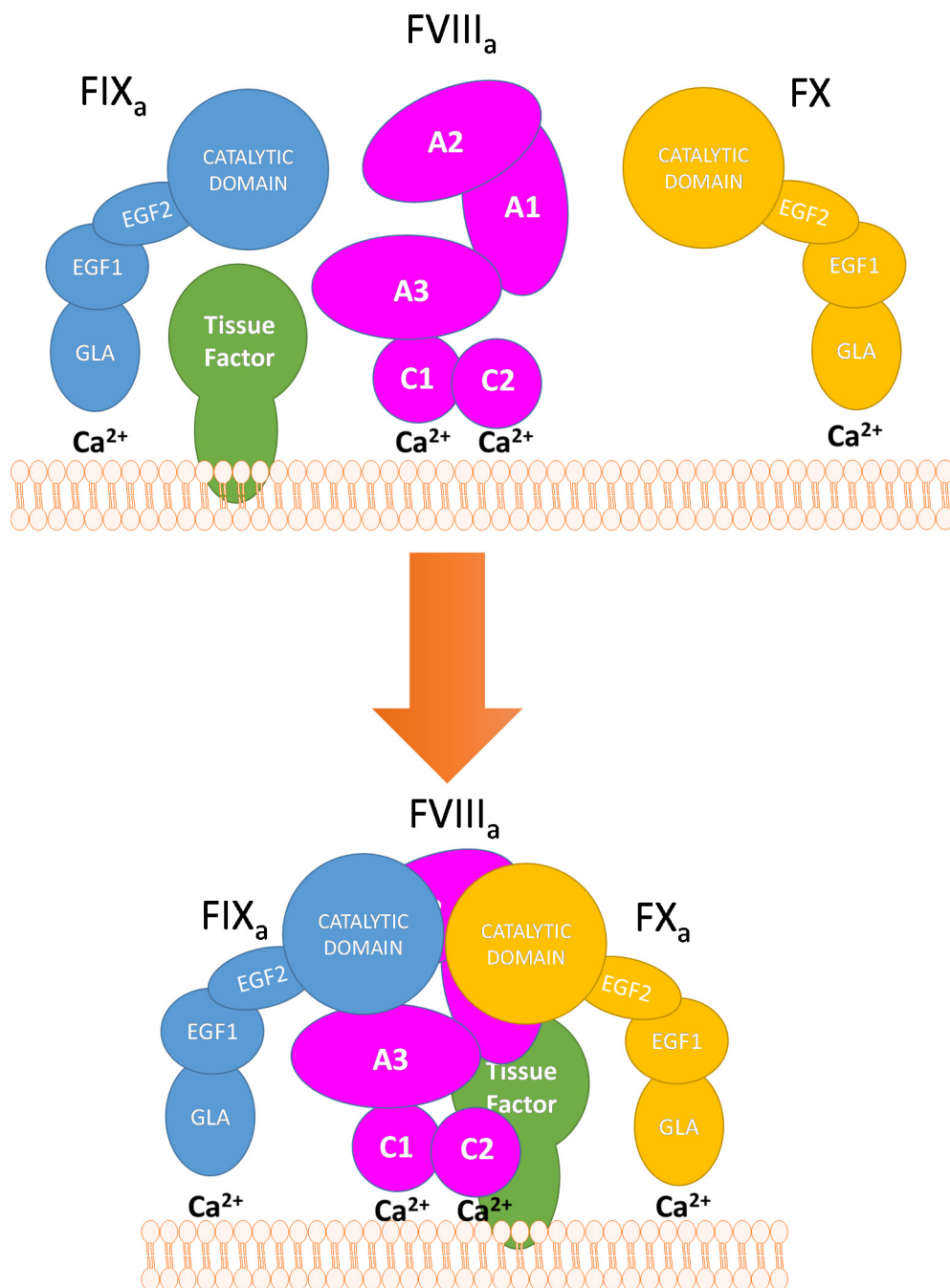


Figure 1.10: Calcium facilitates the formation of the Factor X activating complex on the surface of activated platelets. The negatively charged phosphatidylserine in the phospholipid layer of activated plates coordinates calcium which then binds to the GLA domain of the coagulation proteases. Modified from [62].

1.6 Nanoparticles for Biological Applications

1.6.1 Superparamagnetic Iron Oxide Nanoparticles

1.6.1.1 *Background*

Superparamagnetic iron oxide nanoparticles (SPIONs) are iron oxide nanoparticles in the size range of 1 to 100 nm and are constructed of magnetite (Fe_3O_4) and its oxidised form maghemite ($\gamma\text{-Fe}_2\text{O}_3$). Due to their magnetic properties, as well as ease of modification, SPIONs are one of the most promising materials with applications in nanomedicine as dual agent therapeutic-diagnostics (theranostics) systems for magnetic resonance imaging.

The ferromagnetic properties of a material result in it being capable of responding to a magnetic field, whereby the magnetic subdomains, known as Weiss domains, in the material become reordered, resulting in the formation of a permanent magnet. Ferromagnetic materials have the ability to undergo a transition to a paramagnetic state above their Curie temperature. At this temperature, the materials retain their magnetism in the presence of a magnetic field, but lose their ability to form permanent magnets. In the case of superparamagnetism, this transition occurs below the Curie temperature, when the material consists only of a single magnetic domain, that is, the magnetic domains of the material are ordered in one direction only [63, 64]. This only occurs when the particle diameter is below a critical, material dependent size, typically 20-30 nm for iron oxide nanoparticles [64].

1.6.1.2 *Synthesis and Functionalisation of SPIONs*

Numerous methods exist for the synthesis of iron oxide nanoparticles including co-precipitation, microemulsion and thermal decomposition of organic precursors. Co-precipitation of Fe^{3+} and Fe^{2+} at an optimum ratio of 2:1 as described by Massart [65] is considered to be the most common synthesis route for uniformly size SPION generation [66]. Larger nanoparticles are then produced in a process known as Ostwald ripening, where smaller crystals dissolve and are redeposited onto larger particles [67]. As such, shorter reaction/nucleation times result in small monodisperse nanoparticles.

In the case of thermal decomposition, iron precursors are heated in the presence of hot organic surfactants to produce SPIONs with a narrow size distribution. During this process, nanoparticles are coated with capping ligands to improve the colloidal stability. To improve the biocompatibility or functionality of the SPIONs, post-synthesis modification of the SPION can be carried out. This can include the formation of core-

shell nanoparticles, for example, by using silica [68], gold [69], or coating with small molecules such as dimercaptosuccinic acid (DMSA) [70], targeting ligands or drugs [71].

One of the most common reagents used in the functionalisation of SPIONs are silanes such as 3-aminopropyltriethoxysilane (APTES), p-aminophenyl trimethoxysilane (APTS), and mercaptopropyltriethoxysilane (MPTES). In this process, the hydroxyl groups present on the SPIONs react with the ethoxy/methoxy groups of the silane to form Si-O bonds as shown in Figure 1.11. The terminal group of the silane is then available for conjugation to other molecules [72].

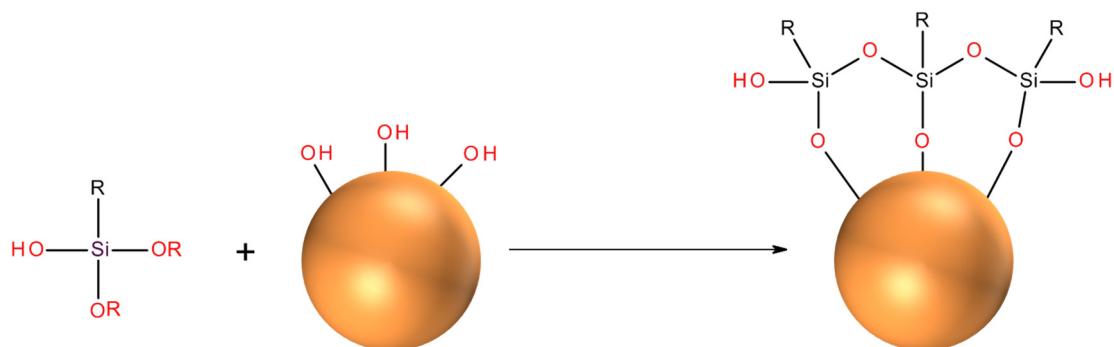


Figure 1.11: Silanation of iron oxide nanoparticles using silanes. Hydroxyl groups on the surface of the nanoparticle displace the alkoxy group (OR groups) forming stable covalent bonds. The remaining variable group (R) allows for further surface group modifications. Modified from [73].

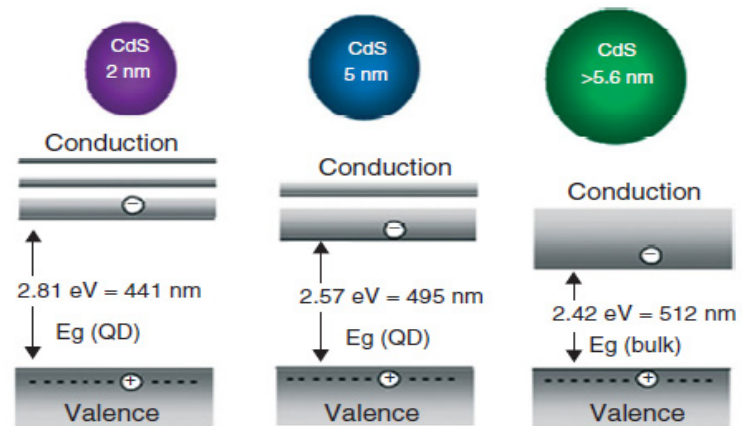


Figure 1.12: The relationship between Quantum Dot bandgap and size [74]. As the bandgap decreases the energy also decrease, changing the colour of the emitted light from high energy blue to low energy red.

1.6.2 Quantum Dots

1.6.2.1 *Quantum Dot Properties*

Semiconducting nanocrystals or quantum dots (QDs) are the focus of extensive research in the field of nanoscience due to their unique physical properties and a wide range of potential applications in photonics, solar cells, biotechnology, medicine and imaging [75]. As the radius of the QD is smaller than its exciton Bohr radius, typically less than 10 nm [76], the excitations are squeezed leading to quantum confinement, and their subsequent size dependent optical properties. The bandgap refers to the energy difference between the top of the valence band and the bottom of the conduction band, and this bandgap increases as particle size decreases, as depicted in Figure 1.12.

An electronically excited state is generated as molecules absorb light, where the excited molecule is energetically unstable with respect to the ground state and can lose this excess energy by radiative transitions. Fluorescence occurs when the charge carriers eventually recombine and the exciton energy is lost as a photon. The emitted light moves from smaller to larger wavelengths as the QD size increases, with smaller QDs emitting in the higher energy blue spectrum and larger in the low energy red.

1.6.2.2 *Biological Application of Quantum Dots*

One of the more promising applications of quantum dots is in cancer diagnosis and treatment. A literature search shows that there are in excess of 90 and 70 publications for imaging and diagnostic uses of QDs, respectively, in 2014 alone [77]. Biological compounds can be conjugated to the quantum dots for the selective targeting of specific cancer cells [78]. The biomedical applications of CdTe quantum dots have been demonstrated by various groups [75, 79-83]. Typically, the use of quantum dots in biological studies is focused around the area of tumour cell labelling. The highly luminescent properties of the quantum dots make them particularly useful for this. The cytotoxicity of the quantum dots themselves is a major focus of interest for the selective killing of these cancer cells [84]. It is thought that the toxicity of these particles stems from the toxicity of the cadmium atoms, photochemical generation of free radicals and nanoparticles aggregation on the cell surface [85, 86]. Unselective targeting of cells could lead to the apoptosis of healthy cells, as a result. Modifications to the quantum dots can also allow for labelling and differentiation of numerous cell types, similar to the use of fluorescent dyes in flow cytometry, for example [87].

1.6.2.3 *Production and Modification of Quantum Dots*

Quantum dots were first discovered by Alexei Ekimov in a glass matrix [88] and by Louis E. Brus in colloidal solutions [89] in the 1980s, and can be produced by an organometallic or aqueous route synthesis. In the aqueous route, non-luminescent clusters are primarily formed, followed by a thermodynamically driven growth of the nanocrystals [90]. The synthesis of quantum dots is dominated by a process known as Ostwald Ripening. In this process dissolution of smaller particles occurs and this material is redeposited on the surface of larger particles [91]. It is thermodynamically driven as larger particles are energetically more favoured than smaller particles [92]. Cadmium ions are classified as 'soft' acids and as such react well with 'soft' bases such as thiols. These interactions are exploited in the stabilization of the cadmium telluride quantum dots as the thiol groups form strong complexes with the cadmium atoms [93]. Other functional groups of the thiolated stabiliser can then be used to functionalize the quantum dots *via* various methods including carbodiimide coupling and ionic and hydrogen bonding interactions [78, 83, 94, 95].

1.7 Nanoparticles and Haemostasis

As indicated in Figure 1.7, nanoparticles have the potential to interact with the coagulation cascade. Interaction of the particles with the zymogens may result in activation or inhibition of components of the cascade leading to thrombosis or haemorrhage. With the increase in use of nanoparticles as vehicles for drug delivery, the potential effects of nanoparticles on the components of the blood coagulation system must be fully investigated. Nanoparticles can be synthesised to have pro-, anti- or no coagulative effects on the body. These effects are most commonly due to the nanoparticle coating and composition, but also size and charge may be important [96-98].

1.7.1 Polymer Based Nanoparticles

Nanoparticles constructed from polymers can have pro- and anti-coagulative effects, as well as being haemocompatible [96, 99]. Lipid-core nanocapsules of varying sizes and charge, synthesised from poly(ϵ -caprolactone), stabilized with polysorbate 80-lecithin and coated with chitosan have been shown to be fully haemocompatible, regardless of coating and surface potential [96]. The PT and APTT were measured and shown to be within normal range. As with many drugs, the volume of nanomaterials correlated to their potential toxicity. The effect of the nanoparticles on platelet aggregation and haemolysis was studied, and the particles were deemed haemocompatible at a concentration of 2% (v/v), with haemolysis and aggregation being noted at 10% (v/v).

The pro-coagulative nature of polymer nanoparticles can be clearly seen following administration of amine terminated PAMAM dendritic nanoparticles [99-101]. Fatal disseminating intravascular coagulation (DIC) like manifestations occurred for both 3.4 and 8.1 nm amine coated dendrimers, whereas carboxyl- and hydroxyl-terminated dendrimers of similar size were tolerated at a 50-fold higher dose. DIC is a systemic thrombo-haemorrhagic disorder involving the generation of intravascular fibrin and consumption of pro-coagulants and platelets, and has been proposed as a possible mode of toxicity of nanoparticle *in vivo* [99, 102]. It is postulated that the positively charged nature of the amine dendrimers could mimic the function of thrombin in a non-specific fashion, thereby activating the coagulation cascade through its interaction with both the intrinsic and common pathways [99]. The effect of the positively charged dendrimers was also not inhibited by the use of antithrombotic or antiplatelet agents [100].

Polymeric nanoparticles such as those synthesised from Poly(D,L-lactide-coglycolide) (PLGA) have been FDA approved for uses in drug delivery, and as such provide a vehicle for the targeted treatment of haemostatic disorders[103]. PLGA NPs have been produced for targeting cerebral thrombosis through functionalisation with Epidermal growth factor 1 (EGF1), enhanced green fluorescent protein (EGFP) and a NF- κ B decoy oligonucleotide. This oligonucleotide inhibits the up-regulation of tissue factor [104], and as such the NPs provide a means to inhibit the coagulation process within a thrombus. Similarly, PLGA and poly(ϵ -caprolactone) nanoparticles have been loaded with low molecular weight heparin, and which has been shown to retain its anticoagulant activity [105]. Polymer based nanoparticles have also been developed for use in surgery following blunt trauma [106].

1.7.2 Polysaccharide Based Nanoparticles

Anticoagulant nanoparticles have also been synthesised from polysaccharide based material such as chitosan, fucoidan and chondroitin sulphate [107]. Fucoidan is a substance found in brown algae that is known to increase the inhibition of thrombin by antithrombin, and as such is an anticoagulant. Upon addition of the fucoidan nanoparticles to plasma, there was an increase in APTT and no significant change in PT, indicating that the nanoparticles act on the intrinsic pathway, in a manner similar to the fucoidan control solution [108]. In fact, the fucoidan nanoparticles had increased anti-coagulative activity compared to the fucoidan solution [107]. This may be due to a conformational change that occurs when polysaccharides are in solution leading to a lower activity, whereas the fucoidan nanoparticles have a fixed conformation, possibly

allowing for a more effective receptor-ligand interaction and binding [107, 109]. The particles were also deemed to be non-cytotoxic following an MTT assay.

Polysaccharide based nanoparticles have been developed for use in haemostatic wound dressing [110, 111]. Nanosheets synthesised from chitosan and alginate spun-coated on a silicon dioxide substrate [110] and chitosan/konjac glucomannan loaded nanoparticles [111], have been developed to adhere to the damaged blood vessel walls and stop hemorrhage in a rabbit model, and for wound healing applications, respectively. It is expected that these formulations will have numerous advantages over current haemostatic wound dressings manufactured from fibrin or recombinant thrombin which aid haemostasis but do not stop bleeding directly [112]. The nanoparticles are biocompatible and show potential in wound healing, most notably by demonstrating increased wound healing performance compared to chitosan alone, in treating bleeding from the hepatic portal vein.

Chitosan based nanoparticles have also been functionalised with the cDNA for the coagulation Factor VIII [113]. These nanoparticles were designed to provide an alternative treatment for haemophilia A, an inherited bleeding disorder caused by a deficiency of FVIII [114, 115]. The oral administration of the FVIII DNA-chitosan nanoparticle resulted in an increase in FVIII expression, along with protection from bleeding.

1.7.3 Inorganic Material Based Nanoparticles

Inorganic materials, such as those synthesised from silica, iron and silver, are currently at the forefront of research aimed at developing novel, biocompatible nanoparticles [99, 116-123]. Iron oxide based magnetic nanoparticles can have both pro-coagulant [116, 118] or anti-coagulant [118, 119] effects, which can be based on the size and coating of the nanoparticles. Unfunctionalised carbon coated iron core nanoparticles caused significantly shorter clotting times compared to the PEGylated nanoparticles, which were comparable to the control [116]. Similarly, 23nm magnetite nanoparticles enhance blood clotting as recorded using a protamine response test [118]. Functionalisation of the nanoparticle with the anticoagulant heparin, results in an increase in clotting time, as expected [118, 124]. Cytotoxicity testing of the nanoparticles on macrophages revealed that the particles were non-toxic at a concentration of 200 µg/mL [118]. To combat thromboembolism events, nanoparticles can be functionalised with coagulation inhibitors such as urokinase [119]. Urokinase is a crucial component of the thrombolysis cascade which activates plasminogen, leading to the degradation of fibrin [125]. Functionalisation of magnetic nanoparticle with urokinase allows for the directing of the particle, by

applying a magnetic field [119]. Application of the magnetic field to the urokinase-nanoparticle conjugate resulted in a 5-fold increase in thrombolytic activity, compared to urokinase alone, and 2.6-fold increase compared to the urokinase-nanoparticle without the magnetic field.

Magnetic iron oxide nanoparticles have also been developed for enhancing wound healing [126]. Thrombin has been functionalised on the surface of iron oxide NPs in order to improve wound healing post-incision. Thrombin is essential for the conversion of soluble fibrinogen to insoluble fibrin [127] and in the aggregation of platelets, to form the so called 'platelet-plug'. Thrombin also has been used in a clinical setting for more than 60 years [127, 128] in applications such as wound management and tissue sealing [126]. However, the half-life of thrombin is extremely short at less than 15 seconds [129] and as such, it is suggested that conjugating the enzyme to nanoparticles will improve the long-term availability [126]. It was shown that the thrombin-bound nanoparticles remained at the wound site, within the fibrin clot for at least 14 days. The skin tensile strength of the wound was also higher for the thrombin-nanoparticle and accelerated wound healing was observed, compared to free thrombin.

The haemocompatibility of silica nanoparticles (SiNPs) remains unclear at present, with some research suggesting that larger particles (<200 nm) may potentially have immediate toxic properties [99], while others suggest that large particles have the least impact on platelet aggregation [123]. The haemolytic nature of silica nanoparticles is widely documented, and is suggested to be caused by pore sizes and surface chemistry of the material [130-133]. The aggregatory properties of SiNPs is possibly due to their ability to stimulate the release of peroxynitrite (ONOO^-) which is linked to platelet activation and aggregation [134]. The porous nature of SiNPs may also allow for the absorption of proteins when introduced into a biological system, possibly making the nanomaterial biologically inert compared to nonporous SiNPs [99, 135].

Silver nanoparticles (AgNPs) have remarkable antimicrobial activity that offers opportunities for their use in nanomedicine [136-138]. They also are noted for their antithrombotic properties [122, 139, 140]. PT testing of 24 nm AgNPs showed no effect on coagulation, while the APTT showed evidence of inhibition of the intrinsic pathway [139], up to a concentration of 67 $\mu\text{g/mL}$. PEGylated 20 nm AgNPs also have an antithrombotic effect that is concentration dependent [122]. As the concentration of AgNPs was increased, there was a significant decrease in platelet aggregation, which as not linked to loss in platelet viability. Activation of platelets and their subsequent

binding to endothelial surface is a key mechanism in thrombus formation. Addition of PEGylated AgNPs resulted in reduced platelet adhesion, again in a dose dependent manner [122]. Probing of the biochemistry revealed that PEGylated AgNPs inhibit key platelet glycoproteins, such as GPIIb/IIIa, and arachidonic acid metabolism, which are major pathways involved in platelet aggregation [141, 142]. Similarly, unfunctionalised 10-15 nm AgNPs inhibit thrombin-induced platelet aggregation, again in a concentration dependent manner [140]. It is thought that it occurs through by inhibiting integrin-mediated platelet function, through direct interaction with the AgNPs, and not silver ions [140].

Gold and silver NPs have also been functionalised with polysaccharide molecules for biological applications [143]. Gold NPs (AuNPs) have photothermal properties which can allow for locally induced heat to result in the release of molecules like glycosaminoglycans (GAGs) such as heparin, heparin sulphate and chondroitin sulphate [143]. The AuNPs and AgNPs functionalised with heparin resulted in a concentration dependent prolongation of APTT, comparable to that of free heparin. These NPs also resulted in inhibition of clot formation, which was not associated to the NPs alone. Chitosan-gold nanoparticles have also been developed for applications in wound dressing [144].

1.7.4 Quantum Dots

Limited work has also been carried out on the prothrombotic nature of quantum dots [145]. Mice injected with amine and carboxyl modified cadmium selenide/zinc sulphide quantum dots have been reported to experience pulmonary vascular thrombosis and almost immediate death [145]. It was demonstrated that carboxyl modified QDs were more potent than their amine counterparts at inducing pulmonary vascular thrombosis. The authors suggest that the carboxyl QDs activate the coagulation cascade through the contact activation pathway, as the thrombotic effect was not detected when mice were pre-treated with heparin [145].

1.7.5 Carbon Based Nanoparticles

Carbon based nanoparticles are becoming widely used in nanomedicine in applications such as biosensors, imaging, drug delivery and photothermal therapies [146-148]. However, as with all nanoparticles for biomedical use, stringent safety testing must be carried out. For example, graphene oxide has been shown to produce a strong aggregatory response in human platelets, comparable to that of thrombin [149], similar to nano carbon [150]. When the nanomaterial is amine-modified it loses its thrombogenic properties while retaining a candidate for photothermal therapy and *in vivo* imaging [151].

Similar to graphene, carbon nanotubes (CNTs) and fullerenes such as C60, are becoming more and more utilised in biomedical applications due to their mechanical strength and high electrical and thermal conductivity[152, 153]. CNTs are also ideal for drug delivery due to their high mechanical stability and can be functionalised on their surface or in the cavity[154]. Single-walled and multi-walled nanotubes have however been shown to be thrombogenic [152, 155]. CNTs have been shown to increase P-selectin expression on platelets and an elevation in platelet-granulocyte complexes. Single walled CNTs were noted to decrease thrombosis time in small mesenteric arteries and in rat carotid arteries [153, 155]. It is thought that the surface characteristics of CNTs might be responsible for the platelet activation and subsequent thrombosis formation [155]. Similar to graphene, CNTs can be modified to become haemocompatible, for example by immobilizing heparin onto the CNT surface or by functionalising the CNTs with carboxyl groups [156, 157]. It is suggested that the covalent functionalisation of CNTs can improve their toxicity profile by enhancing their clearance[158]. Administration of pristine-unfunctionalised multi-walled CNTs results in the activation of the extrinsic coagulation cascade and leads to a rise in both vWF and D-dimer levels [157]. D-dimers are used as a clinical assessment of a hypercoagulative state and are produced by the degradation of cross-linked fibrin [159].

Carbon nanocapsules (CNCs) are similar to other carbon based NPs and have been modified and tested for drug delivery applications [152, 160] and have been shown to be inherently less thrombogenic than CNTs [153] and highly biocompatible[161]. Due to the large surface area of CNC and their biocompatibility, the nanomaterial is suitable for use as a delivery mechanism for haemostatic drugs such as heparin [152]. *In vivo*, *ex vivo* and APTT testing revealed that the heparin retained its activity. The heparin conjugated CNCs increased the time to thrombus formation compared to heparin alone, indicating its possible use as drug delivery mechanism, and the unmodified CNCs were deemed not to increase thrombosis[152].

Other thrombin inhibiting carbon based nanoparticles have also been developed for imaging of acute thrombosis [162]. D-phenylalanyl-L-prolyl-L-arginyl-chloromethyl ketone (PPACK), an irreversible thrombin inhibitor [163], has been conjugated to perfluorocarbon core nanoparticles (PFC NPs) and studied *in vivo* and *in vitro* [162]. The PPACK-PFC NPs outperformed heparin treatment in occlusion studies, and APTT of the control PFC NPs did not differ significantly from saline, whereas the PPACK-PFC NPs resulted in significant lengthening. ¹⁹F magnetic resonance imaging was carried on occluded arteries of mice treated with the PPACK nanoparticles and indicated strong

colocalisation of the particles with the clots. The results indicated that the non-clinically useful antithrombotic PPACK [164] has clinical applications when bound to the PFC NP [162].

1.7.6 Environmental Particulate Matter

Particulate matter (PM) in the environment has also been shown to have effects on coagulation, typically by inducing a pro-coagulant state [165-167]. Workers exposed to airborne PM from a steel production plant have been identified as having shorter PT times but markers for inflammation and fibrinolysis were unchanged [165], and this has been shown to be a function of PM concentration [166]. The APTT of the participants was unchanged indicating its effect on the extrinsic or common pathway, through possible interaction with tissue factor or FVII_a, FX or FV [166]. It has also been suggested that PM causes an increase in tissue factor expression from macrophages [168].

1.8 Nanoparticle Characterisation

To ensure compliance with environmental protection guidelines [169] nanoparticles produced, either directly or indirectly, must be fully characterised [170-172]. This is fundamental in all areas of research and industry as the size of nanoparticles (NPs) often has a profound effect on their properties. It has been extensively reported that NP response to the surrounding environment is size dependent [173, 174] due to their large surface area that interacts with their surrounding matrix, and this can influence their reactivity with toxicity targets in the cells [175]. This has been found to be particularly relevant when the NPs are used for targeted applications. For instance gold NPs have been used in medical application as contrast agents or nanocarriers [176, 177], polystyrene NPs are a good model for diagnostics and environmental applications due to their relatively well defined size and low cost respectively [178, 179], silica NPs as drug delivery carriers due to their size dependent toxicity [180, 181] and iron oxide nanoparticles (SPIONs) as therapeutics carriers [182]. Therefore when working with NPs it becomes crucially important to fully characterised the NP physico-chemical properties and their interaction with the surrounding matrix or environment [183, 184]. This is particularly true when investigating the efficacy and biodistribution of NPs *in vivo* where it is extremely difficult to validate any mechanism of interactions or kinetics from the biodistribution data without having the accurate particle size distributions [185]. Therefore the dispersion state of the NPs in solution becomes a main parameter to be investigated which can be correlated to particle stability, and subsequent shelf life and efficacy [170]. Thus, the implications are not only limited to biological applications but environmental and ecological perspectives [186, 187].

Several techniques have been developed for measuring particle size, shape and dispersity from a suspension of particles; electron microscopy (EM), dynamic light scattering (DLS), disc centrifugation, coulter principle and Nanoparticle Tracking Analysis (NTA) amongst others. Some of these are labour intensive and time demanding whereas others are cost effective and user friendly. The most frequently used and user friendly NP size characterisation technique is DLS [188]. This technique measures the fluctuations in scattered light intensity caused by NPs moving under Brownian motion [189]. At present DLS is considered the first point of entry in the characterisation process, due to the ease of use. However the interpretation of results can be ambiguous as the analysis is weighed towards larger particle size, and as a result the presence of NP aggregates will result in a biasing of particle size distribution, resulting in a loss in accurate size determination. The use of NTA, with a lower detection limit compared to DLS, analysing NPs on a particle by particle basis, offers a new method for the visualisation and characterisation of NPs in suspension.

1.8.1 Nanoparticle Tracking Analysis (NTA)

NTA is a technology platform that utilises the properties of both light scattering and Brownian motion in order to obtain particle size distributions of samples in liquid suspension. A laser beam (of arbitrary wavelength but typically those available from laser diodes operating at 635nm, 532nm, 488nm or 405nm) is passed through a prism-edged glass flat within the sample chamber. The angle of incidence and refractive index of the glass flat is designed to be such that when the laser reaches the interface between the glass and the liquid sample layer above it, the beam refracts resulting in a compressed beam with a reduced profile and a high power density. The particles in suspension in the path of this beam scatter light in such a manner that they can be easily visualised via a long working distance, x20 magnification microscope objective, fitted to an otherwise conventional optical microscope. Onto this is mounted a CCD, EMCCD (Electron Multiplied Charged Coupled Device) or high-sensitivity CMOS camera, operating at 30 frames per second (fps), which captures a video file of the light scattered by particles moving under Brownian motion, within a field of view of approximately 100µm x 80µm x 10µm (Figure 1.13).

Within the field of view, particles are seen moving under Brownian motion, either directly by eye using the microscope oculars or via the camera. The proprietary NTA software records a video file (of typically 30-60 seconds duration) of the particles viewed and then simultaneously identifies and tracks the centre of each particle on a frame-by-frame basis. The image analysis software then determines the average distance moved by

each particle in the x and y directions. This value allows the particle diffusion coefficient (D_t) to be determined from which, if the sample temperature, T , and solvent viscosity, η , are known, then the sphere-equivalent hydrodynamic diameter, d , of the particles can be identified using the Stokes-Einstein equation

$$D_t = \frac{TK_B}{3\pi\eta d}$$

where K_B is Boltzmann's Constant.

As NTA observes Brownian movement in two dimensions a modified Stokes-Einstein equation is used, where the diffusion coefficient can be calculated by the mean square displacement of a particle in two dimension, such that

$$\overline{(x,y)^2} = \frac{4TK_B t}{3\pi\eta d}$$

One crucial advantage that NTA has over other measurement techniques is that it is not biased towards larger particles or aggregates. The software is based on the tracking of single particles, whereas typical DLS techniques place a strong bias on the largest particles present in the sample [188]. This allows for the detection of secondary peaks, which may not be detectable using other traditional measurements.

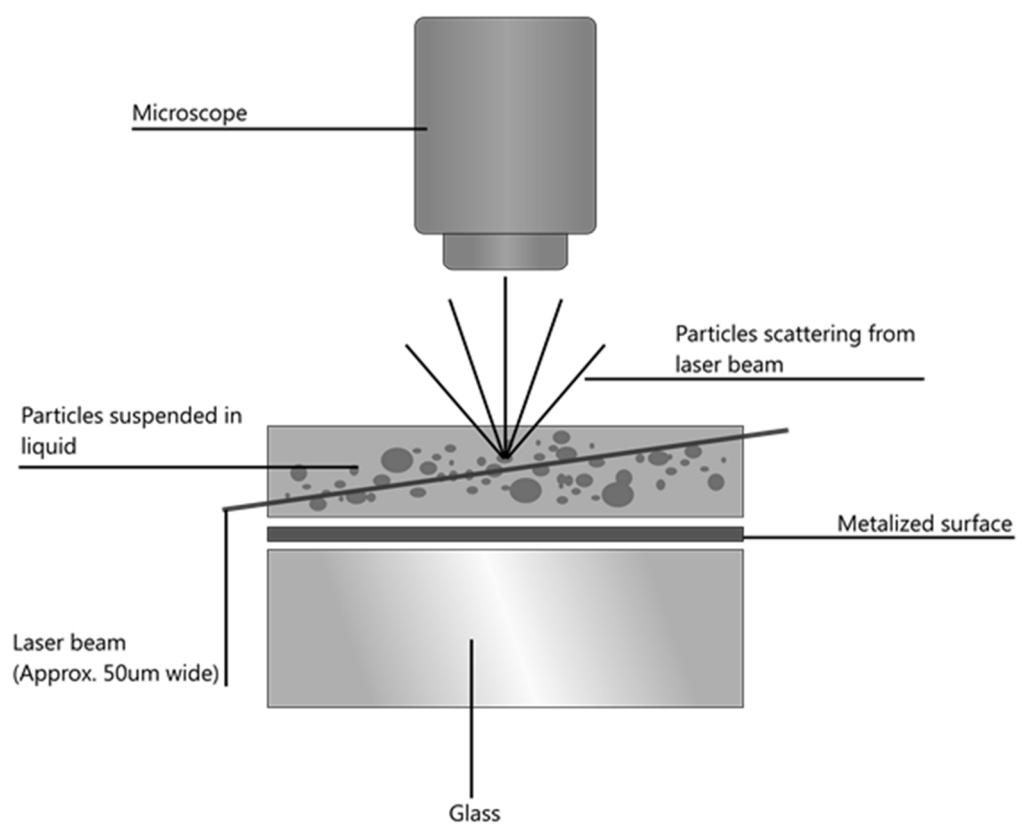


Figure 1.13: Schematic of the optical configuration used in NTA from [172]. The system utilises a top-plate where the nanoparticle solution is injected, a laser to induce light scattering and microscope to record this scattered light.

1.9 Aims

Based on the above literature, the presented thesis aims to:

1. Optimise the characterisation of nano-objects using Nanoparticle Tracking Analysis
2. Utilise these optimised procedures to characterise multiple sample types in the nano-size range in simple and complex dispersal media
3. Identify a suitable iron oxide nanoparticle for use as a possible carrier in the treatment of coagulation disorders
4. Functionalise this nanoparticle with a haemostatically active molecule to interact with the coagulation cascade in order to develop a novel approach for the treatment of coagulation disorders
5. Investigate the interaction of cadmium telluride quantum dots with the components of the coagulation cascade

Chapter 3 of this thesis will present several examples where the optimisation and refinement of the NTA platform for nanoparticle sizing. As part of several academia-industry collaborative projects, NTA was then used to assess the colloidal stability, aggregation state, and nanoparticle size and concentration of materials such as gold nanoboxes, bismuth ferrite nanoparticles, iron oxide nanoparticles, and biological nano-objects such as exosome and virus particles.

Chapter 4 of this study will focus on the selection of a candidate nanoparticle for potential use in the treatment of coagulation disorders. Here, a number of candidates were investigated for their effect on coagulation, with the chosen particle being functionalised with low molecular weight and unfractionated heparin. The functionalised particles were then characterised using NTA *via* the SOPs and techniques developed in Chapter 3, and subsequently their anticoagulant activity was assessed.

Finally Chapter 5 will centre on the investigation of the anticoagulant properties of cadmium telluride quantum dots. Here, two sizes of quantum dots were analysed using numerous diagnostic tests to try to identify the mechanism of action underlying their anticoagulant activity.

Chapter 2

Materials and Methods

2 Materials and Methods

2.1 Materials

Dimercaptosuccinic acid (DMSA) stabilised superparamagnetic iron oxide nanoparticles were sourced from Instituto de Ciencia de Materiales de Madrid, Consejo Superior de Investigaciones Cientificas (ICMM-CSIC). Their stock concentrations are listed below in Table 2.1. All solutions were made up to a 500 µg/mL iron concentration for assessment of their effect on coagulation. The concentrations of the SPIONs used for functionalisation are listed below in the relevant experimental sections.

Table 2.1: Stock concentrations of DMSA stabilised SPIONs

Nanoparticle Sample	Stock Concentration
DMSA coated 5 nm SPION	10.0 mg/mL iron concentration
DMSA coated 7 nm SPION	5.0 mg/mL iron concentration
DMSA coated 9 nm SPION	3.0 mg/mL iron concentration
DMSA coated 15 nm SPION	2.2 mg/mL iron concentration
DMSA coated 22 nm SPION	2.5 mg/mL iron concentration

Unfractionated heparin, Multiparin (Wockhardt UK, stock concentration 5,000 IU/mL) and low molecular weight heparin (Innohep, Leo Pharmaceuticals Ltd., stock concentration 20,000 IU/mL) used in this study were provided as gifts from Michelle Lavin, National Centre for Hereditary Coagulation Disorders, St James's Hospital Ireland. Heparin functionalised SPIONs were made up to 200 µg/mL stock solutions for assessment of their anticoagulant properties.

Adenovirus samples used in Nanoparticle Tracking Analysis experiments were provided by Dr. Silke Krol (Istituto Neurologico, Carlo Besta, Italy) and exosome samples were provided by the National Centre for Biomedical Engineering Science, National University of Ireland Galway as part of collaborative projects.

Cell culture medium, phosphate buffered saline and fetal bovine serum were purchased from Gibco (Bio-Sciences Ltd., Dublin, Ireland). All other reagents used were purchased from Sigma-Aldrich (Dublin, Ireland) unless otherwise specified.

2.1.1 Nanoparticle Functionalisation

Superparamagnetic iron oxide NPs (SPIONs) were selected as the most appropriate candidates for functionalisation. The SPIONs, 15 nm in diameter (± 1 nm) as measured

by Transmission Electron Microscopy (TEM), dimercaptosuccinic acid (DMSA) stabilised, with a mode hydrodynamic diameter of 96 nm (NTA), were functionalised using unfractionated (Multiparin) and low molecular weight (Innohep, Leo) heparins through the formation of amide bonds using (3-Aminopropyl)triethoxysilane (APTES) and 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) coupling, a route previously used in the conjugation of heparin to nanomaterials [75], as shown in Figure 2.1. A fluorescent immunoglobulin G (IgG) was used to confirm the functionalisation procedure.

2.1.2 Preparation of Amino-Terminated Silanised SPIONs

APTES (10 μ L) was added to isopropanol (900 μ L) and ammonium (10 μ L). Amine functional groups were introduced on the stock SPIONs (50 μ L) by addition of the APTES solution (50 μ L) and allowed to react for 24 hours at 4 °C. Particles were washed three times by centrifugation in DI water and dispersed by sonication.

2.1.3 Antibody-Functionalised SPIONs Preparation using APTES and EDC

To ascertain the ability of the chosen nanoparticle to be functionalised, a fluorescent AlexaFluor 488 tagged anti-HER2 immunoglobulin G (IgG) molecule (Biolegend Ltd.) was conjugated to APTES modified SPIONs produced using the procedure listed in the above section. Briefly, following APTES modification, the IgG molecule was pre-activated with EDC (300 μ L, 1 mg/mL) for 20 min at room temperature. The activated antibody was then added to the APTES modified SPIONs (100 μ L), and incubated for 24 hours at 4 °C. Functionalised particles were then washed three times using DI water by centrifugation. Particles were stored at 4 °C until required. A control sample where no EDC was used was also produced, whereby DI water replaced EDC.

2.1.4 Unfractionated Heparin-Functionalised SPIONs Preparation using EDC

Stock DMSA stabilised SPIONs (200 μ g/mL) were pre-activated with EDC (300 μ L, 1 mg/mL) at room temperature for 30 min. Unfractionated heparin (1000 IU/mL) was then added, followed by incubation with vortexing for 24 hours at 4 °C. Functionalised particles were then washed three times using DI water by centrifugation. Particles were characterised using NTA and stored at 4 °C until required.

2.1.5 Low Molecular Weight Heparin-Functionalised SPIONs Preparation using EDC

Stock SPIONs (200 μ g/mL) were pre-activated with EDC (300 μ L, 1 mg/mL) at room temperature for 30 min. Low molecular weight heparin (1000 IU/mL) was then added, followed by incubation with vortexing for 24 hours at 4 °C. Functionalised particles were then washed three times using DI water by centrifugation. Particles were characterised using NTA and stored at 4 °C until required.

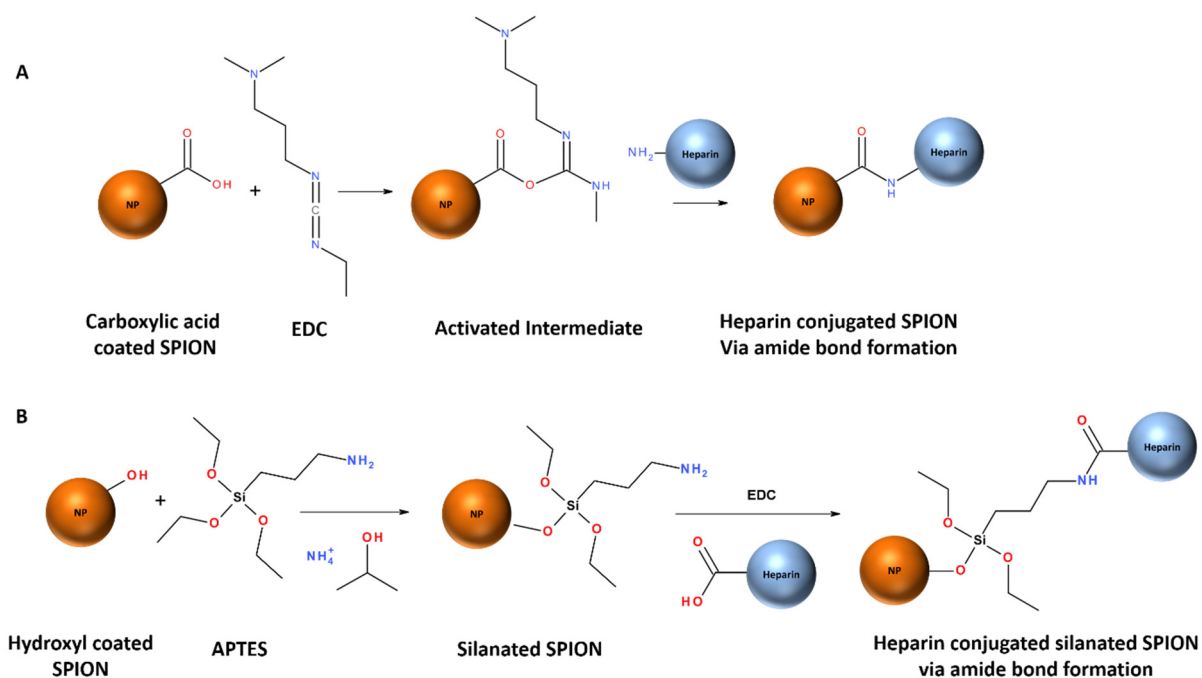


Figure 2.1: Routes for nanoparticle functionalisation. A: Formation of an amide bond between carboxylic acid coated SPIONs and the terminal amine group of heparin using EDC coupling. The initial reaction with EDC should take place under alkaline conditions, with acidic conditions being used for conjugating activated intermediate with amine containing components. B: Silanation of a SPION followed by amide bond formation between the terminal amine group of the SPION and the carboxylic acid group of heparin.

2.1.6 Unfractionated Heparin-Functionalised SPIONs Preparation using APTES and EDC

Unfractionated heparin (1000 IU/mL) was pre-activated with EDC (300 μ L, 1 mg/mL) at room temperature for 30 min., followed by the addition of the APTES functionalised SPIONs, as synthesised above. The reaction mixture was incubated with vortexing for 24 hours at 4 °C. Functionalised particles were then washed three times using DI water by centrifugation. Particles were characterised using NTA and stored at 4 °C until required.

2.1.7 Low Molecular Weight Heparin-Functionalised SPIONs Preparation using APTES and EDC

Low molecular weight heparin (1000 IU/mL) was pre-activated with EDC (300 μ L, 1 mg/mL) at room temperature for 30 min., followed by the addition of the APTES functionalised SPIONs (as above). The reaction mixture was incubated with vortexing for 24 hours at 4 °C. Functionalised particles were then washed three times using DI water by centrifugation. Particles were characterised using NTA and stored at 4 °C until required.

2.1.8 Heparin Functionalisation using EDC and NHS

Stock SPIONs (33 μ L) were activated in a 200 μ L solution containing NHS (100 μ L, 0.2 M) and EDC (100 μ L, 0.2 M) at room temperature for 30 min. Unfractionated (1000 IU/mL) or low molecular weight heparin (1000 IU/mL) was then added, followed by incubation with vortexing for 24 hours at 4 °C. Functionalised particles were then washed three times using DI water by centrifugation. Particles were characterised using NTA and stored at 4 °C until required.

2.1.9 Heparin Functionalisation of Gelatine Coating of SPIONs

To coat SPIONs with gelatine, stock 15 nm SPIONs (60 μ L) were suspended in bovine gelatine (0.1 % v/w, 600 μ L) for 4 hours at 37 °C. Particles were then washed with DI water by centrifugation and magnetic separation. Gelatinised SPIONs were then re-suspended in DI water and functionalised with unfractionated and low molecular weight heparin as per above procedures.

2.1.10 Quantum Dots

Thioglycolic acid (TGA) stabilised cadmium telluride (CdTe) quantum dots were synthesised *via* the aqueous synthesis route by Dr. Joseph McCarthy (School of Chemistry, Trinity College Dublin) using previously published procedures [75, 190]. Aluminium telluride was purchased from Cerac Inc., and all other chemicals for synthesis were purchased from Sigma-Aldrich.

3.2 nm (QD A) and 3.6 nm (QD B) quantum dots were utilised in this study and their physiochemical properties are detailed below in Table 2.2. A Shimadzu UV-1601 UV-Visible Spectrophotometer was used to measure QD absorption, with scans carried out in the 300-700 nm range. A Varian-Cary Eclipse Fluorescence Spectrophotometer was used to determine the fluorescence emission/photoluminescence (PL) spectra of QDs. The excitation wavelength was 480 nm and the emission was detected in the range 490-700 nm, and the Quantum Yields (QY) were calculated from the PL spectra using Rhodamine 6G as a reference. Zeta potential measurements were carried out using a Malvern Zetasizer Nano Series V5.10. Five measurements were usually taken for each sample, each made of 10 to 20 accumulations as optimised by the machine.

Table 2.2: Physiochemical properties of 3.2 nm and 3.6 nm Cadmium Telluride Quantum Dots

Property	QD A	QD B
Coating	TGA	TGA
Size (nm)	3.24	3.61
Peak excitation (nm)	550	589
Peak emission (nm)	577	627
Quantum Yield	18 %	22 %
Stock Concentration (μM)	125	71.2
Zeta Potential (mV)	-30	-31

2.2 Methods

2.2.1 Nanoparticle Tracking Analysis

Nanoparticles were characterised using Nanoparticle Tracking Analysis (NTA) developed by NanoSight Malvern Ltd. This characterisation technique was first commercialised in 2006, and is rapid growing technology [188]. Currently there are in excess of 1000 publications referencing NTA in countless fields of research [191]. The equipment utilises the properties of light scattering and Brownian motion to obtain particle size distributions of samples in liquid suspension [172].

NTA was carried out using a NS500 system equipped with a 405 nm laser, running NTA version 3.1. All nanoparticles were dispersed in ultrapure, 20 nm filtered DI water for NTA analysis. Six by 60 second videos were recorded, at a NP concentration sufficient to obtain a minimum of 200 completed tracks per video for statistical significance, ideally between 20-100 NPs per frame, using procedure developed during multiple rounds of interlaboratory comparison with NanoSight and twelve European and US research institutes [172]. Finite track length adjustment (FTLA) was incorporated to improve resolution and accuracy of the size distribution profiles.

Briefly, nanoparticles are serially diluted in the appropriate buffer at pH 7 or 7.4, unless specified, and vortexed to disperse. The sample is then attached to the sample inlet and the fluidics system loads the sample into the chamber. After selecting the appropriate focus and recording the required number of videos, the software analyses the videos and tracks the movement of each nanoparticle on screen. The algorithm then calculates the size of each individual nanoparticle and generates a size distribution graph for the combined videos as shown in Figure 2.2.

The aggregation state of NPs, not only SPIONs, but also gold nanoboxes and second harmonic generating bismuth ferrite NPs, in complex dispersal media was also assessed *via* NTA, where NPs were dispersed in complete cell culture medium and fetal bovine serum (FBS) [192, 193].

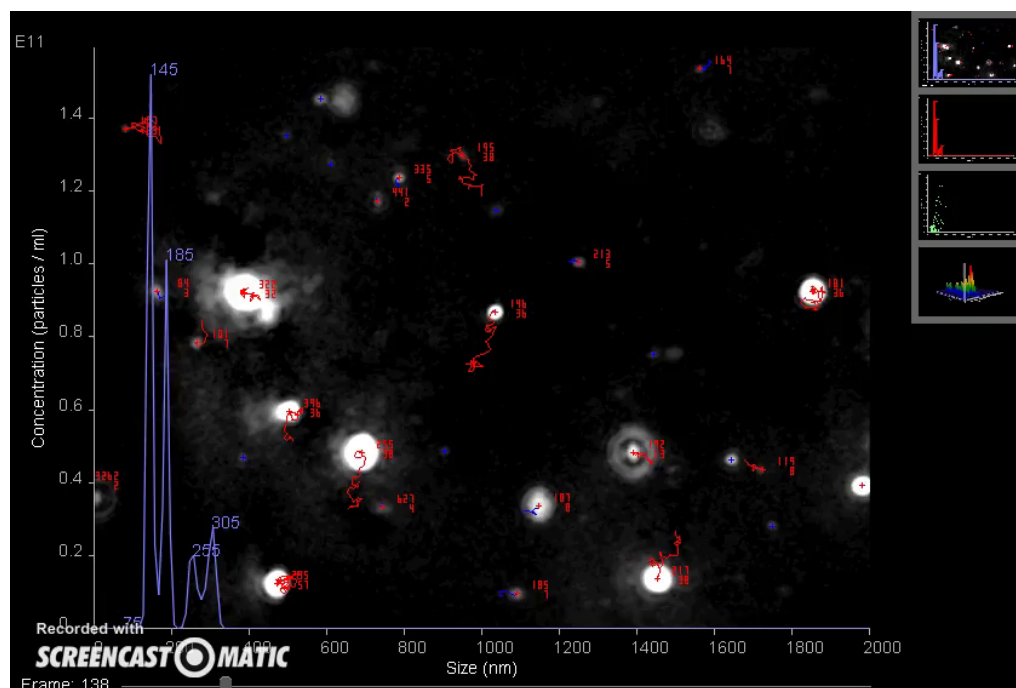
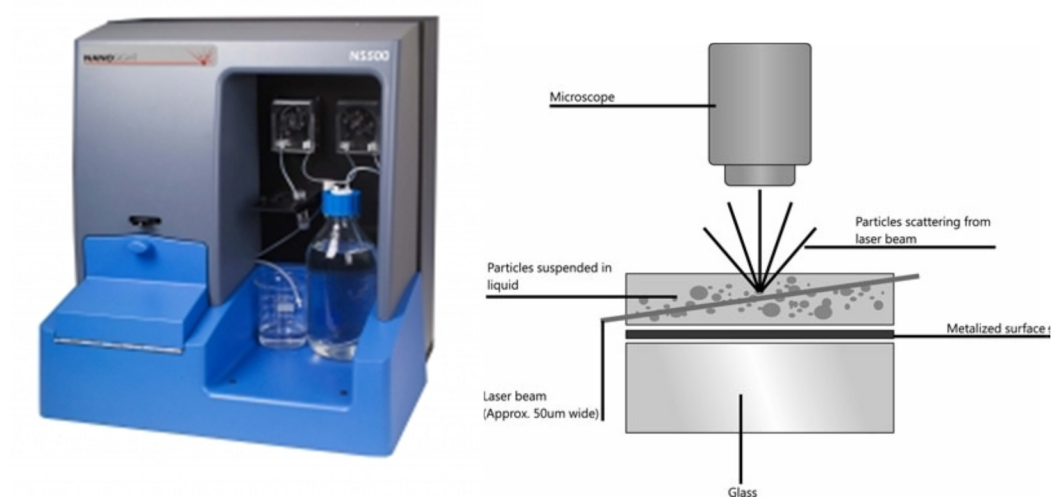


Figure 2.2: The NS500-NTA system, modified from [172]. NTA records and tracks the movement of nanoparticles under Brownian motion and calculates the mean and mode particle size, generates particle size distribution graphs and calculates nanoparticle per ml concentrations.



Figure 2.3: KC4 Delta Coagulometer, complete with auto-start pipette. Samples are prepared in micro-cuvette racks and placed on rotating heated aluminium block to incubate for desired time.

2.2.2 Plasma Preparation for Coagulation Assays

2.2.2.1 *Reference Plasma Preparation*

Human platelet-poor pooled normal plasma (Coag-Norm, Diagnostica Stago, France) was reconstituted as per manufacturer's instructions and allowed to rest for 30 min at room temperature prior to use.

2.2.2.2 *Blood Sample Collection*

Blood samples were collected in 0.109 M trisodium citrate (9:1 v/v) from healthy volunteers who had not taken any drugs known to affect platelet function for at least 14 days prior to study using a 21 gauge needle.

2.2.2.3 *Preparation of Platelet Rich Plasma and Platelet Poor Plasma*

Blood samples were centrifuged for 10 minutes at 20 °C (150g, acceleration 4, deceleration zero). The platelet rich plasma (PRP) layer was collected and stored at room temperature and used within 4 hours of collection. Platelet poor plasma (PPP) was prepared by centrifuging PRP for 10 minutes at 20 °C (3000g).

2.2.3 Coagulometer Setup

Coagulation times are assessed using a KC4 Delta coagulometer (Amelung Trinity Biotech) (Figure 2.3). Following addition of a stainless steel ball and the sample of interest to a cuvette, the sample is incubated at 37 °C for an appropriate incubation period (see below for assay specific incubation times), followed by placing the sample rack into the measuring well of the coagulometer. The measuring well rotates slowly causing the cuvette to rotate along its longitudinal axis. As the cuvette is positioned at a slight angle, gravity and inertia always position the ball at the lowest point of the cuvette. Exactly opposite the ball-position is a magnetic sensor. With the addition of appropriate reagent, a timer is started. As coagulation takes place fibrin strands pull the ball away from its inertia position which triggers a pulse in the magnetic sensor. This pulse electronically stops the timer.

2.2.4 Standard Coagulation Assays

2.2.4.1 *Prothrombin Time (PT)*

HemosIL recombiplastin 2G (Instrumentation Laboratory Company, USA) was reconstituted as per manufacturer's instructions and pre-incubated at 37 °C. A metallic ball-bearing was added to each cuvette, followed by the addition of reconstituted human plasma (50 µL), and incubated at 37 °C in the rotating rack for 1 minute. Clotting was initiated by the addition of 100 µL of reconstituted recombiplastin. The time until clot

formation was measured and recorded. For samples containing NPs, the NP suspension was added following the addition of human plasma, and incubated at 37 °C, as before.

2.2.4.2 *Activated Partial Thromboplastin Time (APTT)*

CK PREST 5 (Diagnostica Stago, France), an APTT reagent comprising cephalin and kaolin activators, was reconstituted following manufacturer's instructions. A metallic ball-bearing was added to each cuvette, followed by the addition of reconstituted CK PREST 5 (50 µL) and reconstituted human plasma (50 µL) and incubated at 37 °C in the rotating rack for 3 min. Clotting was initiated with the addition of CaCl₂ (50 µL) and the time recorded. For samples containing NPs, the NP suspension was added following the addition of CK PREST 5 and human plasma, and incubated at 37 °C, as before.

2.2.4.3 *Thrombin Time (TT)*

Human thrombin (Sigma Aldrich, Ireland) was dissolved in PBS (1 ml) and placed on ice. Reconstituted thrombin (250 µL) was then diluted in DI water (750 µL). A metallic ball-bearing was added to each cuvette, followed by the addition of reconstituted human plasma (50 µL), and incubated at 37 °C in the rotating rack for 1 min. Clotting was initiated by the addition of 50 µL of diluted thrombin. The time until clot formation was measured and recorded. For samples containing NPs, the NP suspension was added following the addition of human plasma, and incubated at 37 °C, as before.

2.2.5 *Intrinsic Factor Screen (IFS)*

The activities of Factor VIII, IX, XI and XII were measured using a one-stage clotting assay developed by the National Centre for Coagulation Disorders by Mairead Doyle (SOP Number: HAEM-LM-COAG-0095, version 2, St James' Hospital, Ireland). All tests were carried out using an ACL-TOP (Beckman Coulter, USA) and HemosIL SynthASil APTT reagent and calcium chloride (Instrumentation Laboratory Company, USA). Briefly, in the case of Factor VIII activity (FVIII:C), normal reference plasma was incubated with QDs, followed by a 1/10 dilution in PBS to increase the sensitivity of the assay.

Subsequently, the pre-diluted QD spiked normal plasma was mixed in 1:1 dilution in Factor VIII deficient plasma. The use of FVIII deficient plasma results in the APTT becoming specific for the activity of FVIII in the normal plasma. A similar procedure was followed for FIX:C, FXI:C and FXII:C.

2.2.6 Endogenous Thrombin Potential (ETP)

The ETP looks at the balance between pro- and anticoagulant factors in plasma. It refers to the total amount of thrombin generated in the assay (Figure 2.4). Using a fluorogenic thrombin substrate and thrombin calibrator (Thrombinoscope, The Netherlands). The calibrator is added to PPP to account for variables such as filter and lamp aging, as well as the non-linear concentration to signal of the amino-methyl-coumarin fluorophore.

Thrombin generation experiments were carried out in 96 well round bottom plates using the PPP and the PPP-reagent LOW, containing low concentrations of phospholipids and tissue factor (1 pM). For 48 wells, the FluCa solution is created by adding Fluo-Substrate (40 μ L) to one vial of Fluo-Buffer.

For experimental wells, each well contained plasma (20 μ L), PPP-LOW (μ L), FluCa solution (20 μ L, added just prior to starting software). Calibrator wells contain thrombin-cal (20 μ L), plasma (20 μ L) and FluCa solution (20 μ L), i.e. no PPP-LOW. Quantum dots of varying concentration were added and results compared to untreated plasma and plasma treated with an equivalent volume of water.

2.2.7 Coagulation Assays for Phospholipid Activity

2.2.7.1 *Dilute Russell's Viper Venom Time (DRVVT)*

The DRVVT is an assay utilised in clinics to identify the presence of antiphospholipid antibodies. As stated previously, phospholipids are crucial for the formation of the prothrombinase and tenase complexes. Antiphospholipid antibodies, or similar inhibitors, can result in the artificial prolongation of coagulation tests. In the DRVVT, the Russell Viper venom (RVV) is used to induce thrombosis, as it directly activates Factor X, through its metalloproteinase activity [194], thereby bypassing any deficiencies in Factors XII, XI, IX or VIII. The assay is dependent on the concentration of phospholipids, changes in phospholipid levels or activity can be detected.

Briefly, PPP is mixed with diluted phospholipids at 37 °C, followed by addition of diluted RVV and calcium chloride, and the clotting time is measured. The DRVVT was measured over a concentration of QDs, along with an equal volume of water by Mairead Doyle.

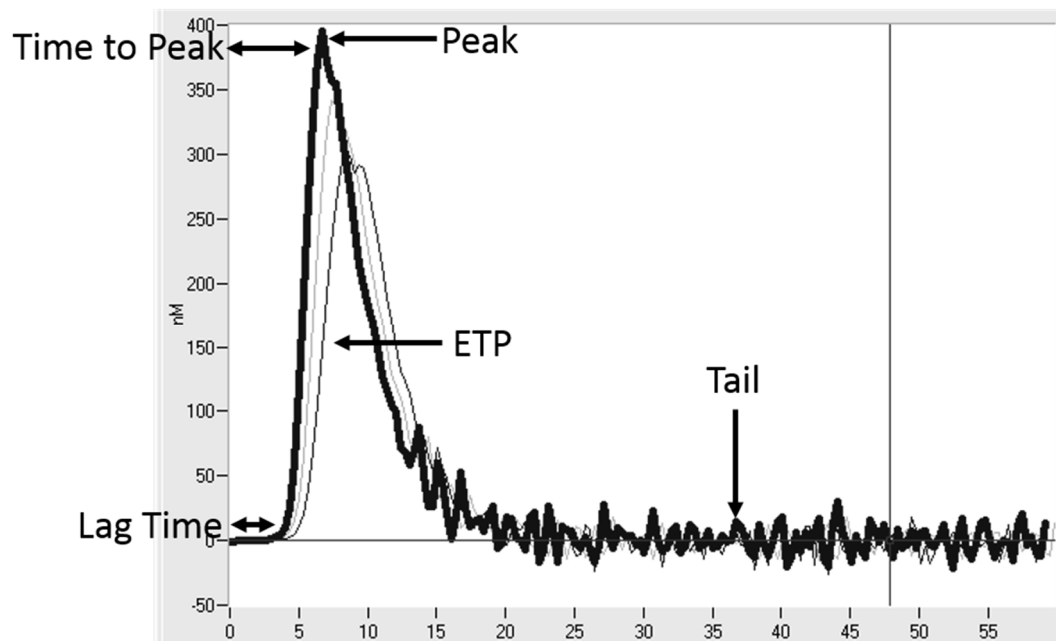


Figure 2.4: Screen clip of the automated Thrombogram. This assay is used to measure the total amount of thrombin generated during the assay, otherwise known as the endogenous thrombin potential (ETP). Frequently measured parameters include (A) the lag time: which measures the initiation of coagulation, (B) the time to peak: the time in minutes required to reach maximum generation, (C) Peak of thrombin generation: the maximum concentration of thrombin generated (nmol/l), (D) Endogenous thrombin potential (ETP): the area under the curve representing the total amount of thrombin generated (nmol/l) and (E): the tail: the time in minutes at which thrombin generation has finished.

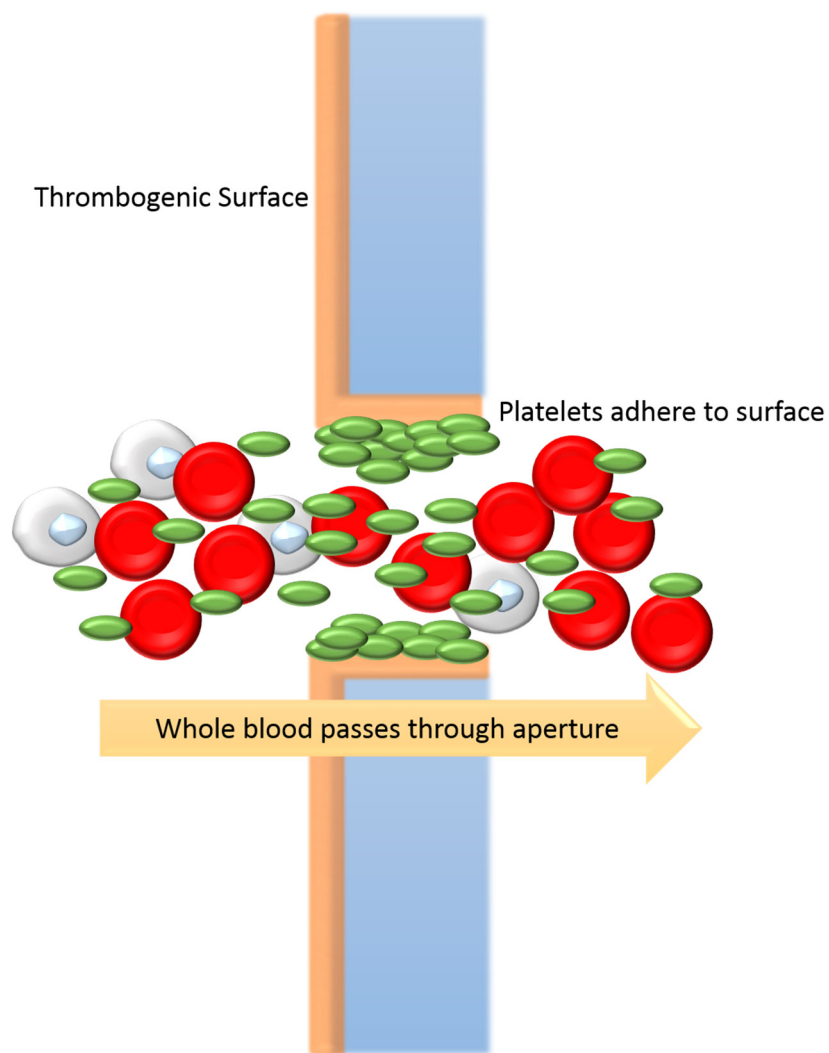


Figure 2.5: Platelet Function Analyser-100. Whole citrated blood is passed through a capillary containing an aperture coated with a thrombogenic surface, either collagen and epinephrine, or collagen and adenosine diphosphate. Platelets become activated by the agonists and aggregate with assistance from VWF to cause the closure of the aperture.

2.2.7.2 *Silica Clotting Time (SCT)*

The SCT is essentially an APTT assay whereby the kaolin activator is replaced by silica microspheres. It is carried also out using both high and low phospholipid concentrations. The assay is carried using varying concentrations of QDs and again, is used to identify if QDs interact with the function of the phospholipids and assembly of the coagulation complexes. The SCT was carried out on an ACL-TOP automated coagulometer using HemosIL SCT Screen and SCT Confirm by Mairread Doyle.

2.2.8 VWF Activity Assays

2.2.8.1 *VWF Antigen Activity (VWF:Ag)*

The VWF:Ag is an immunoassay that measures the concentration of VWF in plasma, but gives no information pertaining to its function. To measure the VWF:Ag, an enzyme linked immunosorbent assay (ELISA) was carried out.

Briefly, a 96 well flat bottom maxisorb plate was coated with rabbit anti-human VWF was diluted 1/500 in carbonate buffer (ph 9.6, 50 mM) (100 µL per well) overnight at 4 °C. The plate was washed with PBS-Tween (PBS-T) (0.5 % Tween-20), and blocked with bovine serum albumin (3 % w/v in PBS-T) for 1 hour at room temperature.

A standard curve was generated by diluting standard reference plasma (VWF concentration 10 µg/mL) serially from 1/80 to 1/2560 in PBS-T. PPP treated with QDs were diluted serially from 1/100 to 1/6400. 100 µL of these samples were then incubated on the plate for 2 hours at room temperature, washed with PBS-T, and incubated with anti-VWF-HRP (Dako, diluted 1/1000 in PBS-T, 100 µL per well) for 1 hour at room temperature. The plate was washed again with PBS-T and incubated with 3,3',5,5'-Tetramethylbenzidine (TMB, 100 µL). The reaction was stopped using sulphuric acid (50 µL) and read at 450 nm.

2.2.8.2 *VWF Ristocetin Cofactor Assay (VWF:RC)*

The VWF:RC is an assay that measures the ability of a patient's plasma to cause the aggregation of platelets in the presence of the antibiotic Ristocetin using a Sysmex CS2100i Analyser. Aggregation induced by ristocetin can only take place in the presence of VWF multimers, and as such the level of VWF multimers can be quantified using the VWF:RC.

Reagents were prepared as per manufacturer's instructions and carried out using procedures developed by the National Centre for Hereditary Coagulation Disorders

(SOP Number: HAEM-LM-COAG-0100, version 2, St James's Hospital, Ireland) by Mairead Doyle.

2.2.8.3 *Collagen Binding*

The ability of VWF to bind collagen was assessed, with and without QDs, using a standard ELISA. 96 well flat bottom maxisorb plates were coated with 100 μ L collagen III (Cambridge Bioscience) diluted in carbonate buffer (ph 9.6, 50 mM) to a final concentration of 5 μ g/mL, overnight at 4 °C. The plate was washed with PBS-Tween (PBS-T) (0.5 % Tween-20), and blocked with bovine serum albumin (3 % v/w in PBS-T) for 1 hour at room temperature.

A standard curve was generated by diluting standard reference plasma (VWF concentration 10 μ g/mL) serially from 1/80 to 1/2560 in PBS-T. To find a VWF concentration that consistently appears on the midrange of the standard curve, platelet derived VWF was diluted serially from 1/100 to 1/6400. 100 μ L of these samples were then incubated on the plate for 2 hours at room temperature, washed with PBS-T, and incubated with anti-VWF-HRP (Dako, diluted 1/1000 in PBS-T, 100 μ L per well) for 1 hour at room temperature. The plate was washed again with PBS-T and incubated with 3,3',5,5'-Tetramethylbenzidine (TMB, 100 μ L). The reaction was stopped using sulphuric acid (50 μ L) and read at 450 nm.

For experiments with QDs, the optimal dilution of VWF was incubated with varying concentrations of QD A and B to identify any alterations in VWF/collagen binding, using the above procedure.

2.2.9 Platelet Function Analyser-100 (PFA-100)

The PFA-100 system utilises a disposable cartridge with an aperture coated with either collagen and adenosine diphosphate (col/ADP) or collagen and epinephrine (col/epi) to detect problems with primary haemostasis. Whole blood is passed through the system, followed by platelet binding and closure of the aperture as shown in Figure 2.5. The results are dependent on platelet function, as well VWF level, with a normal closure time being less than 180 seconds for col/epi and less than 120 seconds for col/ADP.

PFA-100 collagen/epinephrine and collagen/adenosine diphosphate cartridges (Dade, USA) were prepared as per manufacturer's instructions. Blood from a healthy volunteer was in 1 part buffered sodium citrate (3.8 % w/v) to 9 parts blood. The QDs were then added to the blood (2 ml), with 800 μ L of this being pipetted into the cartridge opening. The cartridges were placed in the instrument and the test was run by Mairead Doyle.

2.2.10 Platelet Aggregometry

Platelet aggregometry was carried out using an Aggregation Remote Analyser Module (AggRAM) following a standard operating procedure developed by the National Centre for Hereditary Coagulation Disorders (SOP Number: HAEM-LM-COAG-0032, version 4, St James's Hospital, Ireland). Briefly, the aggregometer light transmission is set to 100 % light transmission using PPP. PRP (225 μ L) is dispensed into a test tube, followed by the addition of a stir-bar. The tube is then incubated at 37 °C for 2 minutes. Aggregation agonists (25 μ L) are then added directly into the PRP and the aggregation is monitored for at least 6 minutes by Mairead Doyle.

Chapter 3

Nanoparticle Tracking Analysis

3 Nanoparticle Tracking Analysis

3.1 Introduction

One of the key challenges in the field of nanoparticle analysis is in producing reliable and reproducible characterisation data for nanomaterials. This is critical, not only for understanding the mechanism of action of nano-therapeutics, but also to comply with directives, guidelines and regulation requested by regulatory agencies. Leading on from this, it was identified that the development of methodologies, techniques and standard operating procedures for the analysis of nano-objects in liquid suspension is crucial in areas such as chemical, biological, environmental, cosmetic, pharmaceutical, and food industries.

In terms of nanomedicine and nanosafety, it has been shown that nanoparticle physiochemical properties can play a role in the efficacy and biodistribution of the nanoparticle [185]. Similarly, the dispersion state of the NPs in solution is the main parameter when studying the particle stability and subsequent shelf life and efficacy [170]. The interaction of the NP with the dispersion media can also play a role in its biological response [195]. Thus, nanoparticle size can be a starting point for the generation of particle aggregates and the formation of protein coronas, which can result in the loss of NP specificity [196].

As such, the aim of this part of the study was the characterisation of nano-objects *via* NTA. NTA is still a relatively new technique, being first commercialised in 2006, and therefore through this research we were able to refine and develop it further. This was brought about by working in an academia-industry collaborative environment where engagement with different communities allowed us to identify and solve problems associated with particle sizing. More specifically we aimed to:

1. Develop robust SOPs for the characterisation of nano-objects *via* NTA
2. Use these protocols to analyse various particles in simple and complex dispersal media
3. Analyse non-typical nano-objects *via* NTA

Table 3.1: Percentage coefficient of variation for each sample in the first interlaboratory comparison [172]. Four rounds of characterisation were carried out, with the initial round using “in house” optimised protocols, and subsequent rounds building on lessons learned. The coefficients of variation (% CV) drop dramatically once strict standard operating procedure are in place. Data accumulated by Malvern Ltd as part of ILC work. – Represents that a sample was not analysed in a particular round, * average % CV for monodisperse samples in that round.

ILC Round	1 (% CV)	2 (% CV)	3 (% CV)	4 (% CV)
30 nm Gold	54.8	10.5	-	-
100 nm Carboxylate Polystyrene	33.3	9.3	-	-
100 nm Aminated Polystyrene	31.2	15.9	-	-
100 nm Silica	34.5	10.0	-	-
100 nm polystyrene	-	-	3.5	3.1
60 nm Gold	-	-	5.1	-
80 nm Gold	-	-	4.1	-
100 nm Polystyrene + Nutrient Mix	-	-	-	3.7
100 nm Polystyrene + Nutrient Mix + BSA	-	-	-	4.7
80 nm Gold (in bimodal sample)	-	-	-	5.5
200 nm Polystyrene (in bimodal sample)	-	-	-	5.2
Average (% CV)	38.5	11.4	4.2	4.4
Average (% CV) (* only monodisperse samples)	-	-	-	3.8*

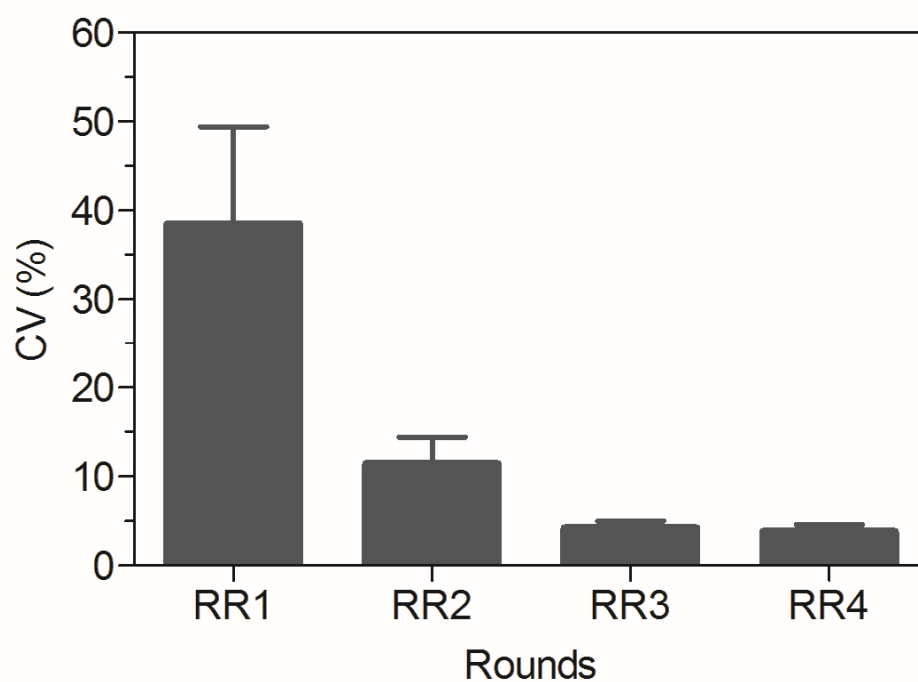


Figure 3.1: Evolution of the average percentage coefficient of variation (% CV) from round 1 to round 4, represented as mean $\pm$ standard deviation. The % CV decreases with each round due to improvements with the standard operating procedures. Data accumulated by Malvern Ltd as part of ILC work.

3.2 Results

3.2.1 Protocol Refinement Results

Nanoparticle Tracking and Analysis (NTA) was used to assess the nanoparticle dispersion quality and aggregation state, and to establish the mean, median and mode NP diameter of materials prior to any use. In order to ensure reliable and reproducible results, NPs were characterised using protocols developed during extensive rounds of interlaboratory comparisons (ILC) led by NanoSight Malvern UK [172]. In all figures below, Trinity College is listed as laboratory 2.

Following four initial rounds of protocol refinement the percentage coefficient of variation (% CV) for multiple sample sized particles, as well as for particles dispersed in complex media was reduced as shown in Table 3.1 and Figure 3.1. Reproducibility analysis was carried out using methods laid out in ISO 5725-2 [197], using classical [average and standard deviation] and robust statistical analysis encompassing median and median absolute deviation (MAD) based statistics, as per ISO 5725-5 [198].

Round 1 of the initial ILC required participants to analyse gold, polystyrene and silica nanoparticles using “in house” optimised protocols. As shown in Figure 3.2 and Table 3.1, this resulted in a large degree of variation between users, with a 54.8 % CV for 30 nm gold nanoparticles, with 2 participants differing greatly from the others. Subsequent rounds of ILC resulted in the evolution of more robust and reproducible protocols for the analysis of nano-objects *via* NTA.

Improvements of the SOP were based around issues detected during previous rounds, including temperature sensors on samples, sample handling and preparation instructions, sample storage, control of measurement parameters and data treatment. Again, this dramatically resulted in the lowering of the % CV as demonstrated in Table 3.1. For example, the % CV of 100 nm polystyrene nanoparticles was reduced from 33.3 % in round 1, to 3.1 % by round 4, a reduction of 90.69 %.

The analysis of bimodal particle mixtures was also carried out in round 4 of ILC 1. Here, 80 nm gold and 200 nm polystyrene NPs were mixed and the modal size distributions obtained as represented in Figure 3.3. Average calculated modal sizes were identified as 84.0 and 190.7 nm, with a coefficient of variation of 5.5 and 5.2 %, respectively.

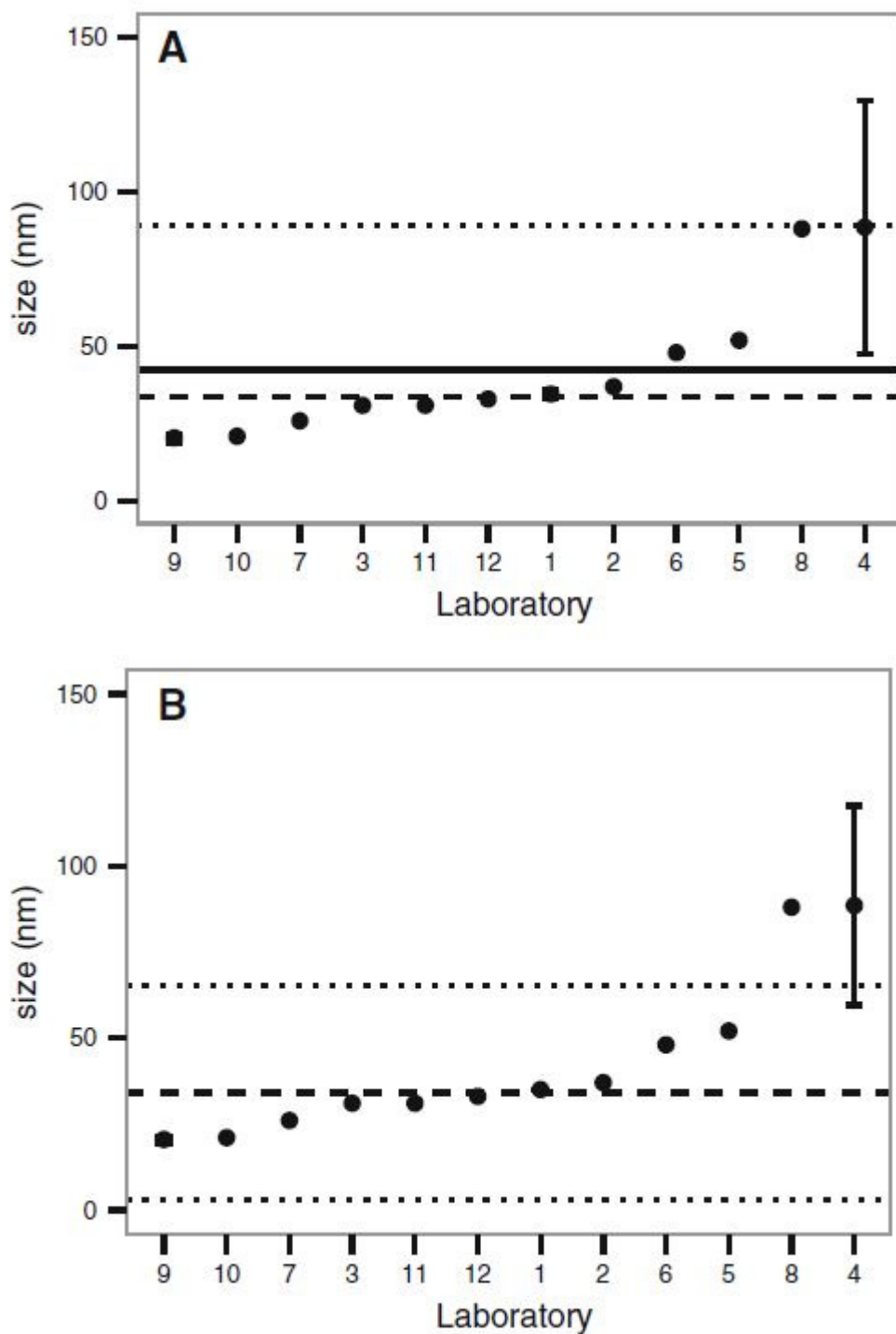


Figure 3.2: Modal particle size for 30 nm gold particles from ILC round 1 results from each partner ordered by increasing modal value based on (A) classical (± 2 SD) and (B) robust (± 2 MAD) statistical analysis. The mean value (solid line), median (dashed line) and 95 % confidence intervals (dotted line) are also shown, where appropriate. As clearly shown a large degree of inter-user variation was identified. Graphs jointly prepared by Malvern, Trinity College Dublin and EMPA Switzerland partners as part of the ILC 2 (under submission for publication).

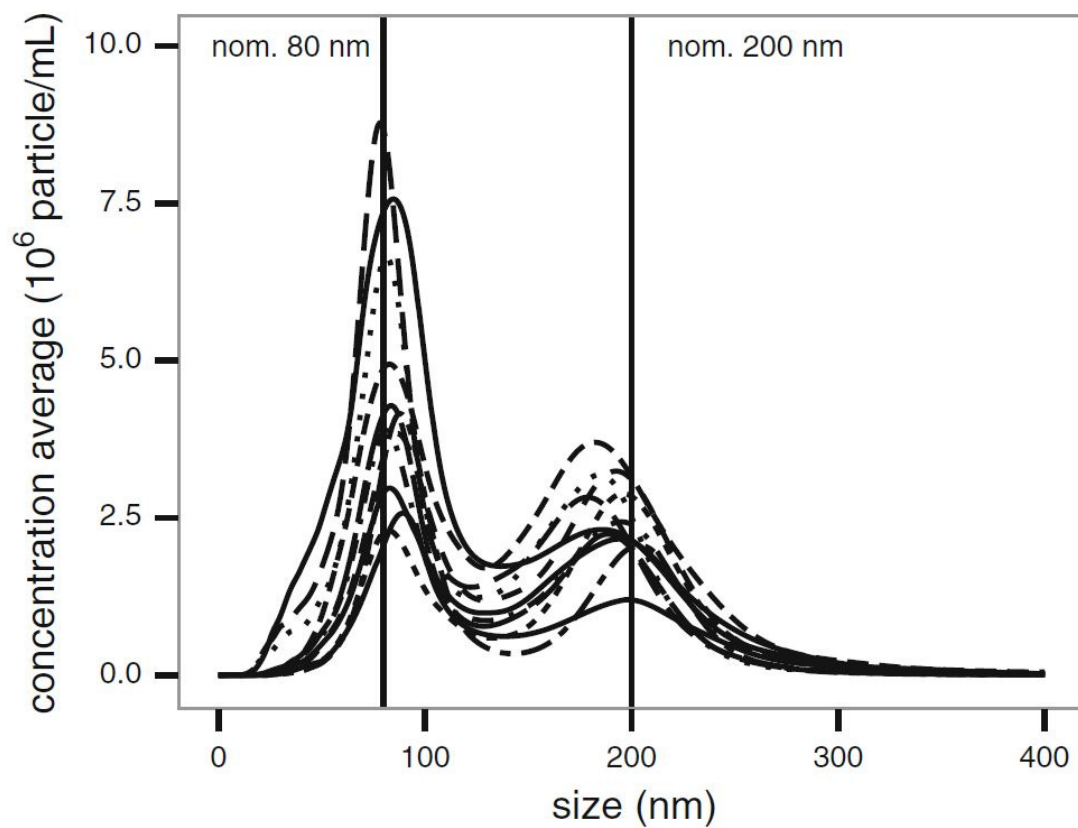


Figure 3.3: Averaged particle size distributions of a bimodal mix of nominally 80 nm gold and 200 nm polystyrene nanoparticles in water analysed by 11 participants in round 4. Graphs jointly prepared by Malvern, Trinity College Dublin and EMPA Switzerland partners as part of the ILC 2 (under submission for publication).

The SOP was also refined to allow for the analysis of nanoparticles in complex dispersal media. In round 4 of ILC 1, 100 nm polystyrene NPs were analysed using NTA following dispersal in cell culture medium and cell culture medium containing 10 % BSA. The % CV was increased from 3.1 to 3.7 % for the medium alone sample, and to 4.7 % when the medium was supplemented with BSA. This increase in % CV was thought to be primarily due to random aggregation of the NPs and absorption of proteins onto the surface to form protein coronas. Interestingly, when compared to the equivalent sample dispersed in water in round 1, the supplement sample had an 85.89 % reduction in % CV.

Following on from the published results of the first ILC, a second ILC (ILC 2) was carried out which contributed to further improvements by the NanoSight (now Malvern Ltd.) research and development team. It brought about improvements in the limits of detection (LOD), incorporation of the finite track length adjustment algorithm (FTLA) and the ability to analyse multimodal samples [199], where TCD and I had a driving role in the discussion and refinement of the experimental work.

Through advances in the software, the LOD has been shown to cover the range from 30-1000 nm. As a result of the jointly synchronised activity of the 11 laboratories involved in the ILC 2, particles in this size range can be accurately and reproducibly analysed, as shown below in Figure 3.4. The reproducibility of analysis of the smaller 30 nm gold nanoparticles can also be improved by increasing the viscosity of the solution. As stated previously, NTA is based around the Stokes-Einstein equation, and as such, once the viscosity of the solution is known, it is possible to calculate the particle diameter. To this end, a 10 % w/v glycerol solution was utilised to increase the viscosity of the sample, decrease the Brownian motion of the NPs and increase the efficacy of the particle tracking, thereby reducing the MAD from 3.2 nm to 2.5 nm as illustrated in Figure 3.5.

In a further refinement of the ILC 2, tri and quadri-modal mixtures of polystyrene latex particles were used to improve the resolution of complex samples in solution. Figure 3.6A and B, illustrate the reproducibility of resolving a 100, 200 and 300 nm mix population of NPs at a ratio of 18:3:2. Figure 3.6C and D take this further and incorporate 100, 200, 300 and 400 nm polystyrene NPs at a ratio of 6:3:3:2. The use of FTLA was instrumental here in the resolution of these peaks as it acts to improve curve fitting and narrowing of peaks as demonstrated in Figure 3.7.

In summary, the interlaboratory comparison studies presented here allowed, as a result of the jointly coordinated work of 11 laboratories, for the reduction in the % CV for samples covering a wide portion of the NTA range, as shown in Figure 3.8A. Dispersion of 30 nm gold particles in 10 % glycerol resulted in a reduction in the % CV from 3.94 %, in water, to 3.54 %, with a corresponding MAD % CV reduction from 0.904 to 0.897. Similarly, % CV [MAD % CV] of 1.69 [0.54], 0.59 [0.548] and 4.01 [2.15] for 100, 200 and 600 nm polystyrene particles. In the case of the multimodal samples, as shown in Figure 3.8B, the % CV for each component of the mixture was calculated. For the 100, 200 and 300 nm trimodal sample, a % CV of 4.94, 2.88 and 2.43 were obtained for the 100, 200 and 300 nm polystyrene NPs, respectively. In the case of the quadri-modal sample, the % CV was recorded as 6.82, 3.88, 2.60 and 3.09 % for the 100, 200, 300 and 400 nm particles, respectively.

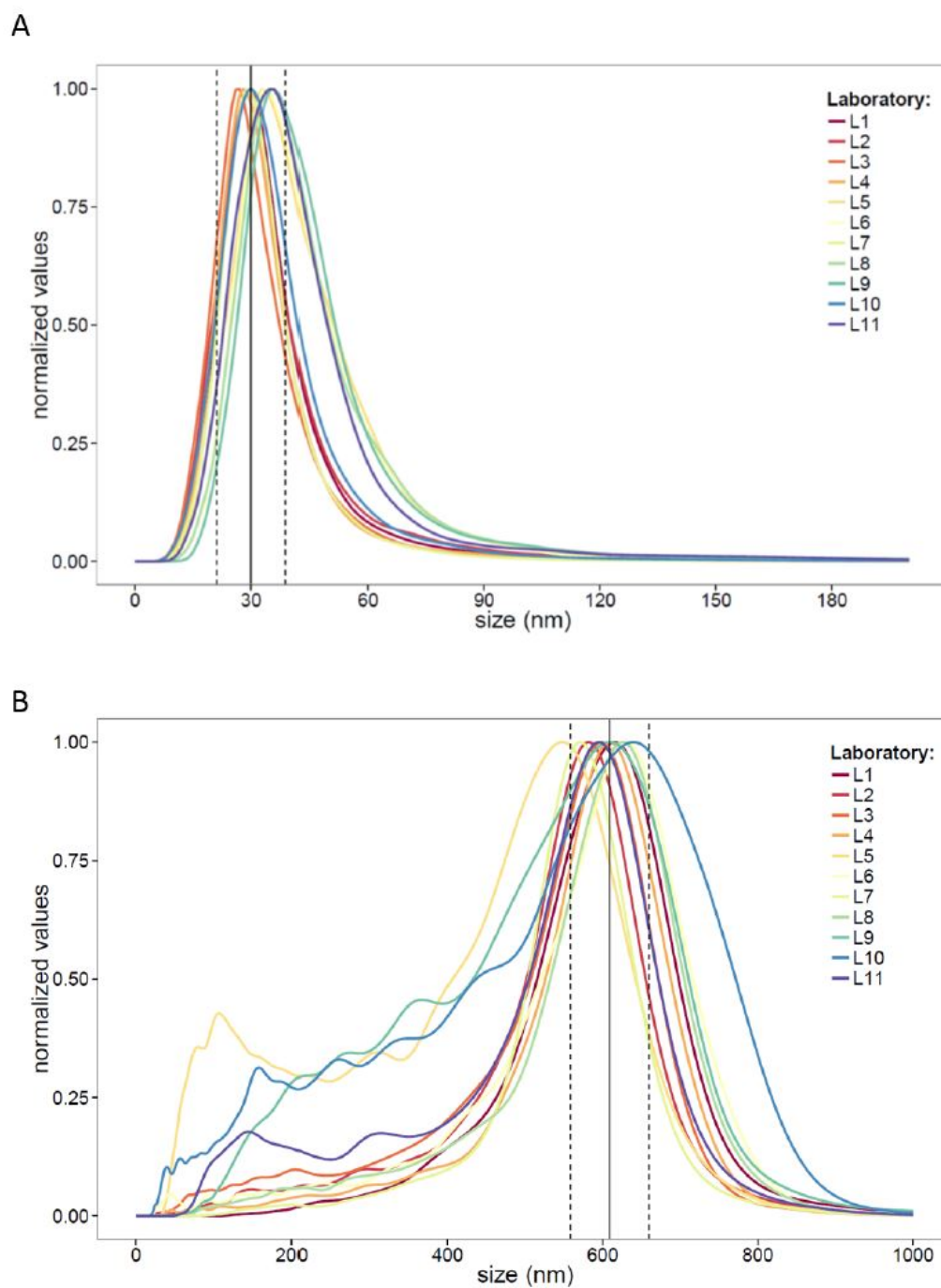


Figure 3.4: Concentration normalised particle size distributions of A: 30 nm gold nanoparticles in water and B: 600 nm polystyrene nanoparticles in water at concentrations suitable for NTA. Graphs and plots jointly prepared by Malvern, TCD and EMPA Switzerland as part of the ILC 2 work.

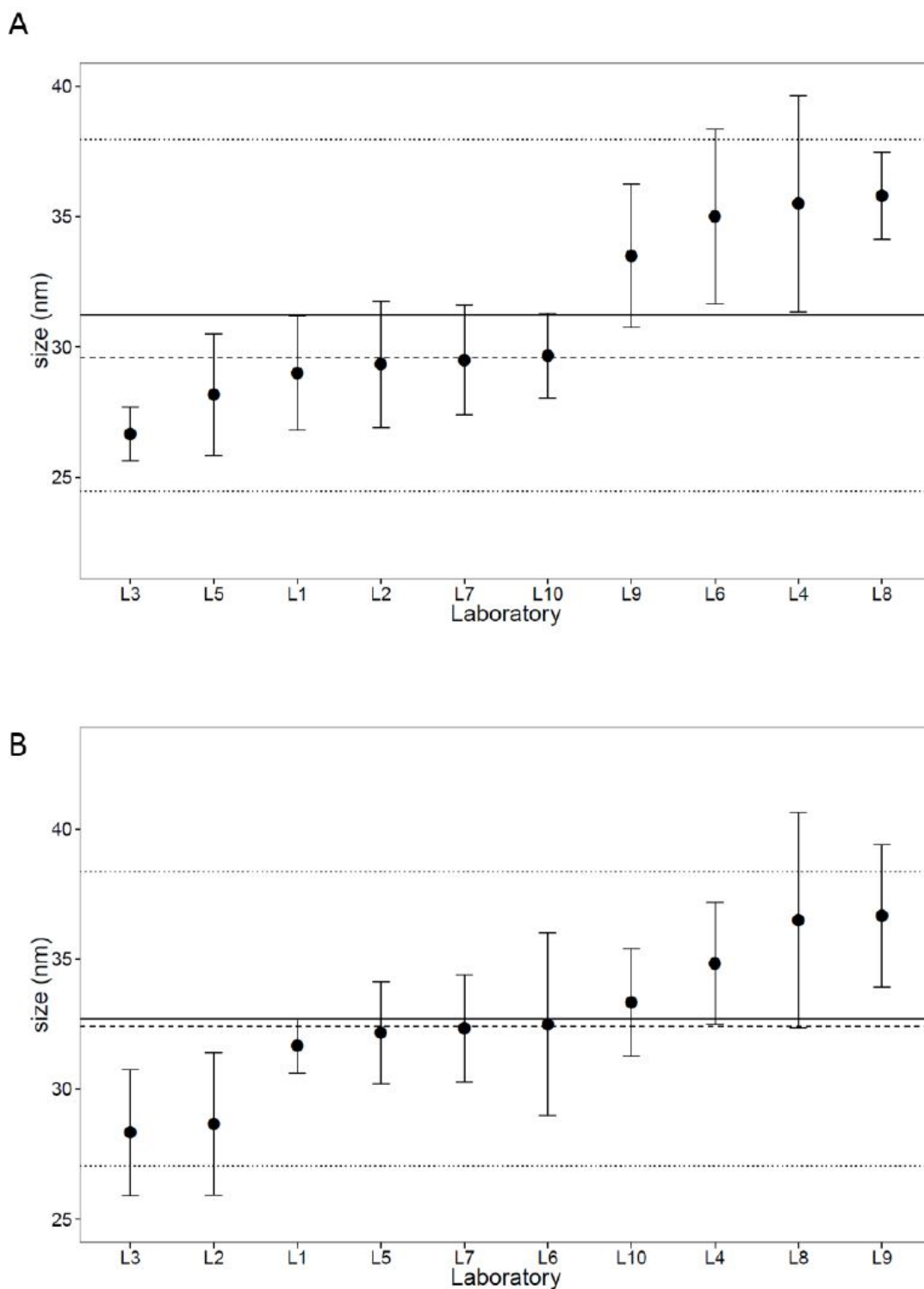


Figure 3.5: Robust statistical analysis of 30 nm gold particles in DI water (A) and in 10 % glycerol (B). Dashed lines represent median value, solid line is mean value and dotted lines are for the upper and lower median absolute differences ($\pm$ MAD). Graphs and plots jointly prepared by Malvern, TCD and EMPA Switzerland as part of the ILC 2 work.

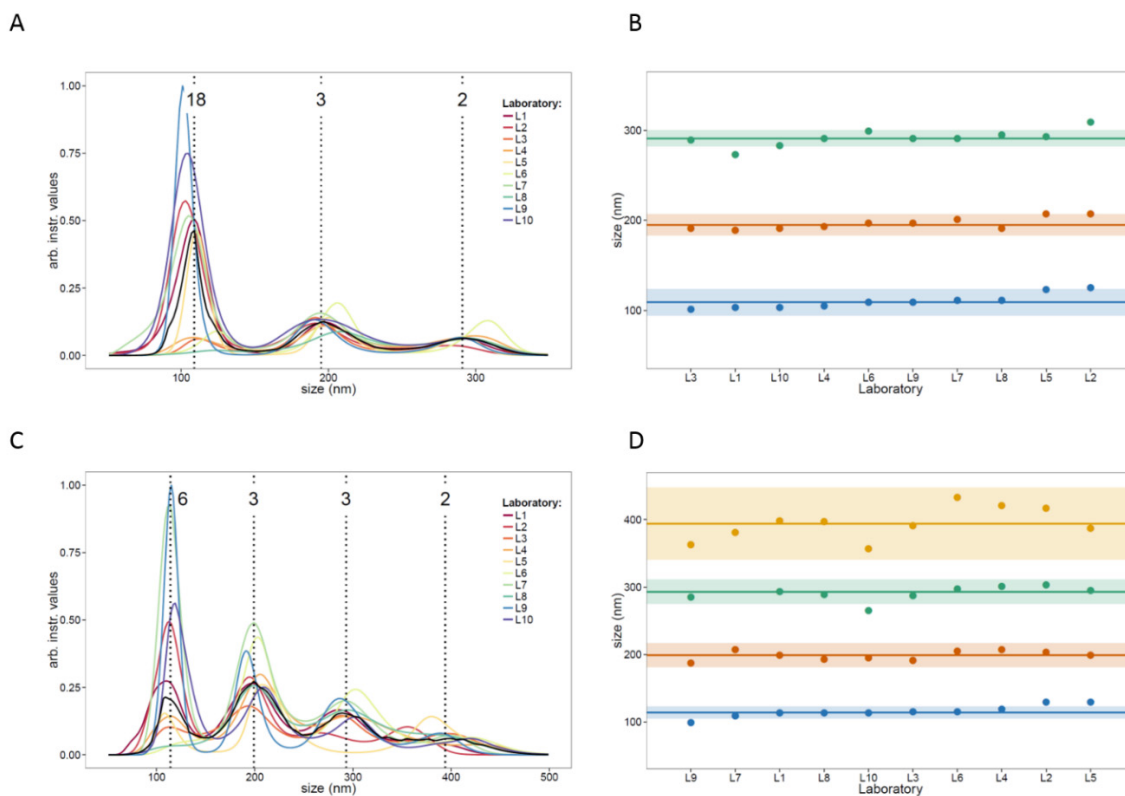


Figure 3.6: Multimodal size distribution graphs and comparisons of mixtures of 100, 200 and 300 nm polystyrene latex (PSL) NPs. A and B plots show the clear tri-modal quantification of PSL NPs (100, 200 and 300 nm) mixed at a ratio of 18:3:2 respectively. C and D plots show clear quadri-modal quantification of PSL NPs (100, 200, 300 and 400 nm) at a ratio of 6:3:3:2, respectively. In figure A and C, particle ratios are shown within each particle peak. Figure B and D report the interlaboratory variance around the chosen NPs in the mixtures. Graphs and plots jointly prepared by Malvern, TCD and EMPA Switzerland as part of the ILC 2 work.

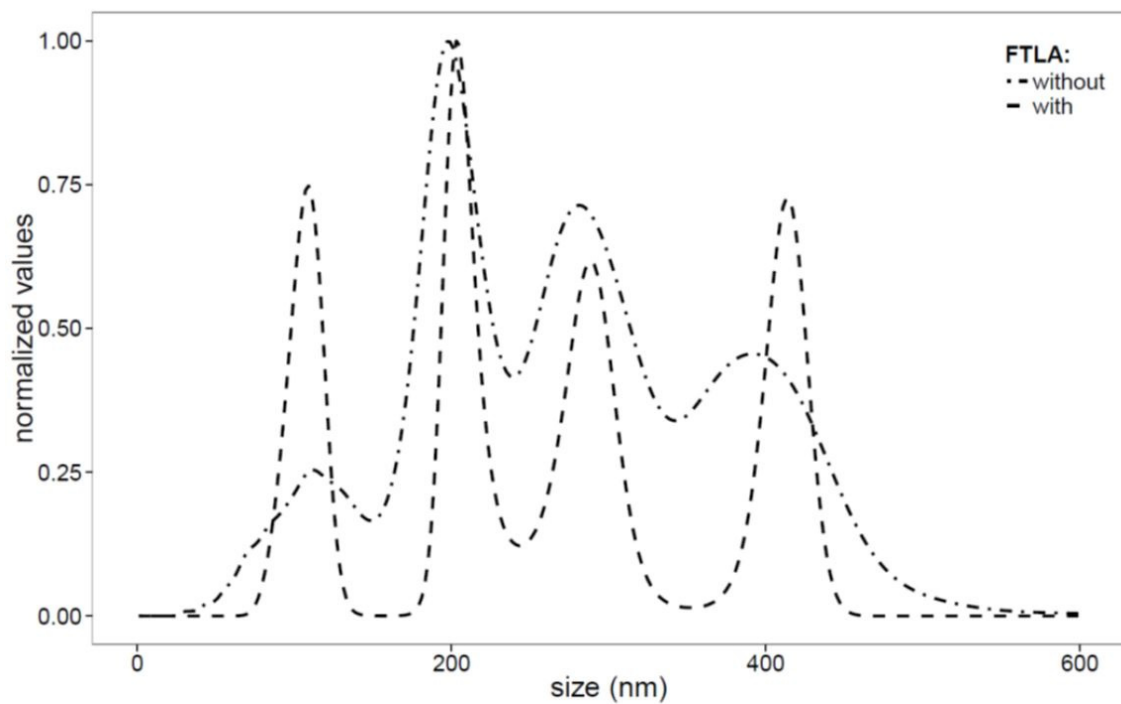
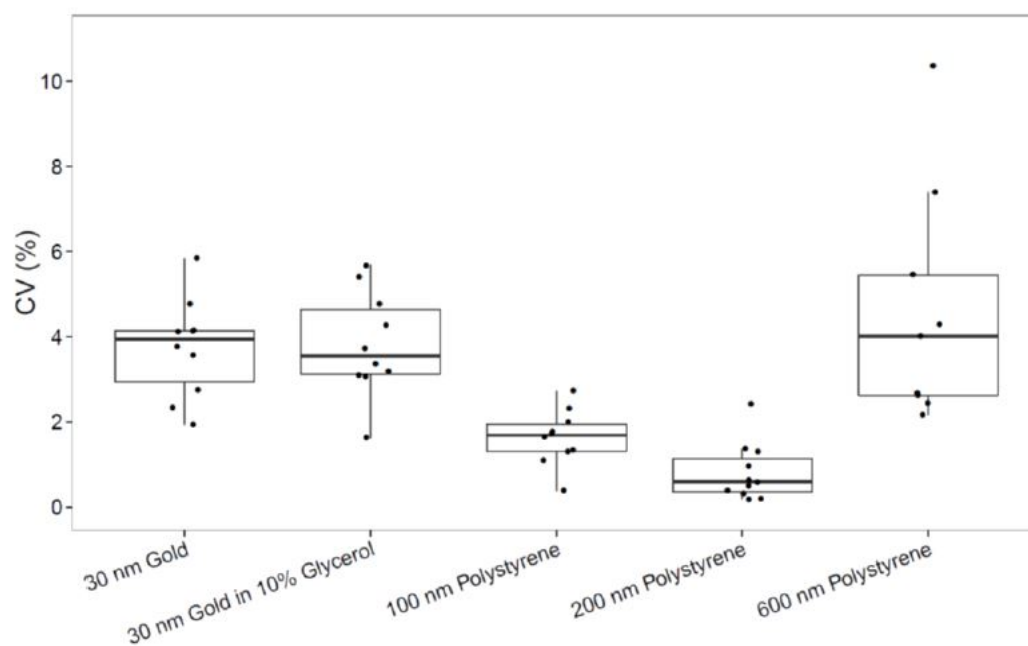


Figure 3.7: Implementation of Finite Track Length Adjustment (FTLA) algorithm improves curve fitting and narrowing of distribution peaks. Here it used to improve the resolution of a multimodal nanoparticle sample containing 100, 200, 300 and 400 nm polystyrene latex particles. As can be seen, particle distribution peaks become more defined when FTLA is applied. Graphs and plots jointly prepared by Malvern, TCD and EMPA Switzerland as part of the ILC 2 work.

A



B

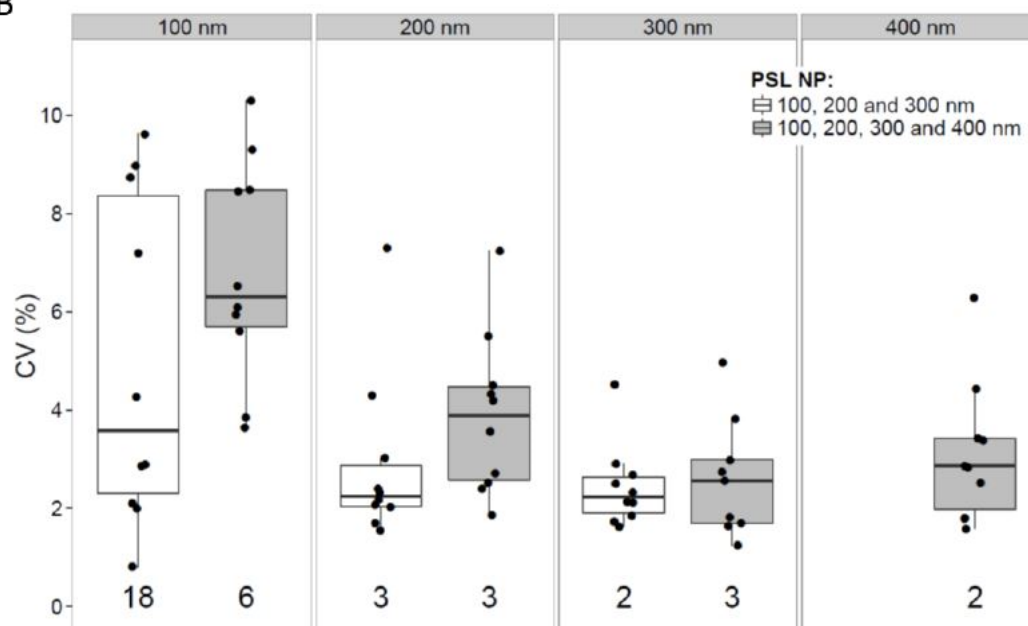


Figure 3.8: Confidence intervals of variance of monodisperse and multimodal samples. A: Scatterplots of median and interquartile range of % coefficient of variance for all laboratories for 30 nm to 600 nm nanoparticles. **B:** Scatterplots of median and interquartile range of % coefficient of variance for mixed population samples. Dashed boxplots represent 100, 200 and 300 nm mixed samples where solid boxplots are for 100, 200, 300, and 400 nm mixed samples. Graphs and plots jointly prepared by Malvern, TCD and EMPA Switzerland as part of the ILC 2 work.

3.2.2 Analysis of Nanoparticles in Simple and Complex Media Dispersions

Following refinement of the standard operating procedures and protocols, NTA was utilised to analyse a multitude of nano-objects in various dispersal media. Gold nanoboxes (AuNBs) of different sizes were analysed using NTA at prior to use and following interaction with the lung cancer cell line, A549 [192]. The zeta-potential of the nanoparticles, i.e. a surrogate parameter for the colloidal stability of the solution was also recorded, and laid out in Table 3.2. Following retrieval from cells after a 24 hour incubation, the AuNBs were analysed again using NTA, as shown in Figure 3.9

Similarly, bismuth ferrite (BFO) nanoparticles were characterised using NTA in various biological media (Figure 3.10) [193]. Here, uncoated particles (U-BFO-NP), and their PEGylated form (PEG-BFO-NP) were dispersed in DEPC treated water, Dulbecco's Modified Eagle Medium (DMEM), Roswell Park Memorial Institute 1640 (RPMI-1640) and Ham's F-12K (F12K), and their supplemented forms contain 10 % fetal bovine serum, using the procedures developed above, at physiological pH 7.4.

Finally, 15 nm SPIONs were characterised using NTA, again to assess their stability in complex dispersal media, with results depicted Figure 3.11. The size distribution plots show narrow, singular peaks indicating monodisperse stable samples. Analysis of these results via unpaired T-tests reveal that any increase in mode nanoparticle hydrodynamic diameter was not statistically significant, demonstrating that dispersal of the SPIONs in complex media will not affect the aggregation state of the particles.

Table 3.2: Hydrodynamic diameter and zeta potential of gold nanoboxes dispersed in water, as measured by NTA. AuNB₁ and AuNB₂ are uncoated gold nanoboxes in two different size ranges from [192].

Particle	Hydrodynamic diameter (in water, nm)	Zeta Potential (mV)
AuNB ₁	90 ± 57	-23.4 ± 8.3
AuNB ₂	105 ± 38	-57.0 ± 8.2

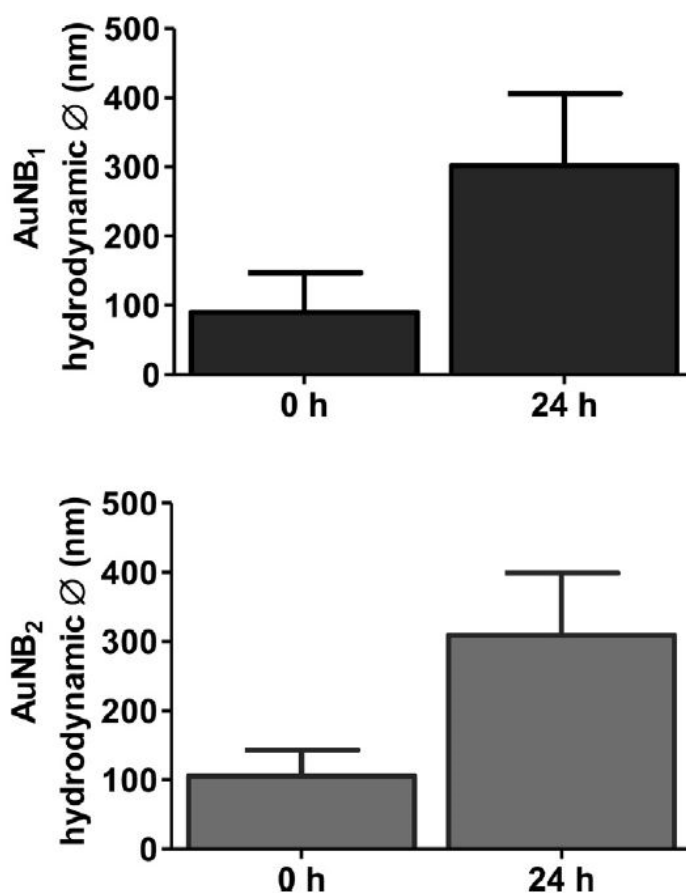


Figure 3.9: Changes in the hydrodynamic size (Ø) of uncoated AuNBs before (0 h) and after incubation with A549 cells for 24 h from [192]. Data are reported as mean ± standard deviation of NTA measurements. The stock AuNBs were vortexed and diluted in DEPC treated ultrapure DI water at a concentration of 1.3×10^{-11} M. Measurements were recorded at pH 7, at room temperature, at six depth positions, recording two videos of 60 seconds at position 1, and 2 by 30 seconds at each subsequent videos, applying a voltage of 24 V ($N = 6$).

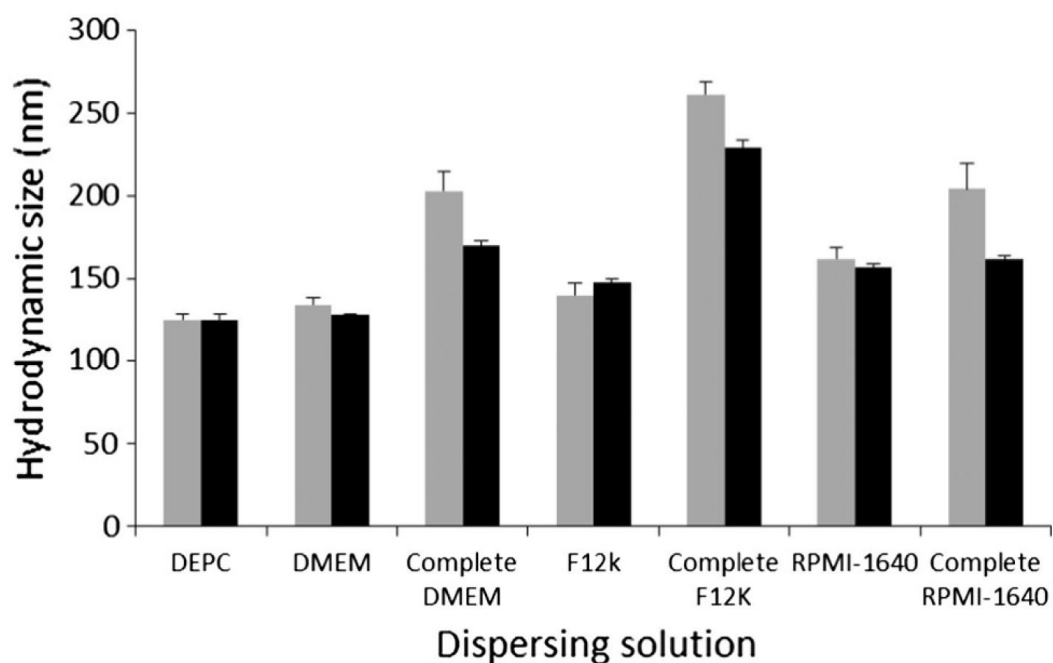


Figure 3.10: Nanoparticle tracking analysis of U-BFO-NP (light grey bars) and PEG-BFO-NP (dark grey bars) in biological media from [193]. Complete: media supplemented with 10% foetal bovine serum (FBS); DEPC: diethylpyrocarbonate; DMEM: Dulbecco's Modified Eagle Medium; F12k: Ham's F-12 K (Kaighn's) Medium; RPMI-1640: Roswell Park Memorial Institute 1640. All measurements were carried out three times at pH 7.4, followed by mean and standard deviation (SD) calculation ($N = 6$).

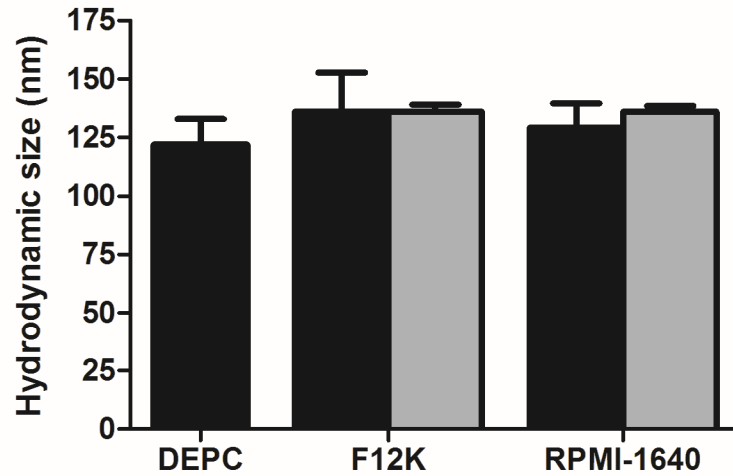
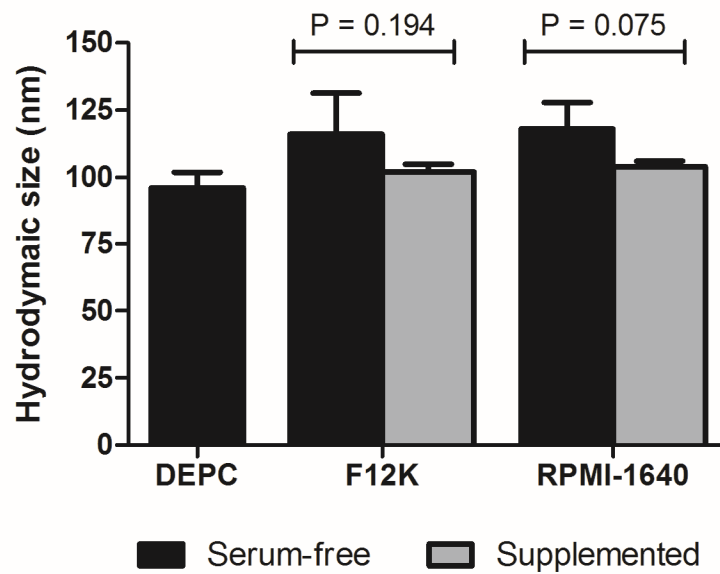
A**B**

Figure 3.11: NTA analysis of 15 nm SPIONs in complex dispersal media, including DEPC water, and complete (containing FBS) and serum free RPMI-1640 and F12K. A: Mean nanoparticle hydrodynamic diameter measured using NTA. B: Mode nanoparticle hydrodynamic diameter. Graphs represent mean $\pm$ standard error. Non-significant changes in mode hydrodynamic diameter determined by unpaired t Test (95 % confidence interval). Data represents mean $\pm$ SEM ($N = 6$).

3.2.3 NTA of Non-Typical Nanoparticle Samples

One of the key benefits of NTA is that it is not confined to the realms of standard nanoparticle characterisation. Refinement of the technique and improved analysis protocols has allowed us to analyse more biologically relevant samples such as virus particles and exosomes.

As part of a collaborative project with a European partner, I have enabled the investigation of the temperature-dependence associated size changes of the adenovirus capsid. The temperature control unit and scripting function allows for precise incremental increases in temperature which can be used to monitor virus size and concentration over a temperature range, in this case, from 20 °C to 45 °C. As shown in Figure 3.12A, at 26 °C a critical point is reached where the virus capsid begins to expand, and this is also apparent in Figure 3.12B where there is a noted decrease in virus particles per ml. As the capsid expands it reaches a critical point where the viral DNA is released. Under normal conditions this DNA is densely packed and allows the NanoSight platform to detect the viral particles. When the DNA begins to be released, it is accompanied by a steady loss in optical density, resulting in less particles being detected, and subsequent decrease in detectable viral concentration.

The interaction of the adenovirus with gold particles was also assessed using NTA (Figure 3.13). These gold particles were below the detection limit of NTA and as such all particle counts can be attributed to the adenovirus. Here it can be seen that gold causes a pronounced increase in the capsid size over the sample temperature range, with 32 °C being the critical temperature that initiates release of the viral DNA.

NTA is steadily becoming of the standard techniques in the characterisation of exosome samples. The technique can be used to analyse samples from various isolation protocols, from different fractions, or to provide data that can be correlated to disease state. A collaborative project with an Irish partner has enabled the investigation of the use of NTA to identify the size of exosomes isolated from different donors using different isolation protocols, as shown in Figure 3.14 A. As can be seen, the low density sucrose gradient fraction 5 results in the isolation of smaller exosomes. Similarly, Figure 3.14B demonstrates the effect of prolonged storage of exosomes isolated from patient samples, over a ten day period.

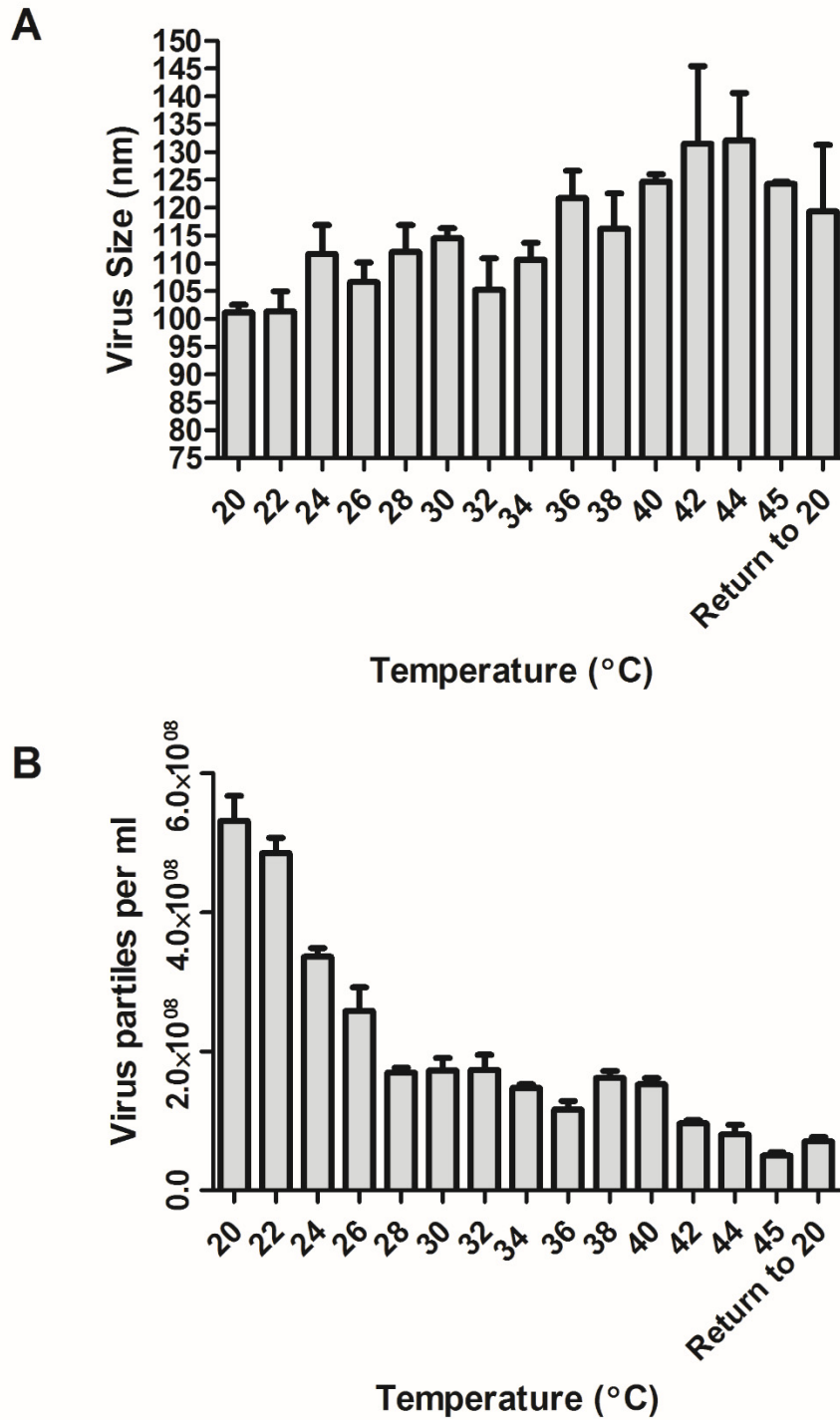


Figure 3.12: NTA of Adenovirus sample over a temperature range from 20-45 °C and back to 20 °C. A: Mode virus hydrodynamic size and B: Virus particles per mL. This collaborative work was established under the QualityNano project (FP7 infrastructure project) with Dr. S. Krol from Instituto Neurologico Italiano, Carlo Besta Italy. Data represents mean $\pm$ SEM ($N = 3$).

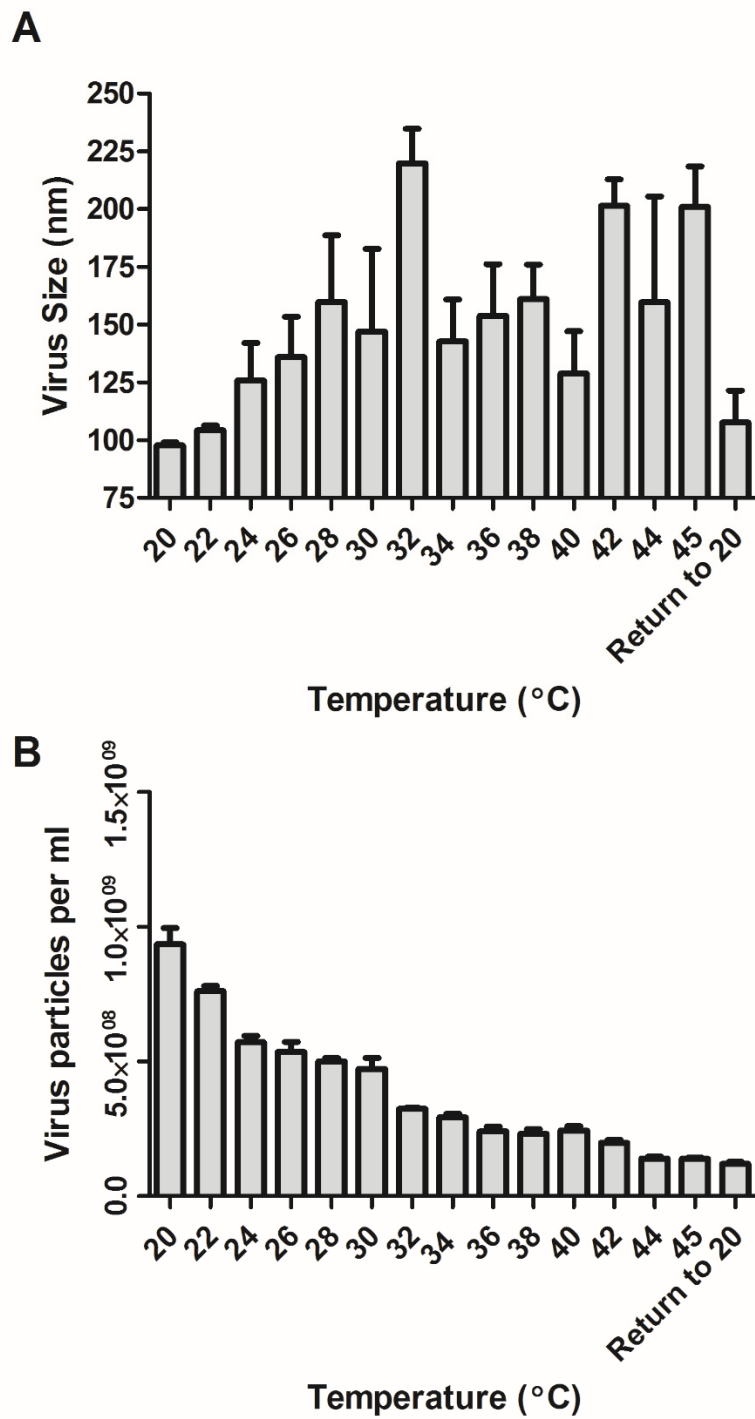


Figure 3.13: NTA of Adenovirus treated with gold nanoparticles over a temperature range from 20-45 °C and back to 20 °C. A: Mode virus hydrodynamic size and B: Virus particles per mL. This collaborative work was established under the QualityNano project (FP7 infrastructure project) with Dr. S. Krol from Instituto Neurologico Italiano, Carlo Besta Italy. Data represents mean $\pm$ SEM ($N = 3$).

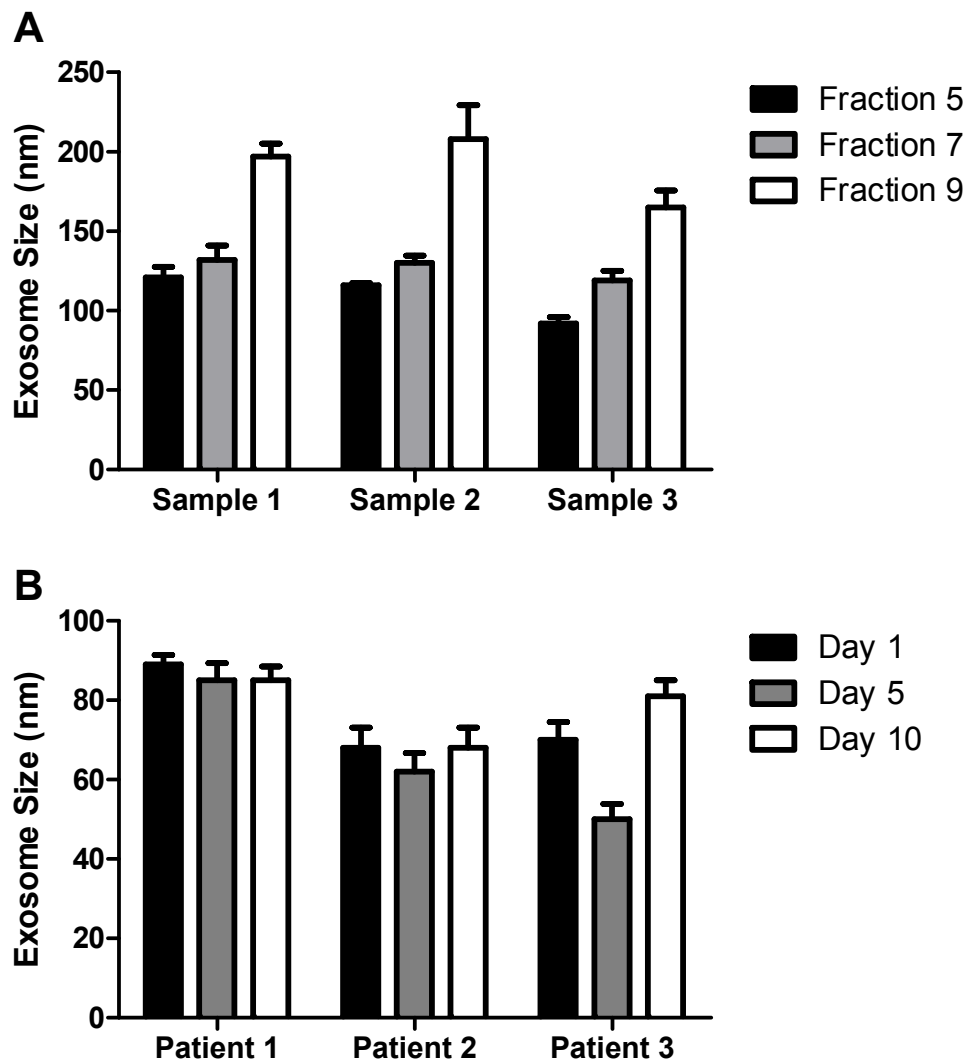


Figure 3.14: Applications of NTA in exosome characterisation. (A) NTA of exosomes isolated from human urine of 3 donors. Fraction 5: low density sucrose gradient fraction 5. Fraction 7: medium density sucrose gradient fraction 7. Fraction 9: high density sucrose gradient fraction 9. (B) Utilisation of NTA to investigate the stability of exosomes following isolation and storage. Data represents mean $\pm$ SEM ($N = 3$).

3.3 Discussion

Accurate and reproducible nanoparticle characterisation is of utmost importance in nanomedicine, and is critical in interpreting results from biological experiments. To this end, NTA provides a means to record, quantify and calculate the size and concentration of NPs in suspension.

Following multiple rounds of interlaboratory comparison, stringent SOPs and protocols have been generated to produce accurate and reproducible sizing data. Initial rounds of published work [172] have resulted in the lowering of the % CV for multiple nanoparticles as demonstrated in Table 3.1. As can be seen in round one, where participants utilised “in house” protocols, the % CV for a 100 nm polystyrene sample was 33.3 %. This was reduced to 3.1 % by round 4. Similarly, bi-, tri- and quadri-modal nanoparticle mixtures were analysed as shown in Figure 3.3 and Figure 3.6. The % CV was greatest for the 100 nm polystyrene particle in the quadri-modal sample, at 6.8 %, but this is still a dramatic improvement from round 1.

In ILC 1 the % CV for a 30 nm gold particle was recorded as 54.8 %. This was reduced to 3.94 % following refinement of the NTA protocol, and further reduced to 3.54 % when particles were dispersed in a 10 % solution of glycerol (Figure 3.5). The upper limits of NTA were also identified through the use of a 600 nm polystyrene sample, with an observed % CV of 4.01 % being recorded (Figure 3.8).

Pivotal to this reduction in % CV across the NTA operating range is the incorporation of the FTLA algorithm. As depicted in Figure 3.7, this algorithm results in the narrowing of size distribution peaks and improved curve fitting, allowing for the improved resolution of multimodal samples, particularly those demonstrated in Figure 3.6. In the case of the tri- and quadri-modal samples, the 100 nm polystyrene particle component had the greatest % CV at 4.94 and 6.82 %. Mode sizes of these particles were 110 and 115 nm, respectively. At the other end of the scale, in the tri-modal sample, the 300 nm particles were undersized to 291 nm, with the 400 nm component of the quadri-modal sample being calculated as 394 nm. This could potentially indicate that NTA oversizes the smallest component of a mixture, and undersizes the largest particles. Further refinements of the curve fitting and FTLA algorithm may improve this.

Following this, the refined and optimised NTA protocols allowed for the analysis of nano-objects in various dispersion media such as gold nanoboxes (Figure 3.9), bismuth ferrite nanoparticles (Figure 3.10), and iron oxide nanoparticles (Figure 3.11).

In the case of the gold nanoboxes, NTA was utilised in the characterisation of two AuNB variants by assessing their hydrodynamic size and zeta-potential prior to use. Similarly, NTA identify changes in the particle size due to protein absorption, following incubation with A549 cells. The hydrodynamic radius of the internalized AuNBs appeared significantly increased when retrieved from A549 cells exposed for 24 h, as compared to the reference, as-synthesized AuNBs (0 h). This indicated protein opsonisation, e.g. the adsorption of proteins such as serum onto the surfaces of uncoated AuNBs, possibly suggesting an immune response. These data were used to support further physiochemical characterisation such as helium ion microscopy [192].

The stability of BFO and SPIONs in complete and serum-free cell culture media was also assessed. The NTA results from these samples demonstrated the ability of the NPs to withstand aggregation in protein rich solutions, and thus may remain in their “nano” state *in vivo*.

Specifically in the case of BFO, PEGylated BFO withstood aggregation in supplemented media, compared to the uncoated particles. These data, along with cytotoxicity data measured through high content screening (Data Not Shown), suggest that for biological application of BFO, the NPs must be coated with a biologically compatible polymer in order to be stabilise the NP in suspension, when in contact with biological media [193]. PEGylated BFO preferentially withstood aggregation and protein absorption in the FBS supplemented RPMI-1640, followed by DMEM. For medium alone samples, i.e. in the absence of FBS, both uncoated and coated BFO were stable in DMEM, with minimal change in size compared to DEPC water. The ionic strength (IS) of the dispersant can play a role in the aggregation of nanoparticles [200], with the IS of DMEM and F12K being higher than that of RPMI [200-202]. Typically, this increased ionic strength would cause and increase in aggregation and subsequent particle size. However, in the case of coated and uncoated BFO, the media with the lowest IS resulted in the greatest increase in particle size. Protein absorption onto both BFO particles was greatest in the FBS containing F12K.

When a 15 nm iron oxide particle is dispersed in RPMI and F12K, and their supplemented equivalents, non-statistically significant increases in particle size and aggregation were detected. Narrow size distribution plots were obtained indicating that the particles were stable and monodisperse, not only in DEPC water but also cell culture medium. In the presence of serum, no protein absorption was detected as demonstrated by no changes in particle diameter. These results are in conflict with other published data where the size

or iron oxide particles increased when the dispersal media was changed from DI water to cells culture medium [203].

In general, these results show that different nanoparticles synthesised from different materials have different aggregatory properties in complex media under physiological conditions. To this end, particle characterisation will be required for specific particles under appropriate conditions to best understand cytotoxicity and biological responses, as both surface coating and chemistry, and material types are fundamental parameters.

Finally, biological nano-sized particles were analysed using NTA. Here we analysed the temperature dependent rupture of the Adenovirus capsid, and the size and stability of exosomes isolated and stored under different conditions.

In the case of adenovirus, the virus size and concentration was also assessed following the interaction of the virus with gold particles (Figure 3.12, Figure 3.13). NTA sizes correlate well with the literature that quote values between 70-100 nm [204]. As can be seen, the presence of gold particles increase the temperature at which the virus capsid ruptures and DNA is released. In the absence of gold particles, the capsid begins to rupture at 26 °C, increasing to 32 °C in the presence of gold. As shown in the above figures, as the temperature increase the virus capsid begins to swell until it ruptures at a critical temperature, release the DNA. This release in DNA causes a reduction in the optical density and subsequent diminished light scattering properties, and apparent reduction in particle concentration. One point of note is that it has been reported that NTA underestimates the viral count by approximately 15 %, compared to standard haemagglutination procedures [205].

Exosomes are micro-vesicles that were first discovered during the 1980s [206], and by convention they are between 30-120 nm in size [207] with this size range making them suitable for analysis *via* NTA. They are secreted by all cell types and are also found in body fluids such as blood and urine [207] and can be isolated using ultracentrifugation and density gradients. As demonstrated in Figure 3.14, the density of the sucrose gradient used in the isolation of exosomes yields exosomes of various sizes, with low sucrose gradients yielding the smallest exosomes. As NTA tracks single particles, the concentration of the exosome fractions can also be calculated and used to identify the optimal density gradient for a specific subset of exosomes of interest.

Similarly, NTA was used to investigate the stability of exosomes following isolation and storage. Figure 3.14B illustrates the effect of prolonged storage at -80 °C on the aggregation or degradation of exosome samples. As such, NTA has applications in the quality control and assessment of exosomes and results may contribute to the identification of disease states.

In summary, it is clear that by incorporation of stringent and shared guidelines and protocols, and the transfer of knowledge between experienced and novice NTA users, accurate and reproducible nanoparticle size and concentration data can be obtained. As demonstrated in the above, once SOPs are in place, the variation among users can be reduced to as low as 0.59 % for monodisperse samples. Subsequently, these protocols are instrumental in the characterisation of multiple nano-objects in varying dispersion media- a crucial feature in the interpretation of results obtained from biological testing.

Chapter 4

Heparin Functionalised Iron Oxide Nanoparticles

4 Heparin Functionalised Iron Oxide Nanoparticles

4.1 Introduction

Superparamagnetic iron oxide nanoparticles (SPIONs) are some of the most promising nanomaterials for applications in biology and medicine. Due to their magnetic properties they have applications in sensors and as dual agent therapeutic diagnostic systems (theranostics) for magnetic resonance imaging. Variations in their size can result in vastly different biodistribution and toxicological profiles, with smaller (less than 10 nm) particles entering and clearing the liver at faster rates than their larger counterparts [208]. Similarly, 40 nm PEGylated SPIONs have been shown to accumulate to a higher extent in solid tumours due to their size dependent extravasation and capture by macrophages [209]. It has also been shown that smaller nanoparticles have longer circulation times in blood than their larger variants [210].

Due to the bleeding risks associated with anti-coagulant treatment [211], heparin was chosen as a candidate drug for conjugation to nanoparticles. It is anticipated that conjugation of heparin to a SPIONs may allow for improved biodistribution and pharmacokinetics providing a possibility for less frequent monitoring. Similarly, the magnetic nature of the SPIONs can allow for targeting them to discrete regions of the body [212], particularly in the prevention of pulmonary embolisms in DVT patients [213]. Heparin's large size can also act as a model to identify the size limit of molecules to conjugate effectively to nanoparticles, and due to the sensitivity of a number of laboratory based assays to the presence of heparin, any changes in activity can be measured.

Previous work in this area has shown some inconclusive results about the efficacy of heparin nanocomposites. Heparin conjugated gold and silver particles have been shown to have antiangiogenic and anticoagulant properties [143, 214] and heparin coated quantum dots retain their ability to bind to macrophage receptors [75], but also heparin conjugated carbon nanocapsules have been shown to prolong the APTT in *ex vivo* experiments to a minimal non-biologically relevant level, but not *in vitro* [152]. This suggests that the anticoagulant activity of heparin nanocomposites may be nanomaterial-specific, or is dependent on the size of the nanomaterials.

In summary, in Chapter 4, we aimed to develop a functional iron oxide based nanoparticle that retains the anticoagulant activity of heparin. This was carried out by using a systematic approach detailed below:

1. Screening a number of iron oxide nanoparticles for their effect on coagulation to find a suitable candidate for functionalisation
2. Demonstrating the chosen particle's amenability to be functionalised with biomolecules
3. Functionalising and characterising the selected particle type with heparin using different methods
4. Investigating the activity of the functionalised particles using standard coagulation assays

4.2 Results

4.2.1 APTT, PT and TT Screening of SPIONs and Reagents

APTT, PT and TT assays were carried out on 5, 7, 9, 15 and 22 nm SPIONs, whereby 5 μ L of the nanoparticle suspension was added to pooled normal plasma. Results revealed that the clotting times were not prolonged and were in range of the untreated control as demonstrated in Figure 4.1. These results suggest that all NP candidates are suitably biologically inert carriers (in terms of their effect on coagulation) for further coagulation targeting applications. The 15 nm particle was selected for functionalisation due to the improved stability (NTA data available in Chapter 3 Figure 3.11) and availability compared to the other candidates.

In parallel, a number of the functionalisation reagents were assessed using the APTT and TT assays. This was to identify, whether remnants of the functionalisation process may have effects on coagulation. As illustrated in Figure 4.2, the results indicate that clot formation times were within their respective normal ranges. An increase in the clot formation time in the TT assay for APTES was observed, which nonetheless was deemed not statistically significant, based on an unpaired t-test ($P=0.2226$, $N = 4$). Unfractionated heparin was used as a positive control and both the APTT and TT were prolonged as expected. The assay was manually stopped after 5 minutes.

4.2.2 Proof of Functionalisation Technique using Fluorescent Antibody

An IgG molecule coupled to the fluorescent tag AlexaFluor 488 was used as proof of concept technique for the functionalisation procedure. The fluorescent signal of the anti-HER2 AlexaFluor 488 antibody was measured using flow cytometry. Side scatter and FITC thresholding was utilised to distinguish between uncoupled nanoparticles and their antibody-conjugated counterparts, as depicted in Figure 4.3 below. An increased fluorescence emission and narrow distribution is detected for the nanoparticles conjugated to the fluorescent antibody using EDC coupling, compared to the sample without EDC.

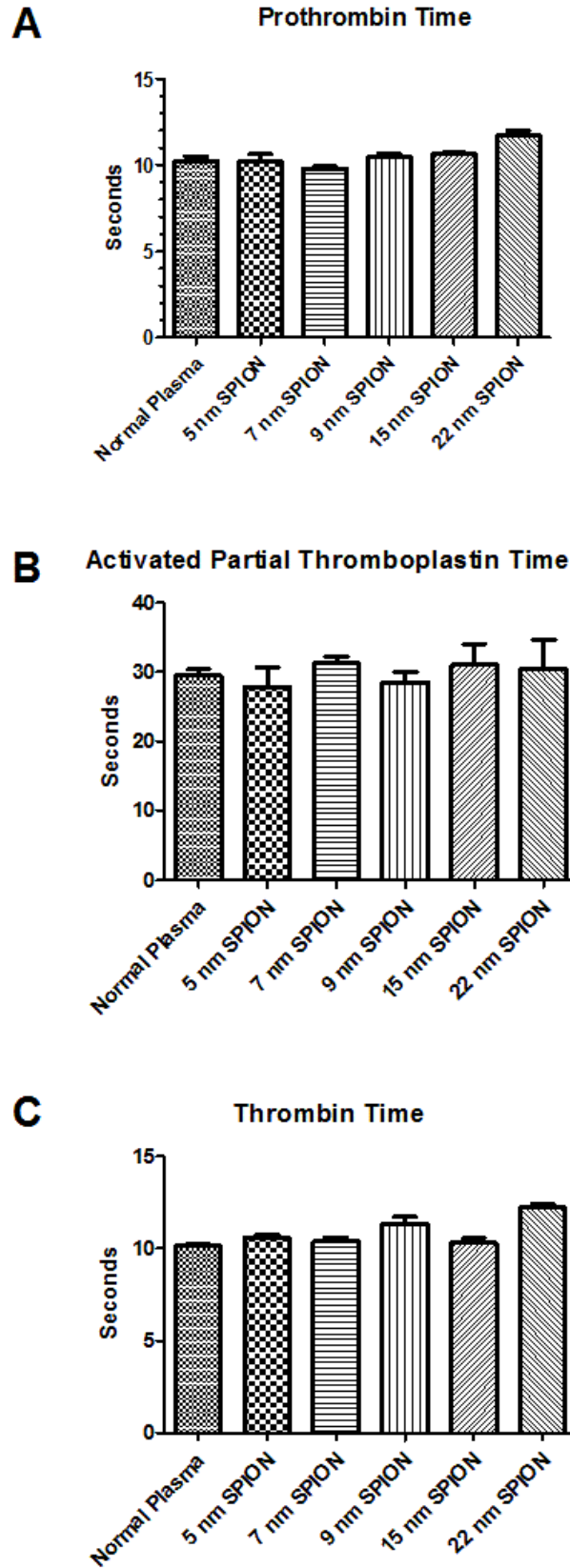


Figure 4.1: Prothrombin Time (PT), Activated Partial Thromboplastin Time (APTT) and Thrombin Time (TT) screening of 5-22 nm bare unmodified SPIONs to assess their suitability as a carrier particle for heparin ($N = 4$).

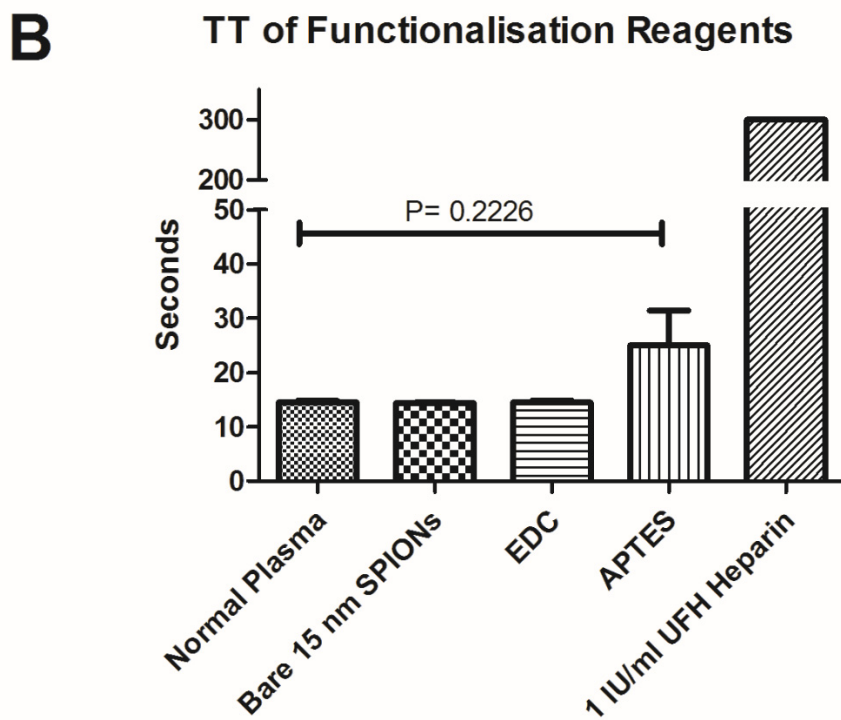
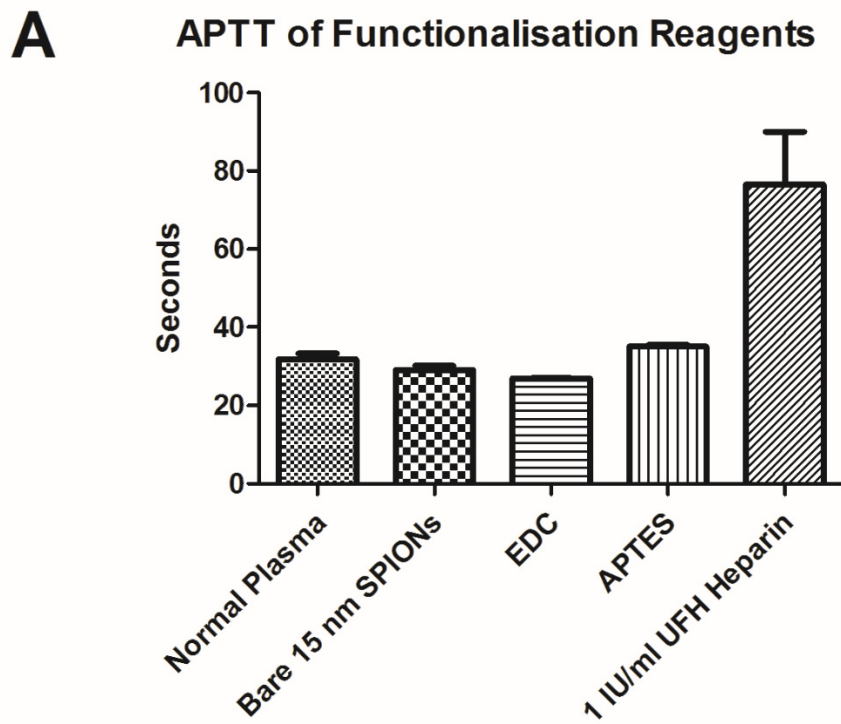


Figure 4.2: APTT and TT of functionalisation reagents to assess the effect of reaction by-products on coagulation. A non-statistically significant prolongation of the TT was observed for APTES with unfractionated heparin prolonging both the APTT and TT, as expected ($P = 0.2226$, $N = 4$).

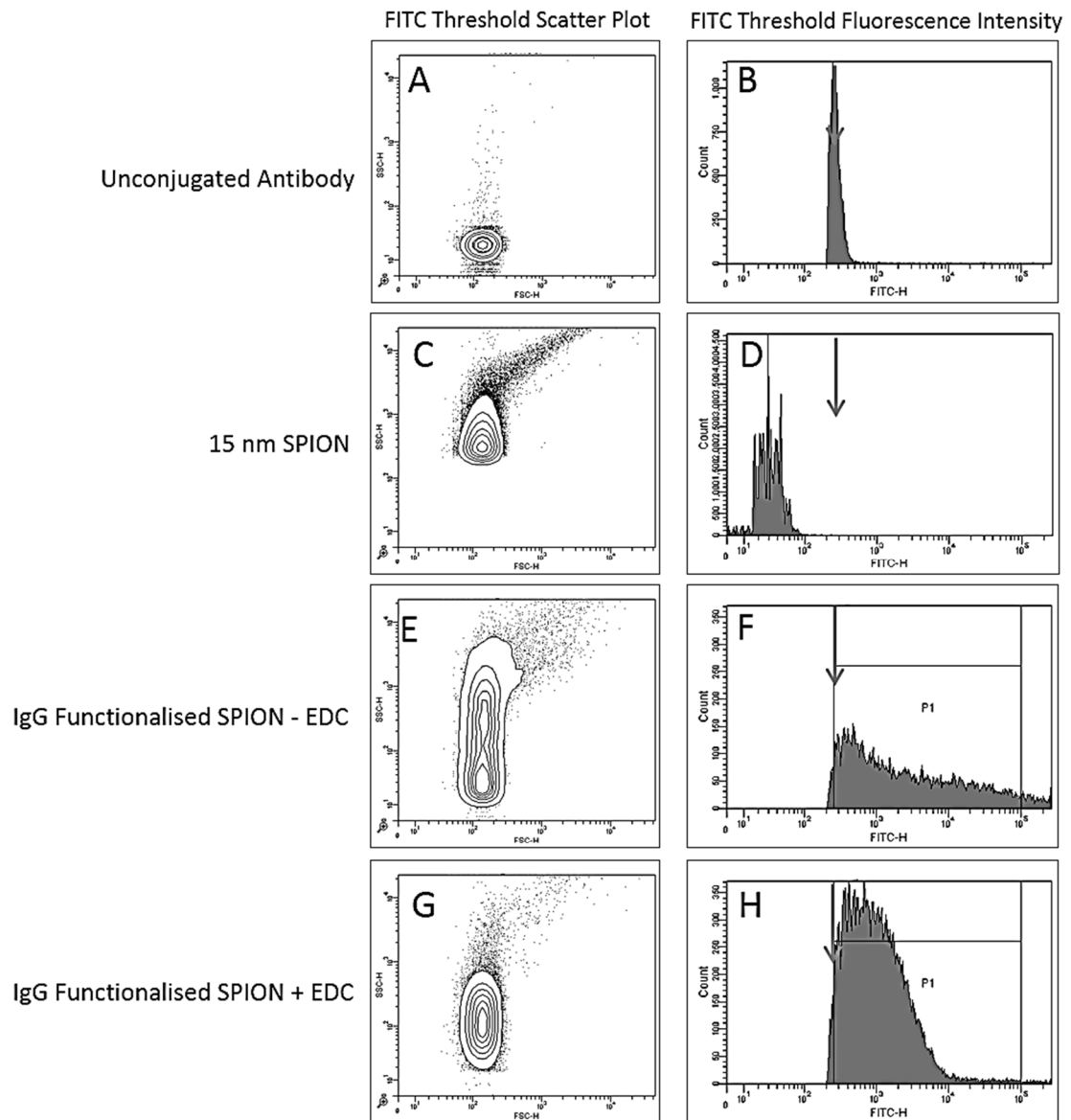


Figure 4.3: Flow cytometric analysis of 15 nm SPIONs functionalised with AlexaFluor 488 tagged IgG antibody. A and B: FITC threshold scatter plot and fluorescence intensity of unconjugated IgG; C and D: Side scatter threshold scatter plot and fluorescence intensity of unconjugated 15 nm SPIONs; E and F: FITC threshold scatter plot and fluorescence intensity of IgG functionalised 15 nm SPIONs without EDC coupling; G and H: FITC threshold scatter plot and fluorescence intensity of IgG functionalised 15 nm SPIONs with EDC coupling. Black arrows indicate maximum FITC-H for unconjugated AlexaFluor 488 antibody. IgG: Alexa Fluor 488 conjugated anti-HER2 antibody, EDC: 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide, FITC: Fluorescein isothiocyanate.

4.2.3 Functionalisation and Characterisation of SPION with Heparin

The 15 nm SPION was functionalised using unfractionated (UFH) and low molecular weight (LMWH) heparin using protocols listed in the materials and methods section. Briefly, two main functionalisation routes were chosen: (1) formation of an amide bond between the carboxylic acid groups of the SPION and amine groups of heparin; and (2) introduction of amine groups to the SPION and formation of amide bonds between the new amine groups and the carboxyl groups of heparin. A third alternative route, where the SPIONs were coated with gelatine prior to functionalisation was also used. Functionalised SPIONs were then characterised using NTA, using the protocols and procedures established, optimised and described in Chapter 3. The various functionalisation routes are depicted in Figure 4.4.

This characterisation revealed information pertaining to the NP size distribution and stability as shown in Figure 4.5 for Route 1 synthesis. Functionalisation of the SPION, both with and without EDC resulted in the formation of aggregates. This was more evident for the low molecular weight heparin samples. Size distribution plots for all functionalisation routes are provided in Figure 4.6.

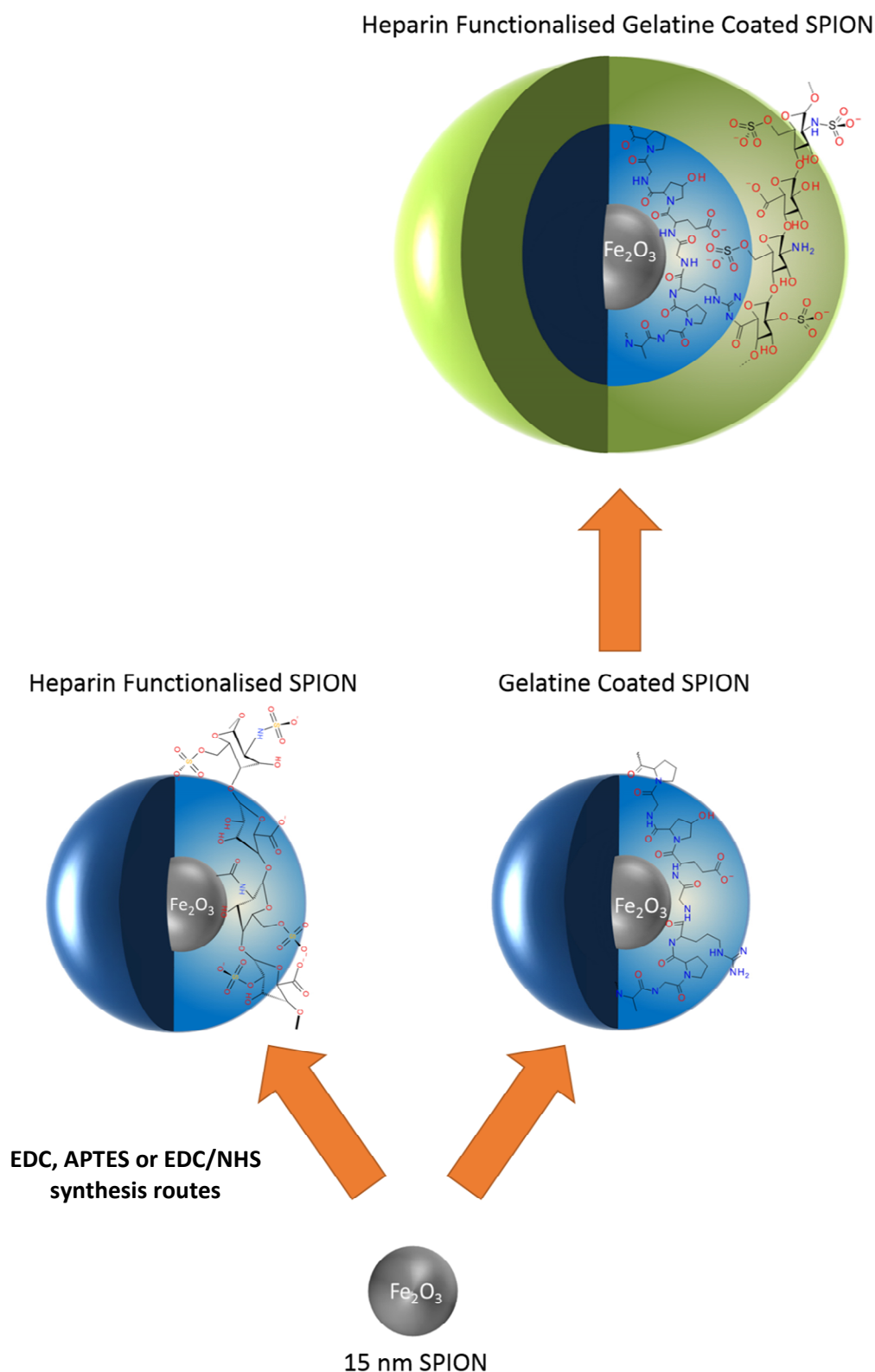


Figure 4.4: Schematic of functionalised SPIONs. The bare SPION can be functionalised through carbodiimide coupling with APTES and EDC/NHS and their variants to yield heparin functionalised nanomaterials. Similarly the SPION can be coated first with gelatine and then functionalised with heparin using similar procedures. It was envisioned that gelatine would provide a scaffold of functional groups to aid in the conjugation of heparin to the SPION.

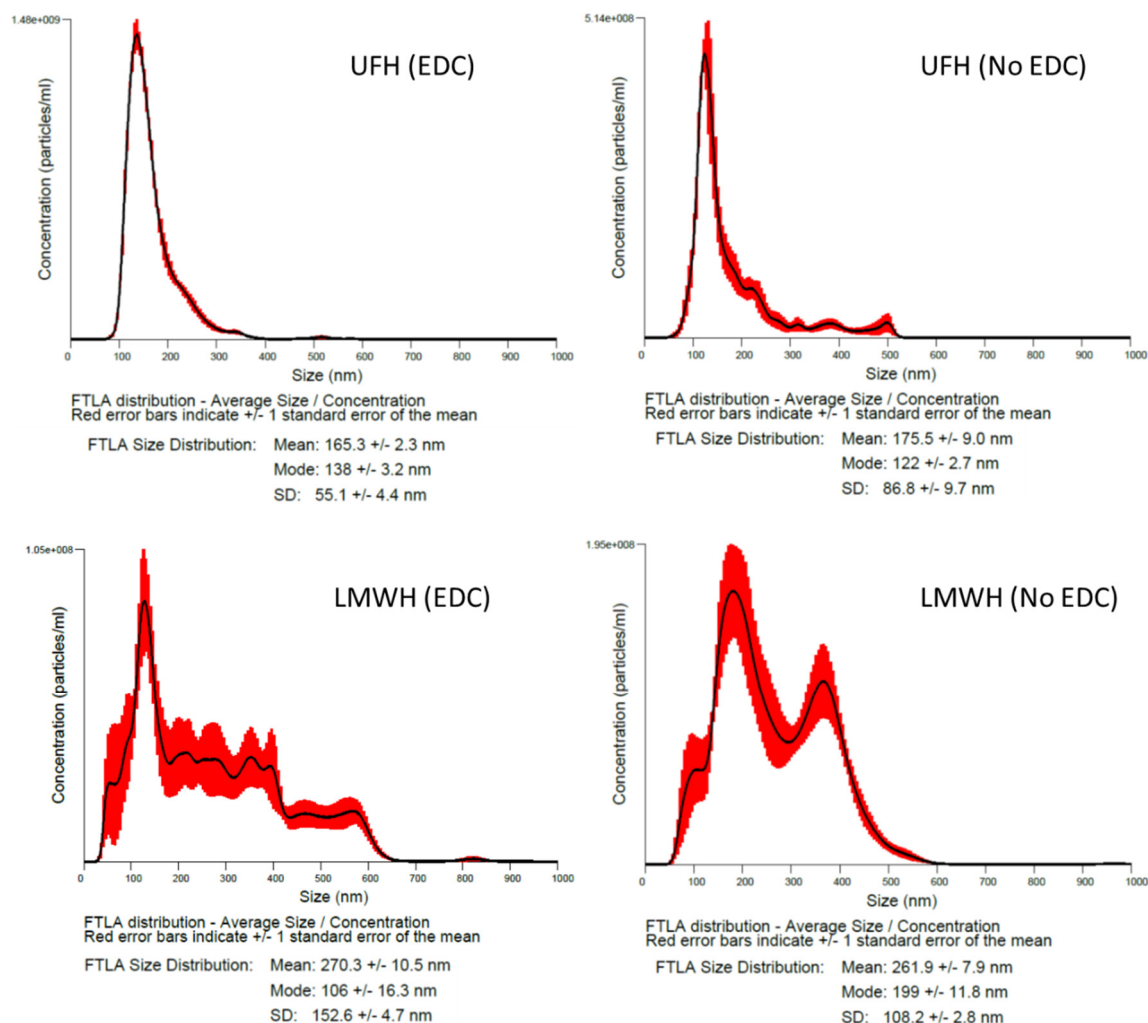


Figure 4.5: NTA analysis of 15 nm SPIONs functionalised with unfractionated heparin (UFH) and low molecular weight heparin (LMWH) using EDC coupling (Route 1). An increase in mode particle hydrodynamic size compared to bare SPIONs when heparin is present indicates that heparin is bound to the surface of the SPION. A notable degree of aggregation is also detected in the LMWH samples as demonstrated by the multiple peaks and large degree of error. Data represents results for 6 by 60 second videos with red error bars indicating ± 1 SEM.

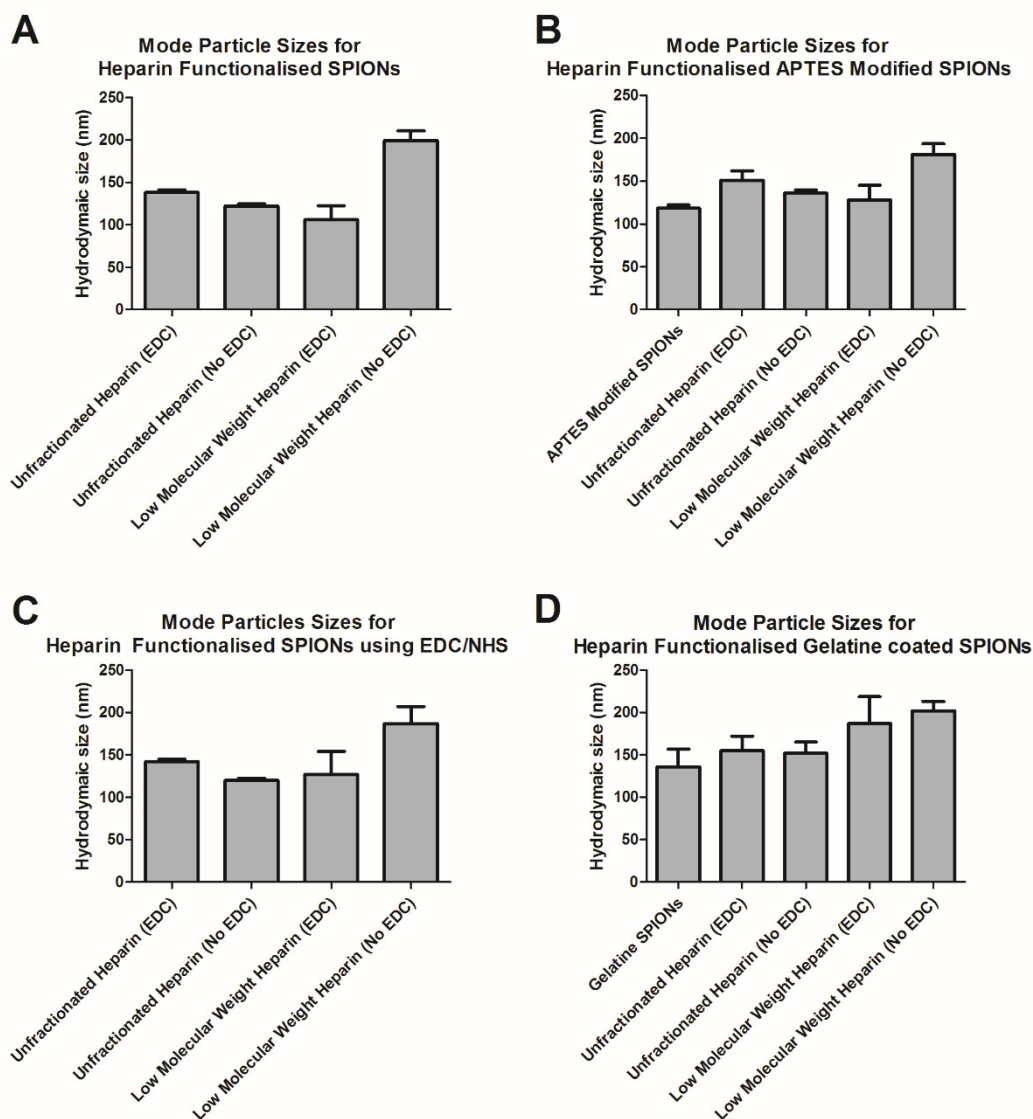


Figure 4.6: NTA size distribution graphs of mode particle size for heparin functionalised SPIONs, using either unfractionated or low molecular weight heparin. A: Heparin functionalised SPIONs using EDC alone. B: Heparin functionalisation using APTES modified SPIONs. C: Heparin functionalisation using EDC and NHS. D: Heparin conjugation to gelatine coated SPIONs using EDC coupling. Data represents mode particle size $\pm$ SEM ($N=6$).

4.2.4 Activity of Heparin Functionalised 15 nm SPIONs

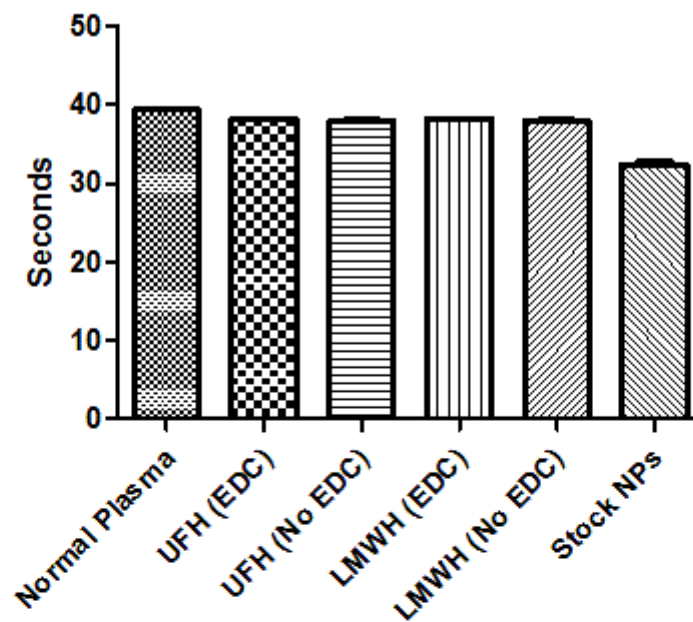
Heparin functionalised NPs were assessed for activity based on APTT and TT assays, two assays that are highly sensitive to heparin. For each assay, 5 μ L of the functionalised SPIONs were added to pooled normal plasma. As shown in Figure 4.7, the activity of heparin was diminished following conjugation to the NP. Similarly, no prolongation in the APTT or TT was detected using the APTES functionalisation procedure (Figure 4.8).

Subsequently, the functionalised SPION suspensions were concentrated by centrifugation. Here, the volume of the sample was reduced by half, thus effectively doubling the concentration. Following this, an APTT assay was carried out again, using 5 and 10 μ L of the samples Figure 4.9. Similarly to the results shown in Figure 4.7, no prolongation in the time to clot formation was observed. In fact, a shortening of the APTT was observed. However these results were still within the normal range.

An activating reagent N-Hydroxysuccinimide (NHS) was also used to aid conjugation. NHS is used as an activating agent for carboxylic acid groups in carbodiimide coupling, particularly when using EDC [215]. Again, there was no heparin activity detected by means of an APTT, as shown in Figure 4.10A, with the exception of the unfractionated heparin without EDC. This prolongation is thought to be due to the electrostatic binding of heparin to the surface of the particle as incubation of the samples in PBS for 1 hour at 37 °C resulted in uncoupling of non-conjugated heparin, as shown in Figure 4.10B.

Finally, heparin was conjugated to gelatine coated SPIONs. It was anticipated that gelatine would act as a scaffold to provide extra functional groups for the conjugation of heparin to the SPION. As demonstrated in Figure 4.11A and B, the APTT and TT was prolonged for the heparin conjugates but not for the SPIONs coated in gelatine alone. To ensure that the heparin was covalently bound through the formation of amide bonds, the particles were suspended in PBS, as before. APTT and TT assays were subsequently carried out, with the prolonged times until clot formation returning to within the normal range, as illustrated in Figure 4.11C and D.

A APTT of Heparin Functionalised 15 nm SPIONs



B TT of Heparin Functionalised 15 nm SPIONs

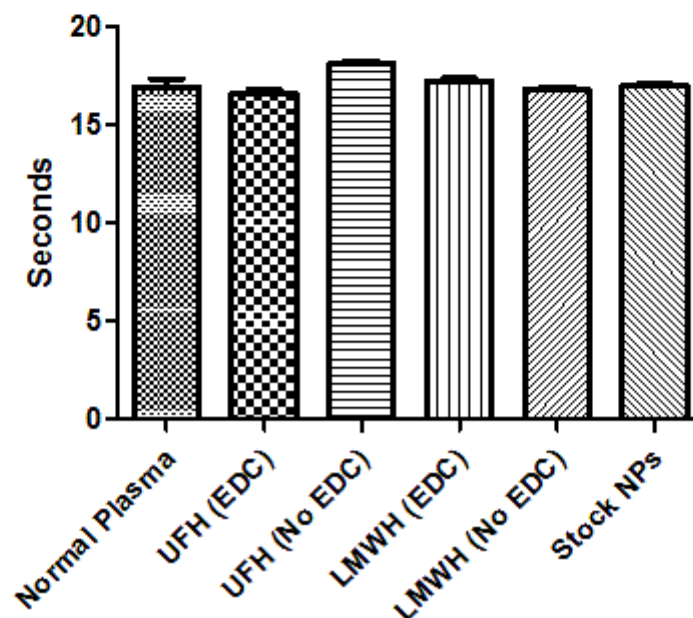
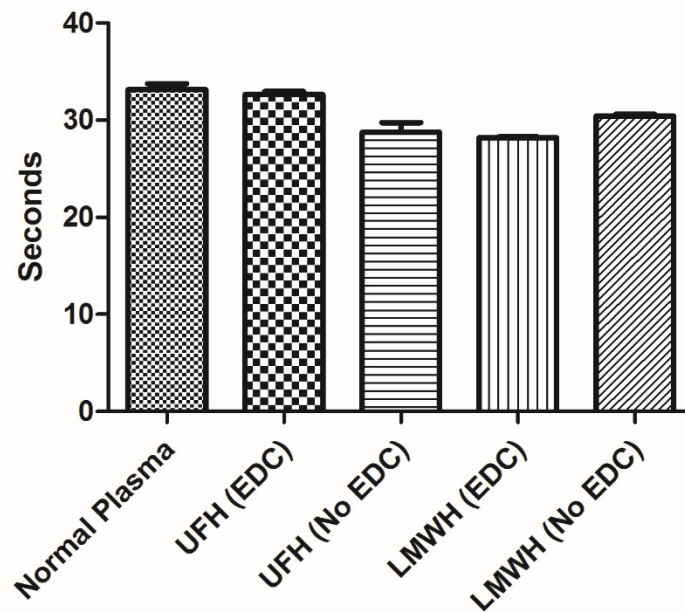


Figure 4.7: APTT and TT of heparin functionalised SPIONs. UFH (EDC) - EDC conjugated unfractionated heparin; UFH (No EDC) - non-conjugated unfractionated heparin; LMWH (EDC) - EDC conjugated low molecular weight heparin; LMWH (No EDC) - non-conjugated low molecular weight heparin. All clotting times are within the normal range indicating no anticoagulant effect associated with the heparin functionalised SPIONs. Data represents mean $\pm$ SEM ($N = 4$).

A APTT of Heparin Functionalised APTES Modified 15 nm SPIONs



B TT of Heparin Functionalised APTES Modified 15 nm SPIONs

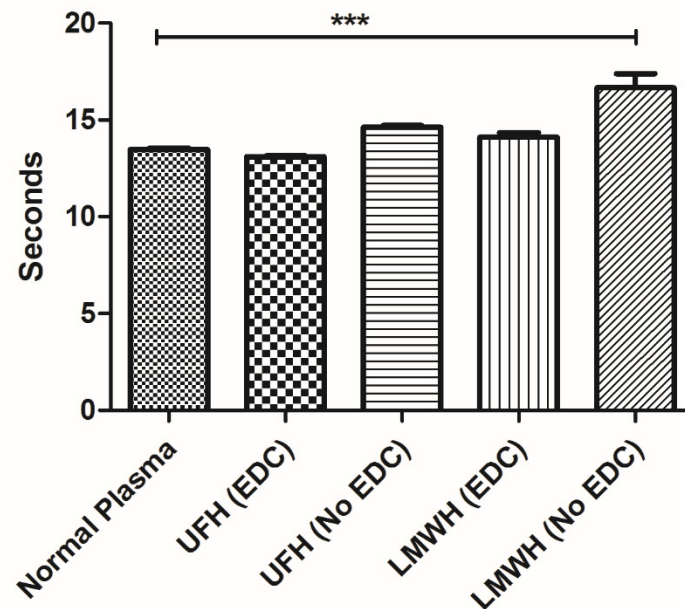
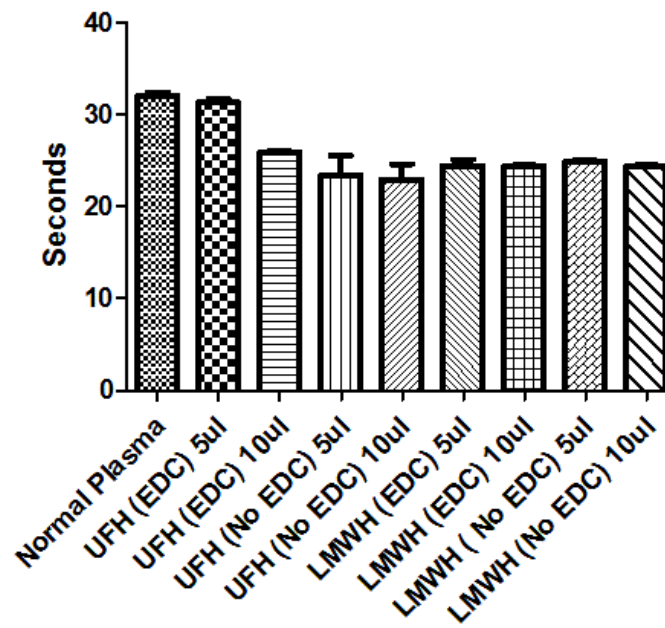


Figure 4.8: APTT and TT of heparin functionalised APTES modified SPIONs. UFH (EDC) - EDC conjugated unfractionated heparin; UFH (No EDC) - non-conjugated unfractionated heparin; LMWH (EDC) - EDC conjugated low molecular weight heparin; LMWH (No EDC) - non-conjugated low molecular weight heparin. No anticoagulant effect is observed as clotting times are within their respective normal ranges. Data represents mean $\pm$ SEM ($N = 3$). *** $P < 0.001$ vs normal plasma (ANOVA with Dunnett's post-test).

**A APTT of Heparin Functionalised NPs:
Concentrated**



**B TT of Heparin Functionalised NPs:
Concentrated**

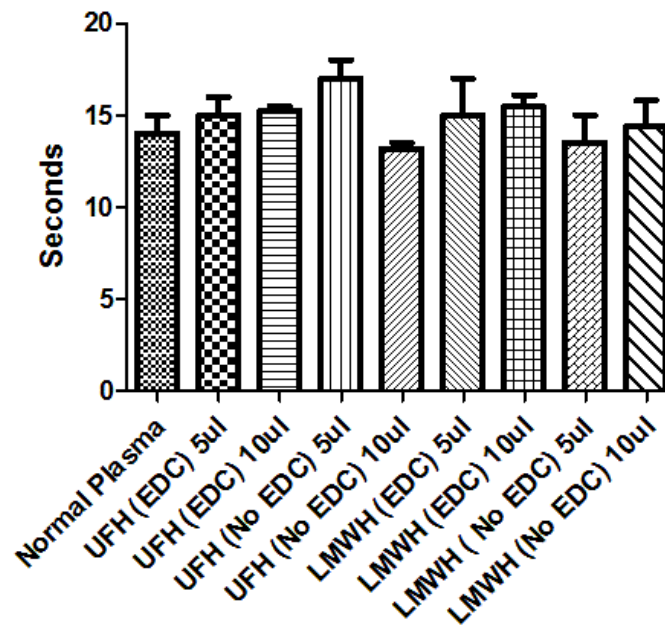
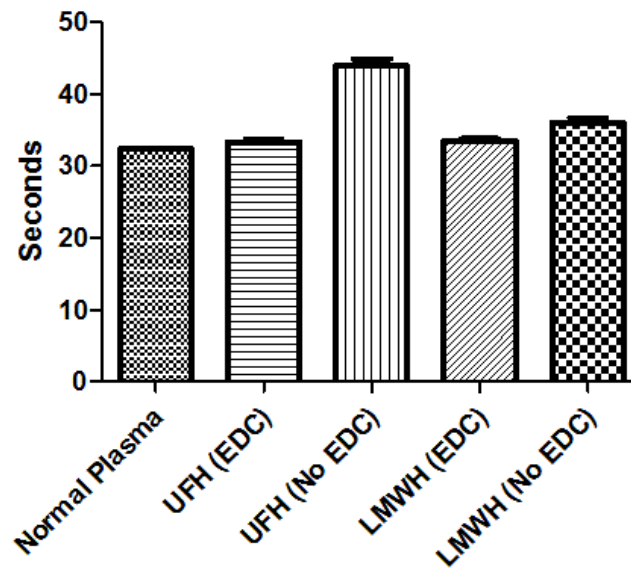


Figure 4.9: APTT (A) and TT (B) of Heparin Functionalised SPIONs following concentration by centrifugation. The volume of dispersant was reduced to half of the original thereby increase the concentration of nanoparticles twofold. The volume of SPIONs added to normal plasma was also increased. All results are still within the normal range. UFH (EDC) - EDC conjugated unfractionated heparin; UFH (No EDC) - non-conjugated unfractionated heparin; LMWH (EDC) - EDC conjugated low molecular weight heparin; LMWH (No EDC) - non-conjugated low molecular weight heparin. Data represents mean $\pm$ SEM ($N = 3$).

A APTT of EDC/NHS functionalised 15 nm SPIONs



B APTT of EDC/NHS functionalised 15 nm SPIONs after PBS incubation

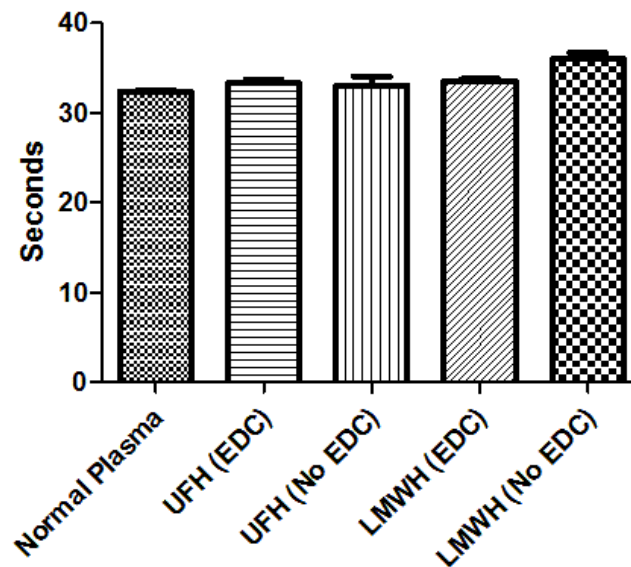


Figure 4.10: APTT of heparin functionalised 15 nm SPIONs using the NHS activator. A: APTT following synthesis. B: APTT following incubation of SPIONs in PBS for 1 hour. UFH (EDC) - EDC conjugated unfractionated heparin; UFH (No EDC) - non-conjugated unfractionated heparin; LMWH (EDC) - EDC conjugated low molecular weight heparin; LMWH (No EDC) - non-conjugated low molecular weight heparin. Data represents mean $\pm$ SEM ($N = 3$).

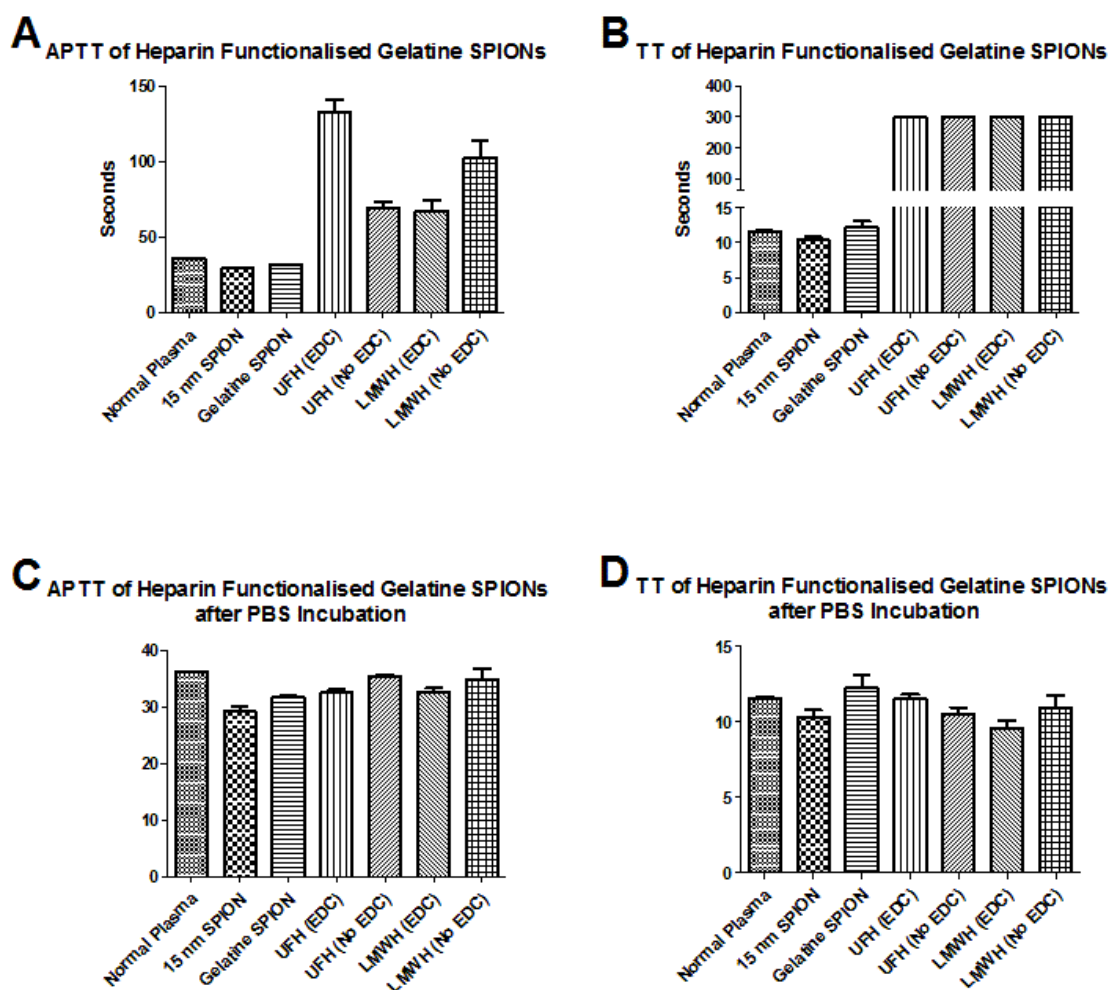


Figure 4.11: Use of gelatine as a scaffold for conjugation of heparin to 15 nm SPIONs. **A** and **B**: APTT and TT assays of as synthesised NPs. **C** and **D**: APTT and TT results of functionalised NPs following incubation in PBS to remove non-covalently bound materials. UFH (EDC) - EDC conjugated unfractionated heparin; UFH (No EDC) - non-conjugated unfractionated heparin; LMWH (EDC) - EDC conjugated low molecular weight heparin; LMWH (No EDC) - non-conjugated low molecular weight heparin. Data represents mean $\pm$ SEM ($N = 3$).

4.3 Discussion

Initially, 5, 7, 9, 15 and 22 nm bare (non-functionalised) SPIONs were screened for their effects in three frequently used clinical coagulation tests, namely activated partial thromboplastin time (APTT), prothrombin time (PT) and thrombin time (TT), as demonstrated in Figure 4.1. These assays reveal the effect of NPs on the intrinsic and extrinsic pathways, and the conversion of fibrinogen to fibrin, respectively. From these tests, it was demonstrated that all NPs had no effect on haemostasis. Previously it has been shown that non-functionalised 22 nm iron oxide nanoparticles caused a statistically significant prolongation of PT and APTT in Sprague-Dawley rats 30 days after exposure [216]. An increase in APTT from 17.46 seconds to 21.13 seconds for rats 30 days post exposure to the nanoparticle was recorded [216]. However, with a reference range of 16-23 seconds [217], this statistical significance does not necessarily equate to biological relevance. Similarly for humans, an APTT and PT reading of between 10-14 and 30-40 seconds, respectively, would be considered within the normal range [15].

In order to demonstrate the amenability of the chosen nanoparticles to functionalisation, a fluorescently tagged antibody was chosen for a proof of concept study. As shown in Figure 4.3, the fluorescent properties of the IgG molecule were retained following EDC coupling to the SPIONs. This fluorescence was diminished when no EDC coupling was used, and yielded a wider distribution with a lower count rate for concentration controlled samples.

As stated previously, due to the bleeding risks associated with anticoagulant treatment and the need for frequent monitoring [211], heparin was chosen as a candidate drug for conjugation to nanoparticles. Characterisation of heparin functionalised SPIONs was also carried out using NTA, as shown in Figure 4.5. In a similar manner in which serum proteins can increase the hydrodynamic size of NPs, functionalisation of NPs with heparin has also result in an increase in NP size. Functionalisation of the NPs with LWMH, with and without EDC, resulted in the largest increase in mean hydrodynamic size. This suggests that in both cases heparin has been incorporated onto the surface of the particle, either covalently using EDC coupling, or through hydrogen and electrostatic bonding, leading to the formation of a corona when EDC is not used. The formation of large aggregates in the low molecular weight heparin samples can also be detected from the size distribution graphs Figure 4.5. In contrast, the use of unfractionated heparin results in the formation of more uniform NPs with a narrower size distribution for both EDC and non-EDC coupled samples Figure 4.5. The increase in modal particle size for the unfractionated heparin-EDC conjugated sample, compared to the non-EDC coupled

NPs suggests that amide bonds have been potentially formed between the amine moieties of the heparin and the terminal carboxylic acid groups of the SPIONs (Figure 4.5)

Assessment of the functionalised NPs using the highly sensitive APTT and TT assays yielded results indicating that upon binding to NPs, heparin loses its biological activity (Figure 4.7). All NPs resulted in clotting times within normal range and in line with the untreated normal reference plasma. It is suggested that conjugation of heparin to the SPIONs results in a conformational change that results in heparin no longer being able to form a complex with antithrombin. Heparin has been conjugated to a number of nanomaterials for applications in nanomedicine, including cellular imaging [75] and angiogenesis inhibition [218], but typically not for exploiting its anti-coagulant properties. Previously, only non-significant changes in clotting time have been reported for heparin functionalised NPs [152]. In the study by Tang *et al.*, [152], heparin was conjugated to carbon nanocapsules and the activity was tested *in vitro* and *ex vivo* using the APTT assay. Results of these assays show that the heparin conjugated nanocapsules did not prolong the APTT *in vitro* [152], indicating that the heparin had lost its activity. Statistically significant prolongation was observed *ex vivo*, but this was still within normal range for mice [152]. These results are consistent with the results obtained to date, possibly suggesting that further avenues must be explored if heparin is to be conjugated to nanomaterials and retain its bioactive anticoagulant properties. Prior modification of the SPIONs with APTES to provide another functionalisation route yielded similar results (Figure 4.8). This again lends credence to the suggestion that heparin loses its biological activity when conjugated to NPs.

The effects of the functionalisation reagents were also examined through the use of the APTT and TT assays. As illustrated in Figure 4.2, neither EDC nor APTES caused an alteration in the clot forming ability of plasma. A prolongation in the TT was observed for APTES, but this was deemed not statistically significant. The variation of the results may be due to the silanes binding to the metallic surface of the magnetic beads that are used in the assay, or binding to the plastic of the micro-cuvettes. This may act to form a complex that has the ability to bind low molecular peptides present in the plasma [219], thus altering the normal clotting pathway. Further studies implementing mass spectrometry or advanced microscopy would be required to prove such possibility.

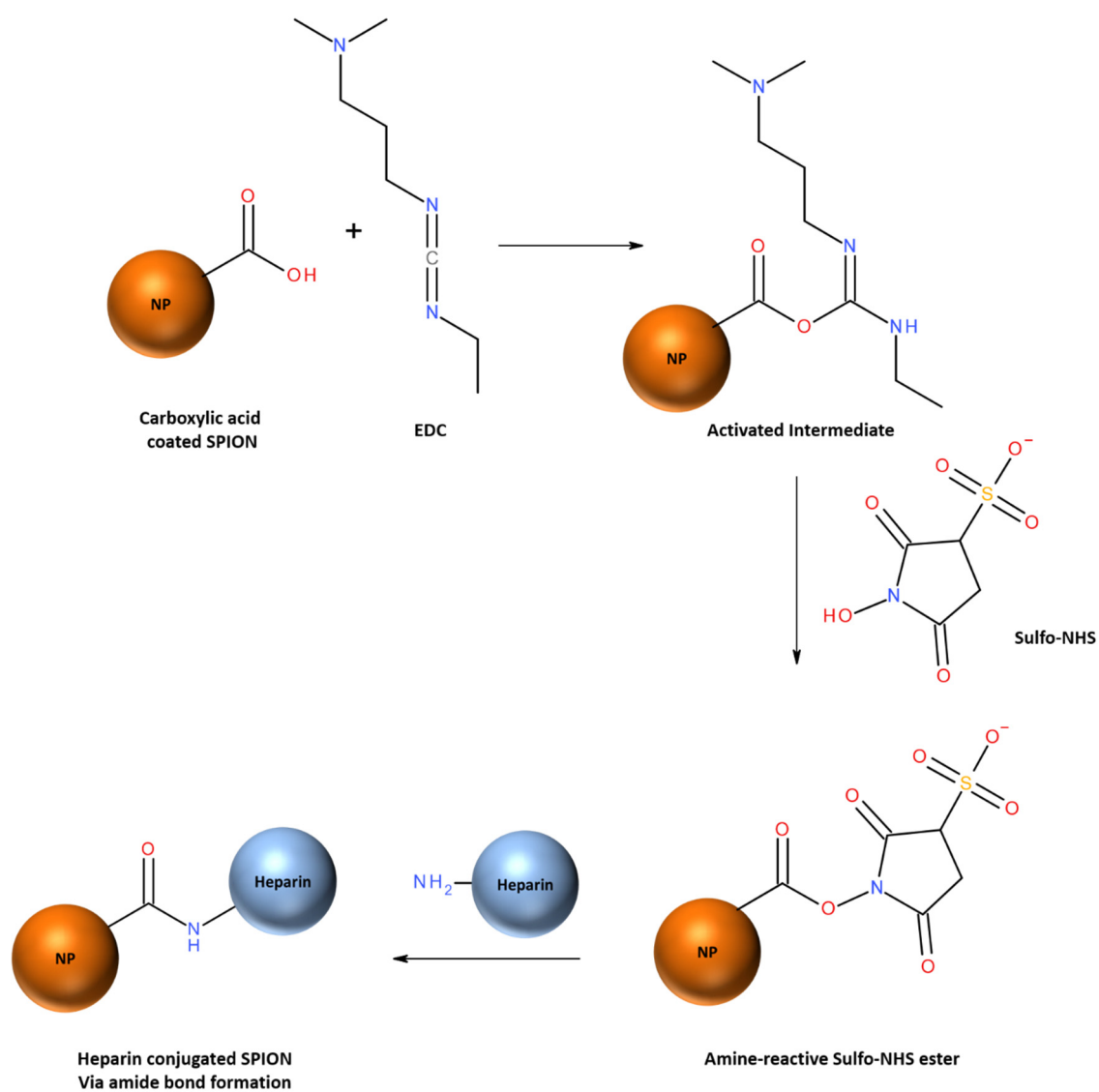


Figure 4.12: Schematic of formation of amide bond using NHS. The carboxylic acid group of the SPION is activated to an activated intermediate using EDC. This then reacts with the hydroxyl group of NHS to form the amine-reactive sulfo-NHS ester. This ester then reacts with amine groups in heparin to form the heparin-conjugated SPIONs.

To identify if the heparin was only present at a low concentration in the as-synthesised samples, the NPs suspensions were concentrated by centrifugation and resuspension in half the volume of DI water. This effectively doubles the concentration of NPs, and in theory, should increase the amount of heparin also present. As illustrated in Figure 4.9, results of the APTT and TT clotting time assays reveal again that heparin, if present is not active in its role as an anticoagulant.

To further validate this, modified synthesis protocols were used where either NHS was used as a carbodiimide coupling activator as shown in Figure 4.10, or the SPIONs were coated with gelatine prior to functionalisation with gelatine as illustrated in Figure 4.11.

The use of NHS in carbodiimide coupling chemistry is commonplace when dealing with biomolecules [215, 220, 221]. It is frequently used alongside EDC when crosslinking peptides and proteins, or when immobilising molecules to surfaces. The schematic in Figure 4.12 demonstrates the mechanism and steps involved in this procedure. As both EDC and NHS are water soluble, organic solvents are not required, thereby reducing possible contamination. However, the reaction of the sulfo-NHS ester with amines is random, as is the reaction with NHS, and so amide bonds will and can be formed where sterically favourable.

As shown in Figure 4.10, the APTT is within the normal range for all samples synthesised using NHS, with the exception of the unfractionated heparin sample created without EDC. The prolongation is thought to be due to electrostatic binding of the heparin to the surface of the NPs as the APTT returns to normal following incubation in PBS, as shown in Figure 4.10B.

Finally, gelatine was used as a scaffold to provide further functional groups on the SPIONs for the conjugation to heparin. This work was based on our previous studies where heparin was conjugated to gelatine coated quantum dots for biological imaging applications [75]. This study demonstrated that heparin conjugated to nanoparticles retained its ability to bind to macrophage receptors, so it was envisioned that it would provide a mechanism to retain the anticoagulant activity of the drug. As shown in Figure 4.11A and B, the APTT and TT, respectively, of the heparin conjugated gelatine SPIONs were prolonged. The SPION coated with gelatine alone retained the haemostatically neutral features of its unmodified form. Again, to confirm that results were due to physically conjugated heparin, the particles were incubated in PBS for 1 hour. As seen

in previous experiments, when incubated with PBS, clotting times returned to within the normal respective clinical ranges for the APTT and TT, as shown in Figure 4.11C and D.

As demonstrated by the results of the functional assays, heparin loses its anticoagulant activity when conjugated to nanoparticles using the above procedures. Any prolongation in clotting times that would be typically associated with heparin treatment were returned to normal when electrostatically bound heparin is removed through incubation with PBS. The high salt concentration of PBS causes desorption of electrostatically bound molecules [222, 223]. It is possible that the random nature of the carbodiimide reaction may result in the crosslinking of heparin molecules. As heparin is a glycosaminoglycan containing many repeating polysaccharide units, EDC coupling using the above procedures may result in the formation of amide bonds between heparin amine and carboxylic acid groups [224]. Each disaccharide group in heparin contains a carboxyl group with approximately 0.3 unsubstituted amino groups per chain [224]. Excess EDC in the reaction may also act to form large agglomerates by crosslinking already functionalised heparin SPIONs. Potential supporting evidence for this can be seen in the results of the NTA in Figure 4.5, where the use of low molecular weight heparin yielded wide size distribution plots, and notable aggregates in the reaction vials.

Similarly, a hexasaccharide template is required for the binding of heparin to thrombin, as discussed in Chapter 1, with a pentasaccharide sequence being required for binding to antithrombin. As such, conjugation of heparin to nanoparticles may prevent either the templating effect to thrombin and sequence dependent binding to antithrombin due to steric hindrance. Specifically in the case of antithrombin, amino acid residues Arg129, Asn45, Lys125, Arg46, Arg47, Lys11, Lys114, Arg13 and Glu113 are heavily involved in heparin binding. These residues form a 50 Å long channel that contains the heparin pentasaccharide. When heparin is conjugated to the 15 nm SPIONs, the physical bulk of the nanomaterial may prevent access to this channel thereby inhibiting interaction with the key amino acid residues.

The negative charge of heparin may also act to prevent effective nanoparticle binding. Heparin is frequently referred to as the biomolecule with the greatest negative charge density, equating to approximately 2.7 negative charges per disaccharide subunit [225, 226]. This negative charge may result in repulsion of heparin from the nanoparticles which will be negatively charged in solution due to their dimercaptosuccinic acid (DMSA) coating [227]. The results of the APTT and TT assays with heparin-functionalised SPIONs through the EDC and APTES routes (Figure 4.7, Figure 4.8 and Figure 4.9)

suggest that this may be the case, as there is no evidence of any electrostatic binding of heparin and the SPIONs. When the NHS activating agent is used this the nitrogen in the succinimide ring is electron withdrawing [228] and may act to counteract the negative charge of the sulphate group. This may then allow for the negatively charged heparin to electrostatically interact with the NHS-SPION complex.

Similarly the complex structure of gelatine may provide numerous active sites for interaction with heparin. Previously it has been shown that gelatine can be used to entrap heparin and yield the formation of microspheres [229]. In the case of the heparin conjugation to the gelatine SPIONs presented here, heparin chains may act to entrap the gelatine SPIONs to form similar nanospheres. Figure 4.11 may lend evidence to support this, since when the nanoparticles are re-suspended in PBS, the heparin activity is lost suggesting that the heparin is only loosely bound to the SPIONs.

Patients undergoing unfractionated heparin treatment are usually monitored for prolonged thrombin times and elevated APTTs [230]. Clinically, heparin dosing is such that it prolongs the APTT to between 1.5-2.5 times that of normal, equating to a plasma concentration of 0.2-0.4 IU/mL [231]. It should be noted that low molecular weight heparin does not usually lead to prolongation of the thrombin time assay, but can do so in high concentrations [230, 232]. As illustrated and demonstrated in the above results, heparin that becomes physically conjugated to the gelatine is suspected to lose its anticoagulant activity, with electrostatically bound heparin retaining its antithrombin and thrombin binding capacity. When the SPIONs are subjected to incubation with PBS, the electrostatically bound heparin dissociates and is lost following the recovery of the SPIONs, thereby yielding normal APTT and TT clotting time results. Further work would be required to fully characterise this mechanism of interaction however. As clearly shown in the above study, results from the APTT and TT demonstrate that the heparin-nanoconjugates synthesised here do not act as an anticoagulant that would be beneficial in a therapeutic setting, and as such heparin conjugated SPIONs may not be suitable for clinical nanomedicine.

Heparin shear-activated liposomes may provide a more targeted and clinically relevant nanotherapeutic, and an opportunity for future work, similar to those reported by Korin *et al* [233]. Under high shear stress, similar to that in the vasculature surrounding an obstruction or thrombus, heparin along with a fibrinolytic agent may act to dissolve the clot and prophylactically prevent reoccurrence. As the drugs will be encompassed in a

liposome, the mechanism of action should not be altered, and thus in principle they should retain their clinically exploitable properties.

Chapter 5

The Anticoagulant Properties of Cadmium Telluride Quantum Dots

5 The Anticoagulant Properties of Cadmium Telluride Quantum Dots

5.1 Introduction

Quantum dots have numerous applications in medicine such as novel fluorescent probes for imaging applications [75, 83], vehicles for the delivery of small inhibitory ribonucleic acids (siRNAs) [234] and as multifunctional targeting, treatment and imaging agents [235]. Previous unpublished pilot research experiments in our lab have suggested that cadmium telluride quantum dots (CdTe QDs) with sizes between 3 and 4 nm had profound anticoagulant effects in a number of standard haemostatic assays including APTT, PFA-100 and Intrinsic Factor Screens. Similarly, negatively charged 2.5 and 5 nm CdTe QDs were also shown to induce platelet aggregation *in vitro* [236, 237].

However due to the high cytotoxicity associated with CdTe QDs [82, 94, 238-240], *in vivo* application of these nanomaterials is limited. Nonetheless, investigation and interrogation of the interaction of CdTe QDs with human plasma and components of the coagulation cascade may allow for the development of nanoparticles with similar characteristics, but with reduced toxicity profiles.

To this end in Chapter 5 we aim to investigate the size dependent anticoagulant properties of CdTe QDs through a number of clinically relevant diagnostic assays.

Specific objectives of this work were as follows:

1. Identify which coagulation pathway the QDs interact with
2. Interrogate the interaction of the QDs with the individual components of this pathway
3. Study the effect of the QDs on primary haemostasis through platelet function assays

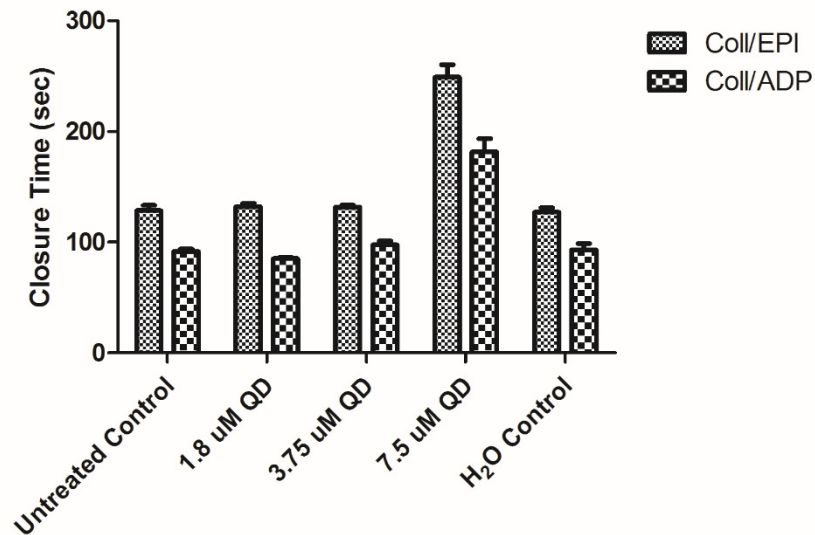
5.2 Results

5.2.1 Effect of Quantum Dots on Primary Haemostasis

In order to assess the effect of CdTe QDs on whole blood and platelets, a platelet function assay (PFA-100) and platelet aggregometry were carried out. The PFA-100 demonstrates the ability of whole-blood to form a platelet plug in an aperture in response to activation by collagen and epinephrine (col/epi) or collagen and adenosine diphosphate (ADP) (col/ADP). As illustrated in the PFA-100 results below (Figure 5.1), the Collagen/Epinephrine agonists demonstrate a 1.8 fold (109 seconds) increase in closure time for the 3.2 nm QDs at a concentration of 7.5 μ M. Similarly, an increase in closure time of 1.92 times (83 seconds) was observed using collagen/ADP for the 3.2 nm QDs. No corresponding effect was observed for the 3.6 nm QDs at the same concentration.

Platelet aggregation was measured by light transmission, whereby as platelet aggregation increases, the % of light transmission also increases. The results obtained for both QDs (Figure 5.2) demonstrated that up to a concentration of 10 μ M there was minimal aggregation, with a maximum % light transmission of 38.6 % and 37.1 %, for the 3.2 and 3.6 nm QDs, respectively.

A PFA-100 Closure Times for 3.2 nm QDs



B PFA-100 Closure Times for 3.6 nm QDs

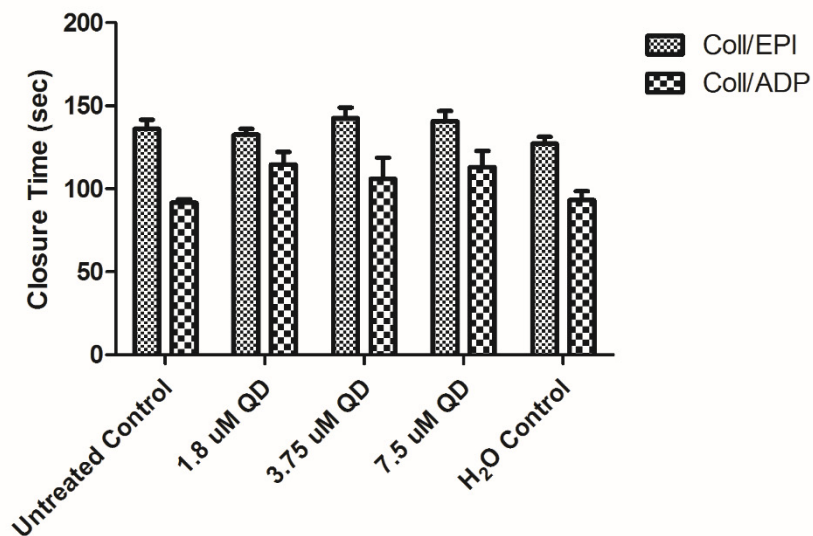
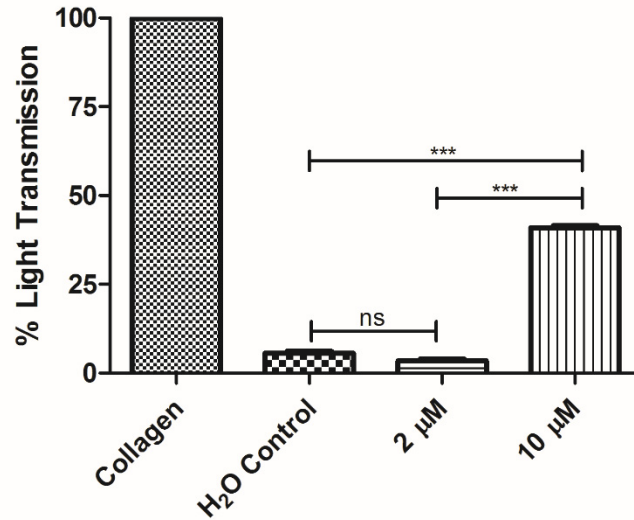


Figure 5.1: Platelet Function Analyser-100 (PFA-100) closure times for (A) 3.2 nm QDs and (B) 3.6 nm QDs over a concentration range up to 7.5 µM. Normal closure times: Col/EPI- 85-165 seconds, Collagen/ADP- 71-118 seconds [241]. Data represents mean $\pm$ SEM ($N = 3$).

A Normalised Platelet Aggregation for 3.2 nm QDs



B Normalised Platelet Aggregation for 3.6 nm QDs

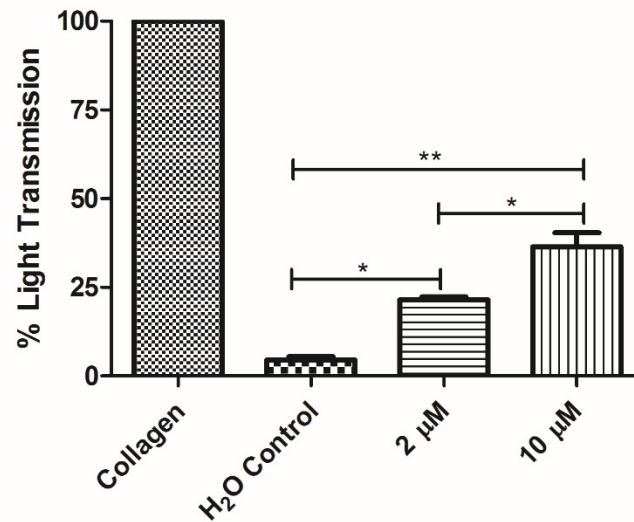


Figure 5.2: Platelet aggregation based on light transmission for (A) 3.2 and (B) 3.6 nm CdTe QDs at 2 and 10 µM. Results are normalised to collagen induced platelet aggregation. Data represents mean $\pm$ SEM, ^{ns} $P > 0.05$, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ (ANOVA with Tukey's post-test, $N = 3$).

5.2.2 Effect of Quantum Dots on Secondary Haemostasis

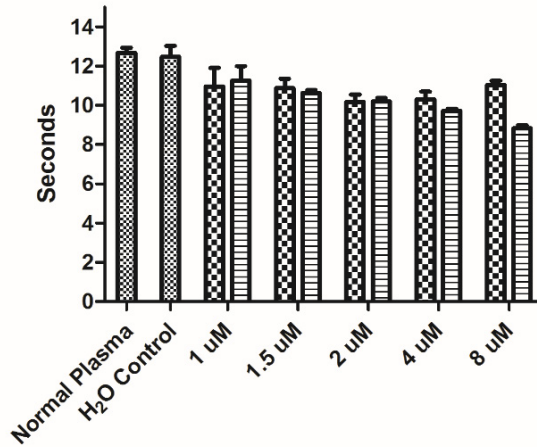
In order to investigate on the effect of QDs on the coagulation cascade, thioglycolic acid-stabilised 3.2 and 3.6 nm CdTe QDs were screened using PT, TT and APTT assays for their effect on haemostasis, up to a concentration of 8 μM , as shown in Figure 5.3. A size- and concentration-dependent prolongation of the APTT was demonstrated, with the 3.2 nm QDs having the most pronounced effect. Compared to the normal control, a sevenfold (146.9 second) and 1.25 fold (6.2 second) increase in the APTT was observed for the 3.2 and 3.6 nm QDs, respectively, at a concentration of 8 μM . Again, a size- and concentration-dependent prolongation of the TT was observed, with a clotting time longer than 5 minutes being observed for the 3.2 nm QDs at 8 μM . No statistically significant prolongation was observed for the 3.6 nm QDs. Similarly, no statistically significant changes above or below the normal range were observed for the PT assay with either type of QD.

These results demonstrated a predominant effect on the intrinsic pathway, and so an intrinsic factor screen (IFS) was conducted to investigate any loss in Factor VIII, IX, XI and XII activity (Figure 5.4). All the factors were within the normal range at a concentration up to 2 μM . Typical reference ranges for the IFS lie between 50-150 % activity [242]. Factor VIII activity was reduced to 50 % upon incubation with 7.5 μM 3.2 nm QDs.

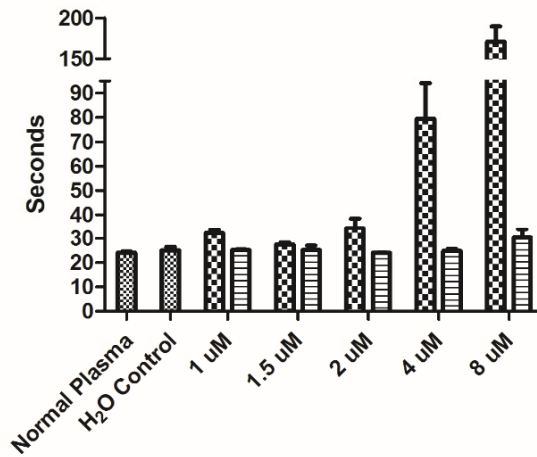
An APTT with varying incubation times were carried out for the 3.2 nm CdTe QDs to identify if the QDs are interacting with the contact pathway, as demonstrated in Figure 5.5. A short incubation time, typically less than 2 minutes, results in the APTT being sensitive to levels and activity of the contact factors [242]. In the case of 7.5 μM 3.2 nm CdTe QDs, short incubation time caused APTT prolongation over the entire incubation range. Similarly, when the incubation time was increased up to 90 minutes, the APTT remained prolonged at 86 seconds, compared to 33.3 for the H_2O control. These data suggest that the 3.2 nm CdTe QDs do not impact the contact pathway factors.

With prolongation of the APTT and TT detected, an automated thrombogram was carried out to investigate if any changes in thrombin generation could be seen. To this end, 3.2 and 3.6 nm QDs were incubated with platelet poor plasma at 1, 4 and 7.5 μM concentrations, as seen in Figure 5.6. As demonstrated, there were no statistically significant changes in the endogenous thrombin potential, peak thrombin, or the start of tail. However, the lag time and time to peak thrombin were significantly reduced for QDs of both sizes. Interestingly, this result was independent of QD concentration used.

PT of QDs over Concentration Range



APTT of QDs over Concentration Range



TT of QDs over Concentration Range

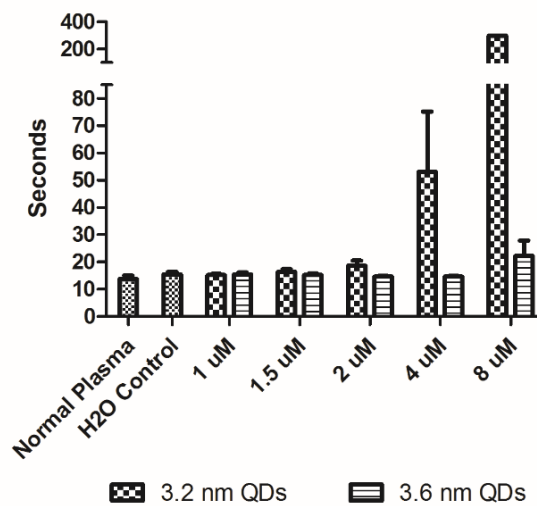


Figure 5.3: Size and concentration dependent effect of QDs on PT, TT and APTT assays. Thrombin time stopped manual after 5 minutes. Normal clotting ranges: PT 10-14 seconds, APTT 25-46 seconds, TT 12-14 seconds. Data represents mean $\pm$ SEM ($N = 4$).

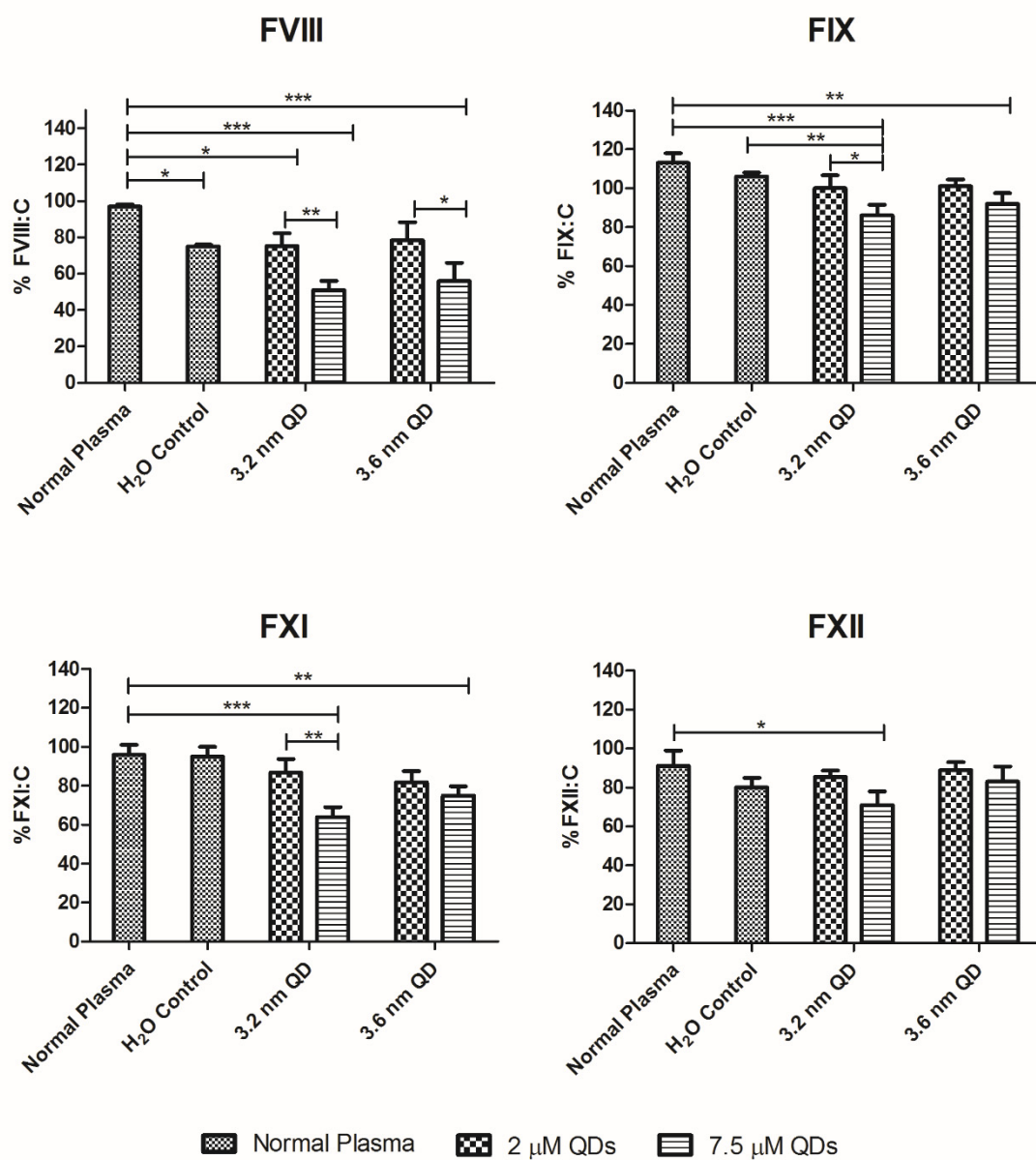


Figure 5.4: Intrinsic factor screen for activity of Factor VIII, IX, XI and XII. The IFS was carried out at 2 and 7.5 μ M QD concentration. Normal factor activities range between 50-150 %. Data represents mean $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ ANOVA with Tukey's post-test ($N = 3$).

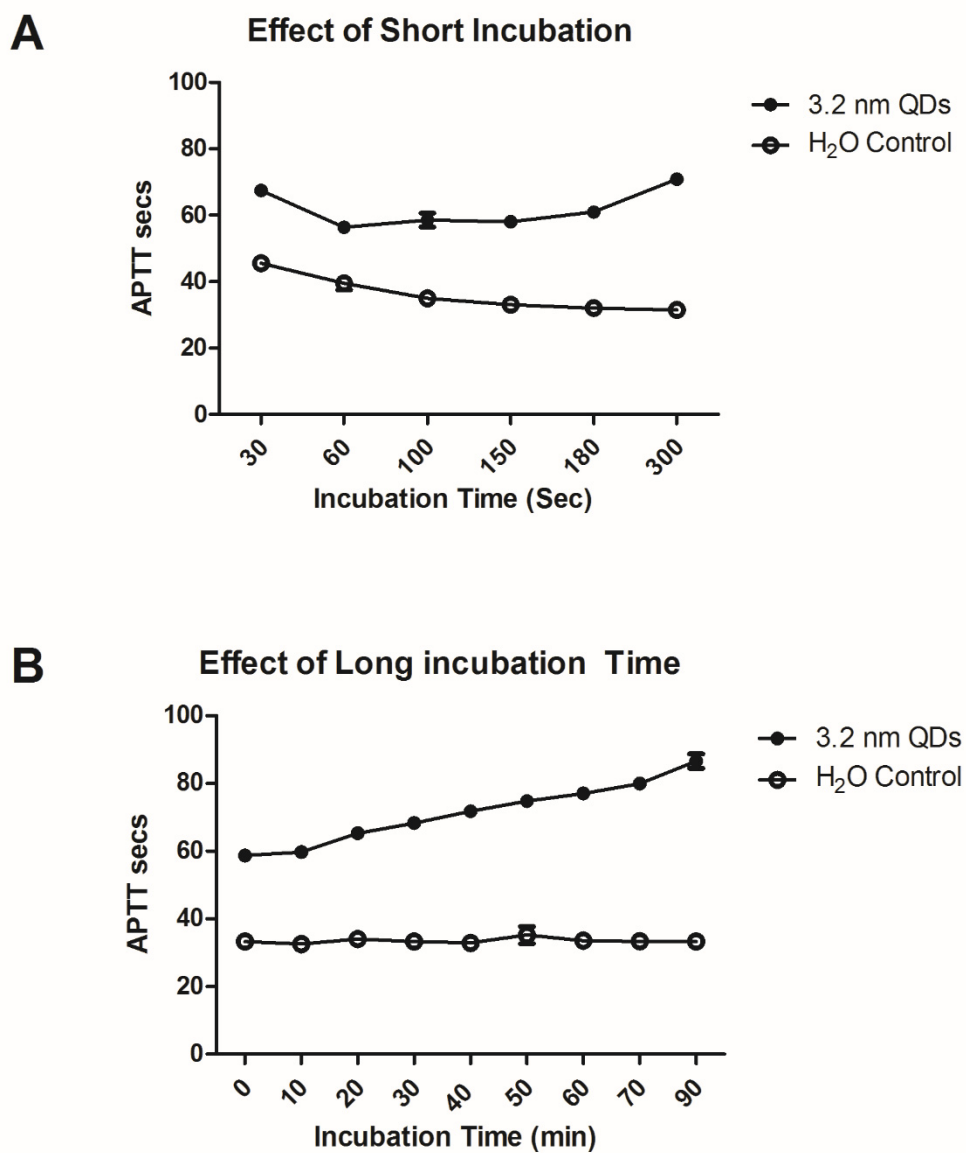


Figure 5.5: Effect of Incubation Time on APTT for 3.2 nm CdTe QDs. A: Short incubation time results in APTT being sensitive to levels and activity of contact factors. B: Long incubation times remove sensitivity towards contact factors. Data represents mean $\pm$ SEM ($N = 3$).

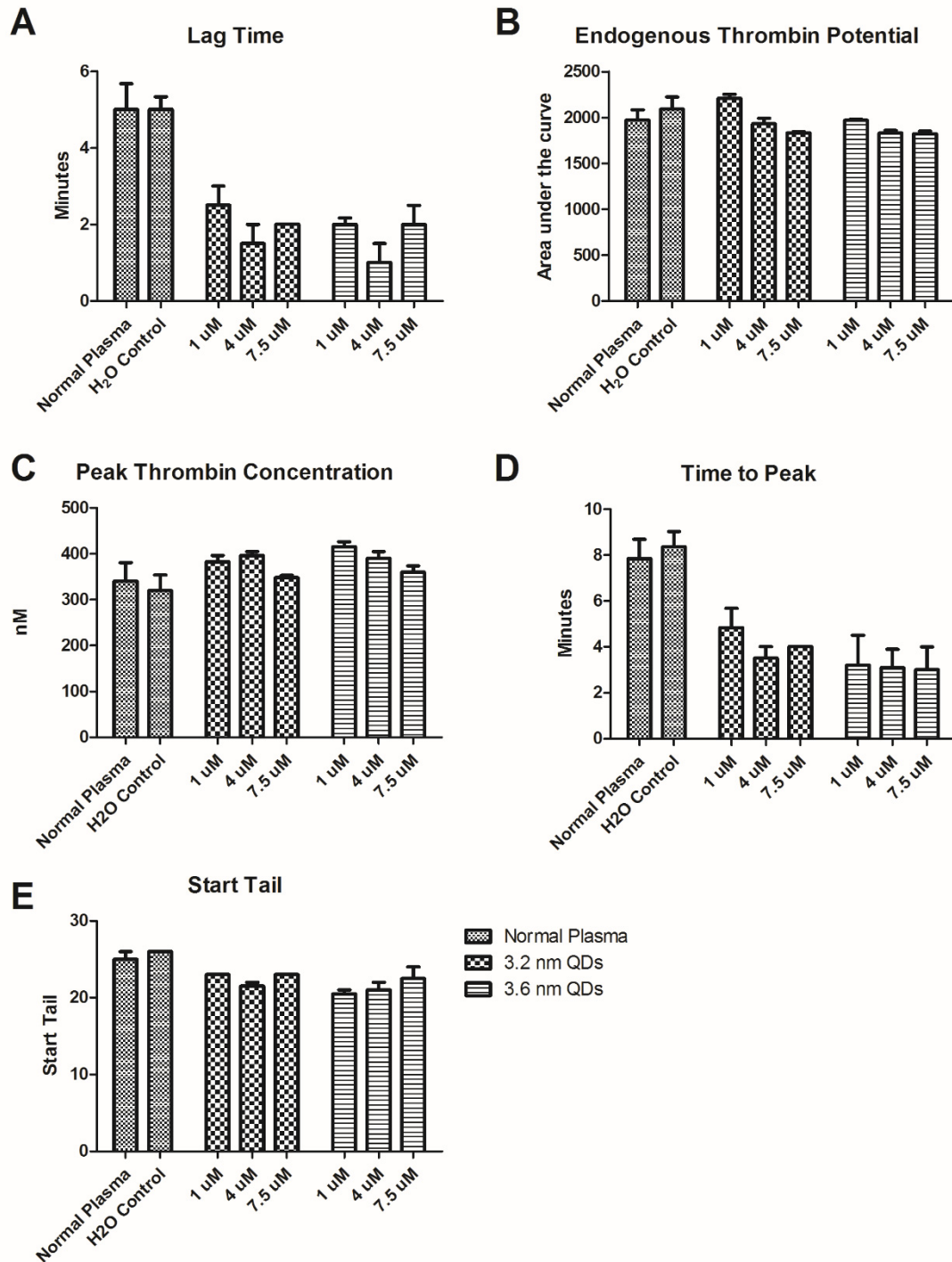


Figure 5.6: Results from Automated Thrombogram for plasma treated with 3.2 and 3.6 nm CdTe QDs at 1, 4 and 7.5 μ M. A: Lag time- time until coagulation is initiated. B: Endogenous Thrombin Potential- the area under the thrombogram curve. C: Peak thrombin concentration- maximum concentration of thrombin produced in nM. D: Time to peak- the time in minutes until peak thrombin concentration obtained. E: Start tail- the time in minutes when all generated thrombin has been consumed. Data represents mean $\pm$ SEM ($N = 3$).

Cadmium toxicity has been shown to be caused by inhibition of calcium channel function and calcium sequestration [243, 244]. With this in mind, it was hypothesised that the cadmium in the QDs may be sequestering the calcium ions necessary for clot formation. To assess this, an APTT was also carried out in an excess of calcium chloride, with the aim that the added calcium would replace the calcium ions lost through binding to the QDs, as illustrated in Figure 5.7. Results of the 2-way ANOVA, with a corrected Bonferroni post-test, demonstrate a highly significant prolongation of the APTT at QD concentrations above 2 μM ($P < 0.001$).

APTT and Calcium Concentration

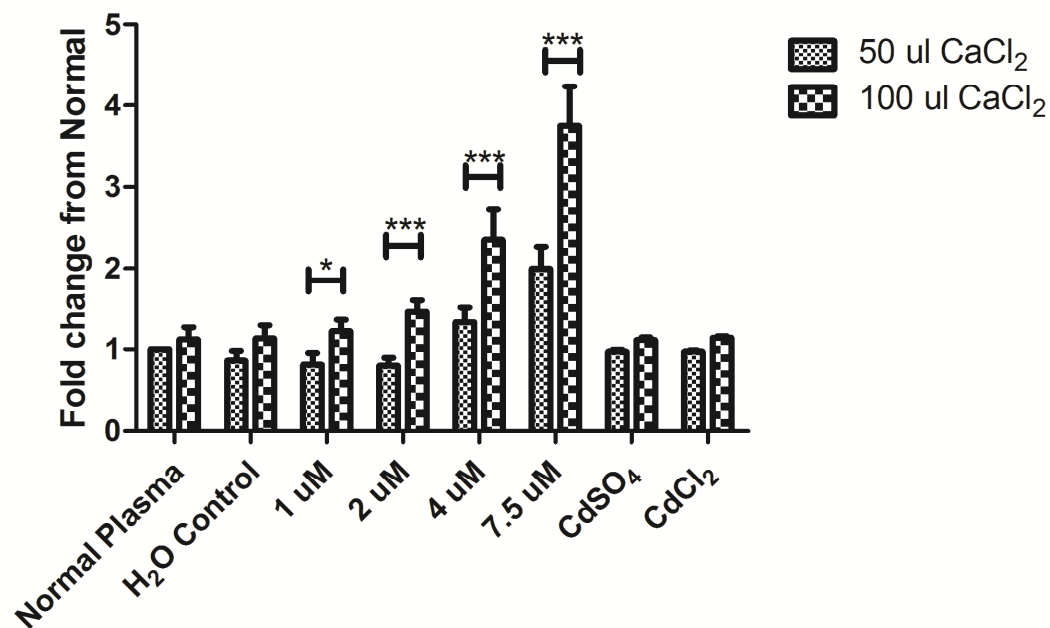


Figure 5.7: APTT of 3.2 nm QDs carried out at standard and double calcium chloride concentrations. Increasing calcium ion concentrations resulted in a doubling of the APTT for the QDs at 7.5 μ M. No change from normal was observed with a control of cadmium sulphate or cadmium chloride at 7.5 μ M. Data represents mean $\pm$ SEM. * $P < 0.05$, *** $P < 0.001$, 2 way ANOVA with corrected Bonferroni post-test ($N = 4$).

5.2.3 Effect of Quantum Dots on VWF

With prolonged PFA-100 and minimal platelet aggregation, we hypothesised that the QDs may be interacting with VWF. To investigate this, VWF antigen level and activity was assessed in the presence and absence of QDs. As seen in Figure 5.8, VWF antigen levels (VWF:Ag) (IU/mL), remained the same following incubation with 7.5 μ M for both sized QDs, compared to untreated plasma. There was no alteration following the 1 hour incubation. Similarly, VWF Ristocetin Cofactor activity (VWF:RC) was also within the normal range following QD treatment. The VWF Collagen Binding to VWF antigen ratios (VWF:CBA/Ag) were also show no change following incubation of plasma with the QDs (Figure 5.9).

5.2.4 Effect of Quantum Dots on Phospholipids in the APTT

The effects of high or low phospholipid concentrations were assessed by measuring the DRVVT and SCT as shown in Figure 5.10. If the QDs were binding to and inhibiting the formation of the coagulation complexes on the phospholipids, it could be feasible that increasing the phospholipid concentration would diminish this effect. No changes from normal were observed at low phospholipid concentration in the DRVVT (DRVVT-S), compared to high phospholipid levels (DRVVT-C) (Figure 5.10A). A slight reduction in DRVVT-C was observed for both sized QDs, and it was independent of incubation time. The SCT is also used to identify changes in phospholipid function. However, it can also be used to identify if the prolongation of the APTT is an artefact due to the contact activator. In the SCT, silica replaces the kaolin contact activator typically used in APTT assays. As shown in Figure 5.10B, the SCT is prolonged for the 3.2 nm QDs at 7.5 μ M. This prolongation is independent on phospholipid concentration and incubation time.

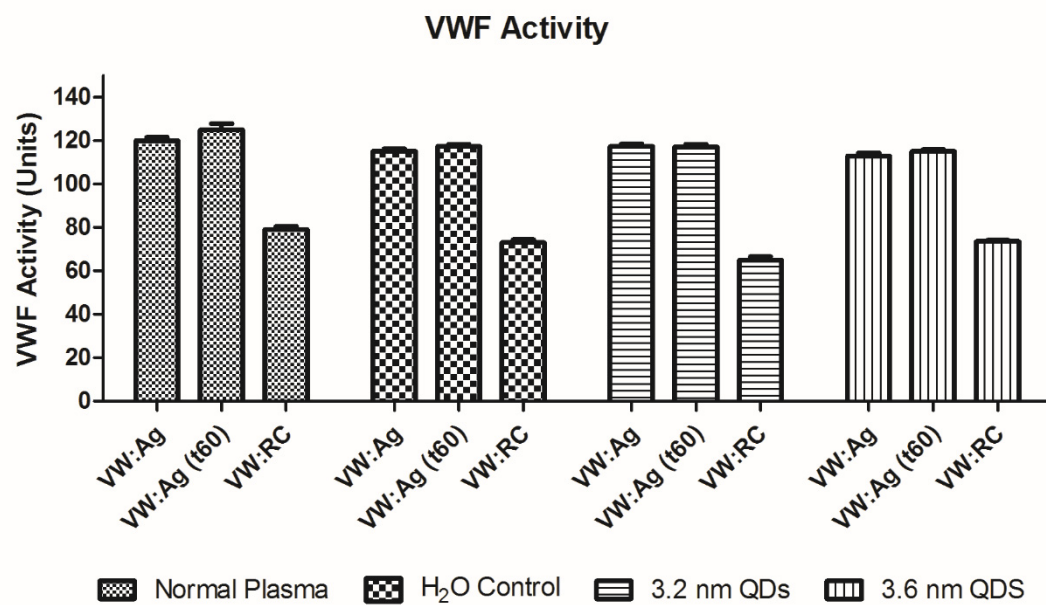


Figure 5.8: Interaction of QDs with Von Willebrand Factor activity measured following interaction with QDs at 7.5 μ M, at time zero and after 1 hour incubation. The results show VWF antigen levels (VWF:Ag) and VWF Ristocetin Cofactor levels (VWF:RC). Data represents mean $\pm$ SEM ($N = 3$).

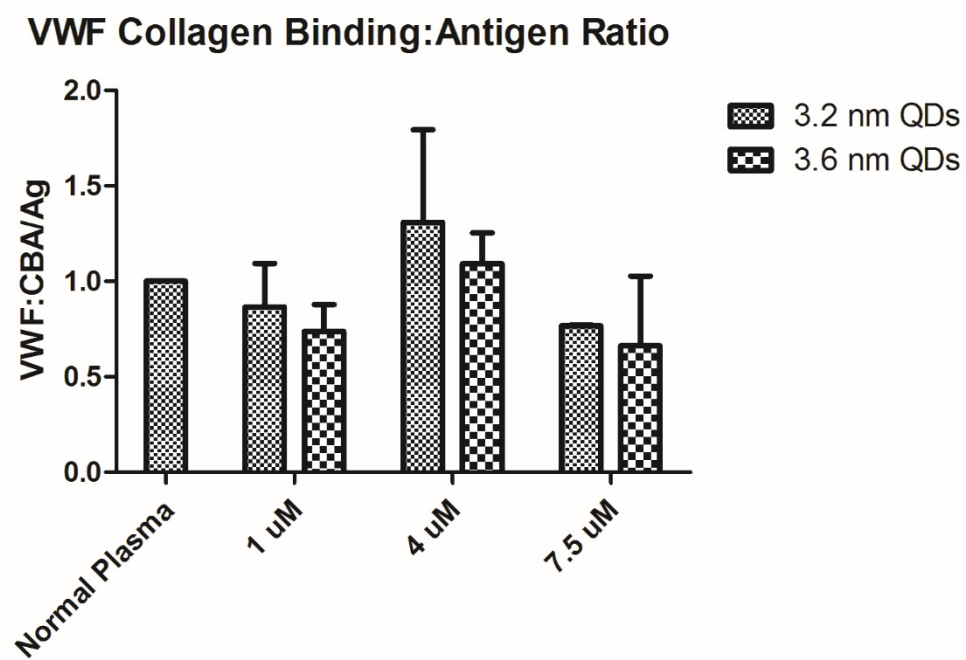


Figure 5.9: Normalised von Willebrand Factor Collagen Binding/Antigen ratios for normal plasma treated with 3.2 and 3.6 nm QDs at varying concentrations. No statistically significant changes were observed. Data represents mean $\pm$ SEM ($N = 5$).

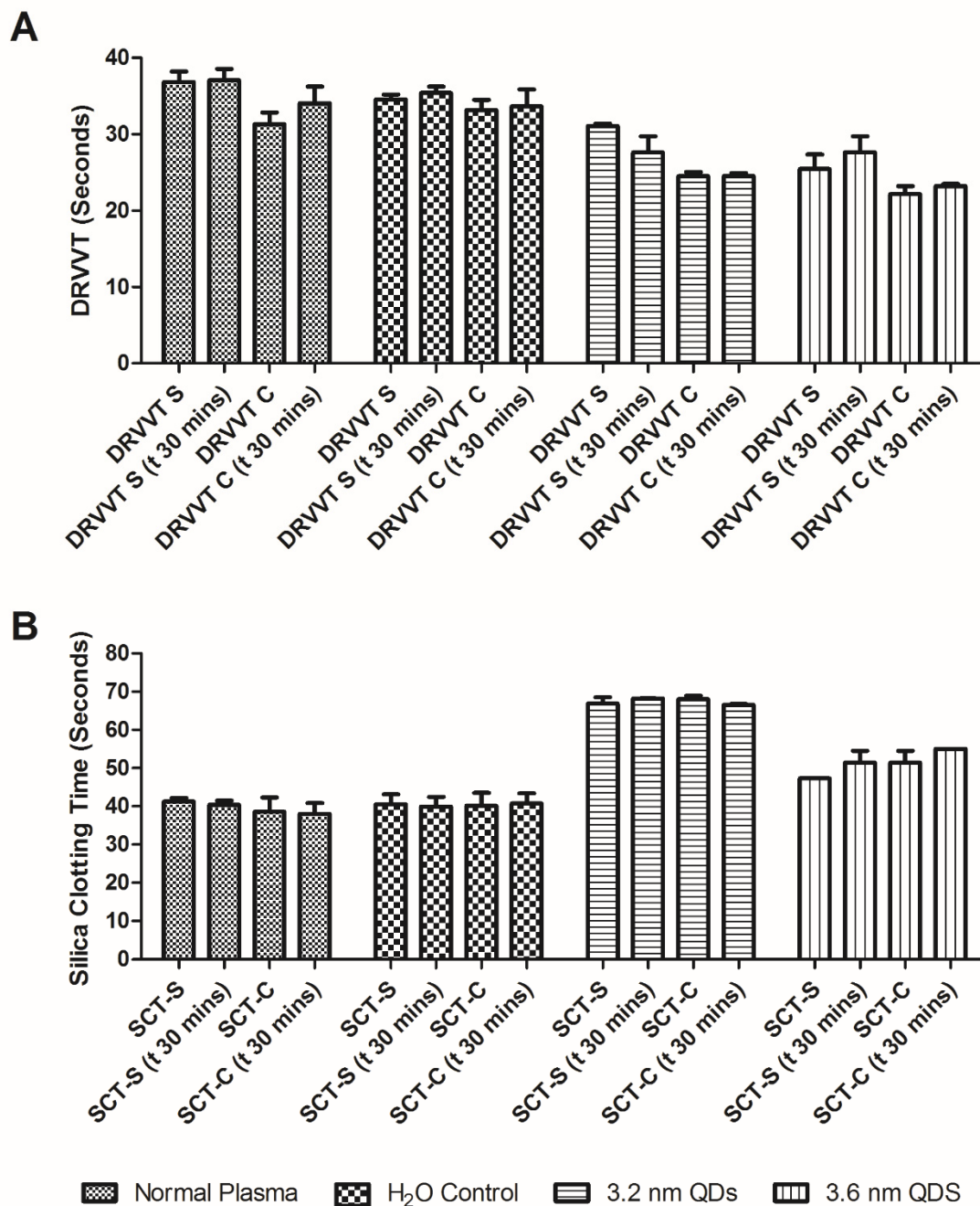


Figure 5.10: Interaction of QDs with phospholipids. A: Dilute Russell's viper Venom Time (DRVVT) for plasma exposed to QDs and measured at time zero and following 30 min incubation. Results are shown for high (DRVVT C) and low (DRVVT S) phospholipid concentrations ($N = 3$). B: Silica Clotting Times (SCT) for plasma treated with QDs at $7.5 \mu\text{M}$. The assay was carried out at time zero and following 30 min incubation with high (SCT-C) and low (SCT-S) phospholipid concentration. Data represents mean $\pm$ SEM ($N = 3$).

5.3 Discussion

Initial screening of cadmium telluride quantum dots for effects on primary haemostasis showed a prolongation in the PFA-100. At a concentration of 7.5 μM of the 3.2 nm QDs, the PFA-100 was prolonged for both collagen/epinephrine and collagen/ADP cartridges. No change from normal was observed for the 3.6 nm QDs. To identify if this prolongation in closure time was due to diminished platelet function, platelet aggregation was measured *via* light transmission for both sizes of QDs at 2 and 10 μM concentrations. Results were normalised to collagen induced aggregation, as collagen is noted as the most thrombogenic component of the sub-endothelium [245]. Both quantum dots induced minimal platelet aggregation at 10 μM , with 40.9 % and 36.5 % aggregation being observed for the 3.2 and 3.6 nm QDs, respectively. These results corroborate other published results for similar sized QDs, where 2.6 and 4.8 nm CdTe QDs induced minimal platelet aggregation in PRP [246]. However, these results differ from other platelet aggregation results caused quantum dots synthesised from other materials [145, 247, 248]. These results suggest that nanoparticle material, as well as size, may play a role in the interaction of quantum dots and haemostasis.

To further investigate the anticoagulant properties of CdTe QDs, various clinically relevant diagnostic tests were conducted. Initially PT, APTT and TT assays were carried out over a concentration range up to 8 μM . Results from the PT assay for both QDs revealed that the function and activity of the extrinsic pathway was unaffected, with both sized QDs yielding results within the normal clinical range. Subsequently, the intrinsic pathway was probed using the APTT. Here, the clotting time of normal plasma was significantly prolonged for the 3.2 nm QDs, at concentrations above 4 μM . No such change from normal was observed for the 3.6 nm QDs. Similarly, the TT was prolonged for the 3.2 nm QDs, with a time in excess of 5 min being observed at 8 μM . These results suggest either a loss in activity of some or all of the intrinsic factors or an inhibition of the coagulation cascade

Following on from the prolonged APTT results, an intrinsic factor screen was carried out to identify any changes in the activity of coagulation Factors VII, IX, XI and XII. It should be noted that normal factor activities are typically between 50 and 150 % [249, 250]. In the case of Factor VIII, normal haemostasis can occur with 25 % activity, and an activity of between 20-40 % of normal is required for the APTT to be prolonged [242]. As shown in Figure 5.4, the activity of Factor VII was reduced to 51 % following incubation of plasma with 7.5 μM of the 3.2 nm QDs. The reduction in FVIII activity caused by the 3.6 nm QDs was deemed not statistically significant. While the activity of FVIII was reduced

quite dramatically, it was still within the normal clinical range. This effect may be additive to a reduction in function in other coagulation factors or altered coagulation complex assembly. For example, Factor XI activity was reduced to 61 % for the 3.2 nm QDs at 7.5 μ M. Results for all other intrinsic factors investigated were within their normal ranges.

To probe further the intrinsic pathway, APTT assays were carried out again, this time with variations to the incubation time. Short incubations, typically less than two minutes, result in the test becoming highly sensitive to levels of the contact factors prekallikrein, kallikrein and Factor XII [242]. On the other hand, longer incubation times result in an assay that is less sensitive to loss in function of these factors. As demonstrated in Figure 5.5A, a short incubation time between 30-300 seconds results in an almost linear prolongation in the APTT at 7.5 μ M of the 3.2 nm QDs. In addition, longer incubation of plasma with 7.5 μ M of the 3.2 nm QDs shows a steady increase in the APTT. Here, the plasma was incubated at intervals up to 90 min prior to initiation of coagulation with calcium.

An automated thrombogram was also used to further investigate the generation of thrombin during coagulation. Here, normal plasma was treated with both 3.2 and 3.6 nm QDs over a concentration range. Illustrated in Figure 5.6 are the results for the lag time, endogenous thrombin potential, peak thrombin concentration, time until peak thrombin and the time when thrombin generation ends. As demonstrated, QDs, independent of size or concentration, caused no alteration in endogenous thrombin potential, peak thrombin or the start of tail. However, the lag time and time to peak were reduced, with no statistical significance being attributed to QD size or concentration. It has been shown previously that lag time, time to peak and start tail can be used as predictors of bleeding risk, with patients with rare bleeding disorders having higher lag times and time to peak measurements [251]. In the case of the QDs, the lag time and time to peak are reduced indicating the QDs speed up the time taken for thrombin to generate. Interestingly these results conflict with the results of the APTT and TT, as rapid thrombin generation would be expected to shorten the clot formation time via generation of a more procoagulant state [252].

As stated previously, calcium plays a crucial role in cadmium toxicity [243, 244, 253]. Cadmium also participates in a number of calcium ion dependent pathways due to its action as a calcium mimetic [254], can also act deplete circulating calcium ions [255] and alter platelet aggregation [256, 257]. To identify if leached cadmium ions from the quantum dots play a role in the anticoagulant effect demonstrated here, an APTT assay

was carried out with an increased concentration of calcium. As shown in Figure 5.7, when 100 μL of CaCl_2 is utilised, the APTT is prolonged even further for the 3.2 nm QDs, compared to the standard 50 μL . A 3.75 fold increase in the time until clot formation is observed at 7.5 μM , compared to the 2 fold change from normal under standard conditions. Cadmium sulphate and cadmium chloride at 7.5 μM were used as Cd^{2+} controls to identify if the effect was solely due to cadmium ions. The experiments with either of these salts resulted in normal APTTs suggesting that it is the formulation of the cadmium in the quantum dots that plays the critical role.

The interaction of QDs with von Willebrand Factor was also assessed. Here, the concentration of VWF following incubation with 7.5 μM of 3.2 and 3.6 nm QDs was determined at time zero and after a one hour incubation. Similarly the function of VWF was assessed by measuring the ability of VWF to cause the aggregation of platelets in the presence of the antibiotic ristocetin. These results are illustrated in Figure 5.8 and demonstrate the concentration and function of VWF is unaffected by the QDs of either size. Further to this, incubation of plasma with the QDs did not impact the ability of VWF to bind collagen. Previously it has been suggested that cadmium can play a role in increasing the response of platelets to aggregatory agonists [258]. Our results however suggest that the QDs do not cause any abnormal reaction between VWF, collagen, ristocetin and platelets, with VWF:Ag, VWF:RCO and VWF:CBA/Ag being within the normal range.

Similar to the ability of cadmium to alter calcium signalling and binding, it also has the ability to act as a facilitator for tissue factor binding to phospholipids [259] and cause the exposure of phosphatidylserine [260]. With this in mind, we aimed to identify if the anticoagulant properties of the QDs was due to interaction with phospholipids. As stated previously, phospholipids are essential for the formation of coagulation complexes as these assemble on the phospholipid layer of activated platelets. To this end, a number of screening tests for phospholipid function were carried out and illustrated in Figure 5.8. The DRVVT assay uses viper venom to activate Factor X with the assay being dependent on phospholipid concentrations. The assay can thus be used to identify phospholipid inhibitors. As shown in Figure 5.10A, high and low phospholipid concentrations were used to identify if the QDs were causing a prolongation in the APTT. Shortening of the DRVVT at high phospholipid concentrations was observed for both 3.2 and 3.6 nm QDs.

Another assay frequently used in the diagnosis of phospholipid inhibitors is the silica clotting time (SCT). This assay is essentially an APTT assay where kaolin is replaced by silica, with the option of including low or high phospholipid concentrations. As shown in Figure 5.10B, the SCT for the 3.2 nm QDs is prolonged regardless of incubation time or phospholipid concentration. Interestingly, the 3.6 nm QDs appear to show their first signs of an anticoagulant effect, with a 12-15 second increase in clotting time being recorded. Again, this effect was independent of incubation time or phospholipid concentration. These data combined with the DRVVT and APTT results suggest that the anticoagulant effect of the QDs is not due to inhibition of coagulation complex assembly as there was no change in clotting time with increased phospholipid concentration, or due to an artefact brought about due to the material used to initiate coagulation.

From the results of this study a clear anticoagulant effect is observed when plasma is treated with 3.2 nm QDs. Preliminary analysis using the APTT and TT assays illustrates that the effect is centred primarily around the intrinsic pathway, with QDs yielding prolonged clotting times for these tests. The PT was unaffected across a concentration range up to 8 μ M. Intrinsic factor screens were then carried out and it was determined that the activity of Factors VIII, IX, XI and XII were all within the normal clinical range. The statistically significant changes observed following treatment with QDs or water control are not to a level that would result in an alteration in clot formation times similar to those seen in the APTT. The activity of FVIII was reduced to 51 %, but it has been reported that an activity level of 25 % will still result in normal haemostasis. As such it is hypothesised that the anticoagulant phenomenon demonstrated in these results may be additive to non-significant reductions in the activities of other coagulation factors. As stated above, cadmium also has the ability to interfere with calcium signalling and phospholipid membrane binding, and so it was thought that prolongation of the APTT and TT may be due to the inability of the tenase or prothrombinase complexes to assemble correctly on the phospholipids required for normal coagulation. Results of clinical assays for phospholipid function revealed that the anticoagulant properties of the 3.2 nm QDs was not due to this mechanism. Experiments with alterations to the concentrations of calcium used also yielded interesting and unexpected results. It was thought that increasing the amount of calcium in the APTT would result in normalising the effect of the QDs and return the clotting time to the normal range. Interestingly this was not the case as increased calcium concentrations resulted in a further increase in the APTT. At a 7.5 μ M concentration of the 3.2 nm QDs, the APTT is normally twice that of normal, when the standard 50 μ L of calcium is used. This is increased to 3.75 times that of normal when an increased volume of calcium is used. No changes in the clotting

times were observed under untreated control conditions, plasma treated with a QDs equivalent volume of water, or plasma treated with a 7.5 μM solution of cadmium salts, indicating that this effect is strictly due to the presence of QDs and not cadmium ions as originally hypothesised. Thrombin generation was also carried out and demonstrated that both QDs caused a decrease in the lag time and time to peak- a result that was deemed independent of QD size or concentration. These data somewhat contradict the results of the APTT and TT, as shortened lag times and time to peaks typically reflect a tendency towards a prothrombotic state. The results from the PFA-100 show a similar anticoagulant feature for the 3.2 nm QDs, with the closure time being prolonged outside of the normal range at concentrations above 7.5 μM . Platelet aggregation was then assessed with results indicating that both 3.2 and 3.6 nm QDs at concentrations of 10 μM induce minimal aggregation. These results combined with the PFA-100 results suggest that VWF function and ability to link activate platelets to the thrombogenic surface of the analyser is diminished. However, results of the VWF:Ag and VWF:RCO were all within the normal range and showed no indication of altered function. These data suggest that QDs are interacting and inhibiting platelet adhesion in a non-typical fashion.

In summary, when the results of the clinically relevant diagnostic screening tests are combined, they indicate that the 3.2 nm QDs affect coagulation through a yet unknown mechanism. It can be suggested that the phenomenon is due to additive anticoagulant effect of the QDs through lowered FVIII activity combined with reductions in other factor activity, altered thrombin generation and possible VWF or phospholipid interaction. It may also be possible that the 3.2 nm QDs are able to bind to or interact with key features of coagulation factors or phospholipids altering their function, with the 3.6 nm QDs being above this critical size. Further molecular modelling and binding studies may provide more evidence for this.

Chapter 6

General Discussion and Future Work

6 General Discussion and Future Work

6.1 General Discussion

Nanoparticles are becoming increasingly utilised in all areas of science, technology and healthcare. More specifically in terms of nanomedicine, the physical and chemical properties of these innovative materials allow for their application in diagnostics, therapeutics, drug delivery and multifunctional therapeutic-diagnostic (theranostic) agents.

However, in order to ensure compliance with environmental and regulatory guidelines, nanoparticles must be fully characterised, with particle size being of the most important starting points. Similarly, the interaction of nanoparticles with blood and haemostasis components should be fully investigated, as this interaction will inevitably occur *in vivo*. Full nanoparticle physico-chemical characterisation will be required to help interpret the biological data obtained from these experiments. Under the current European Commission definition of nanomaterials stating that 50 % of a true nanomaterial sample must lie between 1-100 nm [1], Nanoparticle Tracking Analysis (NTA) provides a method and reproducible platform to cover the characterisation of such nano-objects.

Through the use of extensive inter-laboratory comparisons, a set of standard operating procedures and refinements were developed which acted to reduce the user-to-user variability of the NTA technique. Initially, “in house” developed protocols were assessed and demonstrated a large coefficient of variance (% CV) for multiple sample types. However, through industrial engagement and knowledge transfer, a set of standard operating procedures were developed for the analysis of nanomaterials. This was not only limited to sample preparation and analysis protocols, but also covered other areas such as sample shipment and handling. The % CV for the analysis of a 100 nm polystyrene nanoparticle sample was reduced from 33.3 % to 3.1 % over four iterations. This is a dramatic reduction of 90.69 % and is less than the variation observed when using dynamic light scattering (DLS), NTA’s companion technique. Based on DLS guidelines the % CV for a monodisperse sample with a diameter greater than 20 nm should be less than 20 % [261]. Following two successive rounds of interlaboratory comparison this was reduced further to 1.69 % with a median absolute deviation of 0.54 % by the end of ILC 2. More dramatically, a 200 nm polystyrene nanoparticle sample had a % CV of 0.59 %, again with median absolute deviation of 0.54 %. These results clearly show the improved user-to-user reproducibility that can be achieved following

industrial engagement and the refinement of standard operating procedures when adopting a reliable technology platform that NTA has shown to be.

The analysis of multimodal samples identified an opportunity to further refine the technique, and so NanoSight-Malvern improved the limits of detection through incorporation of Finite Track Length Adjustment algorithm (FTLA). This algorithm helped improve the resolution of size distribution and improved curve fitting, thereby allowing the analysis of more complex, polydisperse samples. In the case of a trimodal sample containing 100, 200 and 300 nm polystyrene particles a % CV of 4.94, 2.88 and 2.43 was obtained for each component respectively. Increasing the complexity further, when a 400 nm sample was introduced to the trimodal sample, the % CV of the 100, 200, 300 and 400 nm nanoparticles were calculated as 6.82, 3.88, 2.60 and 3.09 %, respectively. The FTLA algorithm is instrumental in the analysis of these complex mixtures and dramatically improves the resolution, compared to DLS, as previously demonstrated [188]. It has been suggested that FTLA should not be used when dealing with polydisperse samples due to the possible introduction of artefacts [262]. It can be argued however that the artefacts detected in the study by Van der Pol [262] were due to the use of an earlier version of NTA (version 2.3.5) and therefore no longer apply due to the improvements that resulted from the rounds of ILC which I have contributed to and presented in this thesis.

This improvement in the accuracy and reproducibility of NTA was instrumental in allowing for the analysis of multiple sample types including gold nanoboxes, bismuth ferrite nanoparticles, superparamagnetic iron oxide nanoparticles, exosomes, and virus particles, under physiological conditions. The aggregation state of the particles in simple and complex dispersal media can now also be assessed following optimisation of the technique through rounds of interlaboratory comparisons. This type of analysis would not typically be possible using other characterisation techniques based on inherent limitations. More specifically, one of the fundamental disadvantages in DLS is its bias towards larger particle sizes [188]. This bias would result in a skewing of results where aggregates are present. The dispersion of nanoparticles into high salt or protein containing media resulted in the formation of larger nanoparticles that would bias the size distribution data if it were carried out using DLS rather than NTA. Further to this, transmission electron microscopy imaging of the particles would not yield useful data due to the burning of the organic material forming the protein corona, on the surface of the nanoparticle. Similarly, the preparation of the sample for electron microscopy has been known to induce aggregation of nanoparticles resulting in incorrect size distribution

and stability data. As the solvent evaporates, nanoparticle aggregates can form and as such, will not offer a true reflection on the size and stability of the sample in liquid suspension. As such, NTA offers a fast, reliable, reproducible and accurate means to characterise nanoparticles in suspension where the true size, concentration and stability of the sample can be obtained.

In the case of more biologically relevant nano-objects, NTA can be used to identify the temperature at which the capsid of the adenovirus ruptures. The light scattering properties of the virus provide good resolution for the NTA technique and the monitoring of this scattering can be used to follow the bursting of the capsid over a temperature range. It was demonstrated here that treatment of an adenovirus sample with gold nanoparticles causes an increase in the temperature at which the capsid ruptures. Again, NTA offers advantages here over other characterisation techniques. The ability to separate particle types by their respective diffusion coefficients can allow for the characterisation of a sample containing particles of similar size but with different diffusion properties. Similarly, the scripting function of NTA dramatically reduced the labour intensiveness of similarly styled experiments. Here a list of commands can be created that will allow the NTA platform to follow and run instructions provided to it by the user with the goal of freeing up more time for experimental design and setup. With the addition of an auto-sampler, a large number of nanoparticle samples can be loaded and analysed with little user intervention.

NTA is rapidly becoming the go-to technique in the characterisation of exosomes as it can provide quantitative data about the size and concentration of the sample. Previous methods are time-consuming and semi-quantitative [263] with exosome concentrations typically being calculated by BCA assays [264]. In this thesis, NTA was also used to analyse a number of exosome samples isolated from patients and cell lines in a rapid format and yielded information on their size, stability and concentration simultaneously. The technique was used for quality control purposes to study the size distribution of exosomes isolated using different methods. Similarly, NTA was used to monitor the stability of exosomes following prolonged storage, a critical aspect when storing valuable patient samples. Preliminary results demonstrated that low density sucrose gradients were optimal for the isolation of the smallest and most monodisperse exosomes and also indicated that prolonged storage did not dramatically accelerate the degradation of the samples.

Further, the refined and optimised NTA protocols were crucial in the analysis of heparin-functionalised nanoparticles. Here, we aimed to develop novel nanotherapeutics for the controlled and targeted treatment of prothrombotic disorders. Heparin was chosen as a candidate molecule, as conjugation to nanomaterials may help to improve the biodistribution and pharmacokinetics of the drug. There have also been some previously published though inconclusive results relating to the efficacy of heparin nanoconjugates. With this in mind we set out to systematically identify if heparin could be conjugated to nanoparticles and retain its anticoagulant properties. Through the use of multiple synthesis routes and variants, it was determined that while heparin can be conjugated to the surface of nanoparticles, the anticoagulant activity is lost. Examination of the literature has shown that the reported statistically significant prolongation of the clotting times is in fact not clinically relevant. In a number of *ex vivo* assays, it was determined that potential remnants of the functionalisation procedure would not act in a prothrombotic mechanism which could diminish the anticoagulant activity of heparin. Further to this, an increased volume of the nanoparticles was added to the coagulation assays in order to ensure that sufficient functionalised material was present. It is thought that the loss of heparin's anticoagulant properties is due to the inability of the heparin-nanoparticle conjugate to bind effectively to its target, specifically affecting the formation of the thrombin-antithrombin-heparin complex.

Finally, the anticoagulant properties of cadmium telluride quantum dots were also investigated using clinically relevant diagnostic tests. Here we demonstrated what 3.2 nm QDs exhibit a significant anticoagulant effect based on a number of diagnostic assays. This phenomenon was demonstrated to affect the intrinsic pathway due to prolongation of the APTT and TT tests. Similarly, the PFA-100 assay was prolonged for both collagen/epinephrine and collagen/ADP cartridges at concentrations above 7.5 μ M for the 3.2 nm QDs. No corresponding response was observed for 3.6 nm QDs at equivalent concentrations. A number of hypotheses were tested in this study to identify if the QDs were inhibiting specific coagulation factors or preventing the formation of the coagulation complexes on the phospholipid surfaces. Results from these experiments demonstrated that the activity of Factor VIII was reduced, but not to a level that would have an impact on normal haemostasis *in vivo*.

The anticoagulant effect was also deemed to be phospholipid independent and contact activator independent. The silica clotting time demonstrated that the prolongation of the APTT is not an artefact due to the mechanism of contact formation. In this assay it was established that the prolongation observed in the APTT was not due to the interaction of

QDs with kaolin in the standard APTT or with silica in the SCT. This assay in conjunction with the DRVVT also illustrated that QDs had no effect on the function of the phospholipids in coagulation.

Another hypothesis tested was to determine whether the QDs were binding to and sequestering calcium ions that are necessary for coagulation to take place. Here we added an excess of calcium ions to determine if the increased calcium concentration would return the APTT back to the normal clinical range. However, when an increased volume of calcium was used, the APTT was prolonged further. The mechanism by which this occurs is not known, but is thought to be due to the physical structure of the nanoparticle as cadmium salts at equivalent concentrations were shown to have no effect on the coagulation cascade.

It was also demonstrated that the QDs do not induce platelet aggregation or interfere with the concentration and activity of VWF. Results based on light transmission demonstrated neither 3.2 nm nor 3.6 nm CdTe QDs elicit a platelet aggregation response. Previous studies have shown that cadmium [258], as well as 2.6 and 4.8 nm CdTe [246] can act as a platelet agonist, possibly demonstrating that the results observed in this study are primarily due to the physical size of the nanomaterial and not from cadmium ions leached from the QDs. Similarly, VWF antigen and function assays revealed that the QDs of either size do not affect the concentration of the factor or the ability of VWF to agglutinate platelets in the presence of ristocetin.

In summary, nanomaterials provide an opportunity for use in controlling haemostasis. They can elicit a response on the coagulation cascade such as in the case of quantum dots, or can be functionalised with molecules to interact with some components of this pathway. Further refinement of this will be necessary, most likely through the use of novel anti- or pro-thrombotic agents which retain their activity when conjugated to nanomaterials. As demonstrated in this work, heparin lost its anticoagulant activity when conjugated to iron oxide nanoparticles. However, this is not to say that all nanodrugs will similarly lose their activities. It may simply be a case of systematically identifying candidate molecules and refining the functionalisation procedures.

6.2 Future Directions

The continuous development of the NTA technique through the link between Malvern Instruments and the interlaboratory partners' centres will lead to significant advance in nanoparticle characterisation. Ongoing refinement is leading towards dramatically improved and accurate concentration measurements [265]. It is expected that these results and advancements in NTA will improve the characterisation of nano-objects and increase the number of applications of the technique, particularly in the area of virus titre calculations and analysis of other biological samples.

One area of uncertainty remains around the sheer number of NTA instrument variations. Currently there are three NanoSight instruments on the market: the NS300, NS500 and LM10 versions, with each having a range of configurations with one of four laser wavelengths (405 nm, 488 nm, 532 nm and 642 nm) and one of three camera types (EMCCD, CCD and sCMOS). This leads to a total of 36 possible NTA variants (3 systems x 4 laser units x 3 camera types). In order to fully assess the user-to-user variation and accuracy, experiments should be carried out by a number of users operating the same NTA device. This would yield important results pertaining to the accuracy of the system and if the variation observed between users is strictly due to the inherent error of the device or due to subjective differences in camera level and focus position. Currently NanoSight-Malvern are refining the camera level and focusing ability of NTA and have begun to incorporate an auto-camera level and auto-focus option. These features may not be optimal when dealing with polydisperse or complex samples but have clear advantages when dealing with well-defined and monodisperse materials such as exosomes or virus particles.

The fluorescence and zeta-potential measurement capabilities of the NanoSight system can also provide an opportunity for further study. The laser wavelength of the device can be used to excite fluorophores that can aid in the concentration determination of nanoparticles. Fluorescent antibodies compatible with the user's NanoSight system can be selected for binding to nano-objects of interest. Applications of this are most prevalent in the characterisation of exosomes, where subpopulations or debris can be selectively removed based on their fluorescent signals, and can complement their exclusion based on diffusion coefficients. It should be noted that the binding of the antibody to the surface of the exosome may act to increase the hydrodynamic size of the exosomes, rendering the sizing data inaccurate or non-representative of the pure sample. Research should be conducted on selecting the optimal fluorophores for this work, as well as selecting parameters such as flow rates and filter setups to minimise fluorescent quenching.

Quantum dots may be considered the fluorophore of choice due to their small size and photo-stable properties. The use of single domain antibody-QD conjugates may provide a means to yield data pertaining to the size and concentration of subpopulations of biological nano-objects of interest [266].

Similarly, the NS500 system can be equipped with zeta-potential measurement capabilities. The zeta-potential of a particle is a surrogate parameter for the stability of the particle in solution, with the magnitude of the zeta-potential reflecting the degree of electrostatic repulsion between adjacent particles. The further the value is from zero, the greater the stability of the particle in solution as shown in Table 6.1.

Table 6.1: Zeta-potential measurements as a parameter for colloidal stability modified from [267].

Zeta Potential (mV)	Relative Stability
0 to ± 10	Rapid aggregation
± 10 to ± 30	Incipient instability
Greater than ± 30	Good stability

This option to equip zeta-potential measurement capabilities provides a means to obtain zeta-potential, size and concentration measurements simultaneously. The validity and accuracy of the zeta-potential results should be assessed through interlaboratory comparison, although it may prove difficult to obtain sufficient participant numbers due to its limited availability. The Malvern Zetasizer range is currently the world's most commonly used system for the determination of the zeta-potential of a sample [268], and as such, the economic benefit and market share may not be present to further refine the zeta-potential capabilities of NTA. However in terms of basic research, the multiparametric physiochemical characterisation of nanoparticles using NTA can add to the existing knowledge base and existing characterisation datasets.

Functionalisation of nanomaterials will require further optimisation to develop nanotherapeutics for the treatment of coagulation disorders. Critical to this will be the utilisation of more advanced synthesis protocols where drug candidates are coupled to nanomaterials. These procedures may allow for the selective crosslinking of specific functional groups, rather than the random conjugation observed when EDC coupling is used. At the same time, shear activated liposomes have potential for use in the treatment and lysis of blood clots. These liposomes could be filled with fibrinolytic agents to breakdown blood clots and thromboembolisms. Under high shear stress, such as that

observed in the vicinity of blockages within mammalian vasculature, liposomes can rupture releasing their active components. These particles may have benefits in the clinic by reducing adverse effects associated with systemic delivery of thrombolytics. Similarly, long acting Factor VIII carriers may be designed using nanoparticles. These particles could dramatically improve the quality of life of Haemophilia A patients.

One critical issue with the design of nanomedicines appears to be the selection of the drugs for conjugation to nanomaterials. In simple terms, therapeutic drugs typically bind to a critical site or target to elicit their response, be it the antagonism of an enzyme, the upregulation of specific genes or the synergistic activation of proteins. To simplify this further, in the case of the stereotypical lock-and-key analogy for the binding of a drug to a receptor, the key will no longer open or even fit the lock if it is modified. To this end, drugs for the generation of nanomedicines should not be simply selected off the shelf, but rationally designed so that they can be effectively conjugated to nanomaterials and retain their physiologically active properties. As stated above, the synthetic procedures should be refined so that the active site of the drug remains open, active and not sterically hindered so that it can function as designed. Biopharmaceuticals (biologics) may potentially be new candidates for conjugation to nanomaterials rather than traditional small molecules. These genetically engineered proteins may be synthesised to contain non-biologically active chains that would be suitable for conjugation to a nanoparticle, similar to the PEGylation of proteins [269, 270], or generation of fusion proteins [271].

On the other hand, while the toxicity associated with quantum dots will limit the majority of *in vivo* experiments, the results from this study along with physico-chemical data may allow for the synthesis of other non-toxic variants. Further interrogation of the interaction of 3.2 nm QDs with components of the coagulation cascade may allow for the identification of their definitive mode of action. This information combined with the characterisation data may pinpoint key features that are required to develop potentially less-toxic alternatives. Molecular modelling such as docking or quantitative structure activity relationship (QSAR) studies may reveal information pertaining to key sites that the 3.2 nm QDs can interact with, whereas their relatively larger 3.6 nm counterparts cannot.

6.3 Translational and Societal Impact

6.3.1 Nanoparticle Characterisation

Nanomaterial characterisation is needed not only in basic research but also to comply with environmental and regulatory procedures. With more and more advances in technology, nanoparticle exposure of humans is likely to increase. The unique properties of the nanomaterials that are of interest in industrial and consumer applications may also give rise to unique biological reactivity. A number of studies have shown that this biological response is dependent on the size of the nanomaterial [174, 195, 272, 273], similar to the anticoagulant response elicited by the 3.2 nm QDs that is not seen with 3.6 nm QDs. The interaction of the nanomaterial with its dispersant is also a key parameter that must be studied as the dispersal media may have implications for the NP aggregation state. To this end, a thorough nanoparticle characterisation is required which must not only be accurate, but also consistently reproducible, in order to carry out due risk assessments and to limit exposure to potentially hazardous materials.

6.3.2 Nanomedicines for the Treatment of Coagulation Disorders

As stated previously, nanomaterials provide an opportunity for the development of new therapeutics, diagnostics and combined theranostic devices. They also provide a means to alter the biodistribution and pharmacokinetic properties of existing therapeutics. In the case of heparin treatment, due to the bleeding risks associated with heparin administration frequent monitoring is required. While not heavily invasive, this monitoring requires frequent analysis of patient blood samples with corresponding financial implications. Thus, if a nanotherapeutic drug can be designed with improved kinetics and reduced bleeding risks, patient quality of life can be improved. Similarly, the financial burden on the healthcare system may also be reduced.

While the mortality rate due to bleeding disorders is low with 1,900 deaths being attributed to coagulation defects in the US in 2010 [6], the incidence of bleeding disorders is not. For example, Haemophilia A is associated with 1 in every 10,000 male births. These patients require frequent monitoring and generally require factor replacement therapy throughout their lives. Similarly, Von Willebrand Disease is the most common hereditary bleeding disorder, affecting 1 % of the general population [7]. To this end, nanoparticles may be used as carrier molecules to improve the circulating half-life of deficient coagulation factors in patients. Further to this, the improved kinetic profiles brought about by nanomedicines may result in enhanced patient quality of life, along with the corresponding economic benefits.

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Publications

Interlaboratory comparison of size measurements on nanoparticles using nanoparticle tracking analysis (NTA)

Patrick Hole · Katherine Sillence · Claire Hannell · Ciaran Manus Maguire ·
Matthias Roesslein · Guillaume Suarez · Sonja Capracotta · Zuzana Magdolenova ·
Limor Horev-Azaria · Agnieszka Dybowska · Laura Cooke · Andrea Haase ·
Servane Contal · Stein Manø · Antje Vennemann · Jeans-Jacques Sauvain ·
Kieran Crosbie Staunton · Sergio Anguissola · Andreas Luch · Maria Dusinska ·
Rafi Korenstein · Arno C. Gutleb · Martin Wiemann · Adriele Prina-Mello ·
Michael Riediker · Peter Wick

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Abstract One of the key challenges in the field of nanoparticle (NP) analysis is in producing reliable and reproducible characterisation data for nanomaterials. This study looks at the reproducibility using a relatively new, but rapidly adopted, technique, Nanoparticle Tracking Analysis (NTA) on a range of particle sizes and materials in several different media. It describes the protocol development and presents both the data and analysis of results obtained from 12 laboratories, mostly based in Europe, who are

primarily QualityNano members. QualityNano is an EU FP7 funded Research Infrastructure that integrates 28 European analytical and experimental facilities in nanotechnology, medicine and natural sciences with the goal of developing and implementing best practice and quality in all aspects of nanosafety assessment. This study looks at both the development of the protocol and how this leads to highly reproducible results amongst participants. In this study, the parameter being measured is the modal particle size.

Sonja Capracotta, Zuzana Magdolenova, Limor Horev-Azaria, Agnieszka Dybowska, Laura Cooke, Andrea Haase, Servane Contal, Stein Manø, Antje Vennemann, Jeans-Jacques Sauvain, Kieran Crosbie Staunton, Sergio Anguissola, Andreas Luch, Maria Dusinska, Rafi Korenstein, Arno C. Gutleb, Martin Wiemann, Adriele Prina-Mello, Michael Riediker, Peter Wick have contributed equally to this study.

Keywords Nanoparticle · Interlaboratory comparison · Reproducibility · Polydispersity · Toxicology · Health and safety implications

P. Hole (✉) · K. Sillence · C. Hannell
NanoSight Ltd., Minton Park, London Road, Amesbury,
Wiltshire SP4 7RT, UK
e-mail: patrick.hole@nanosight.com

C. M. Maguire · K. C. Staunton · A. Prina-Mello
Trinity Centre for Health Sciences, Department of
Clinical Medicine, Institute of Molecular Medicine,
St. James's Hospital, James Street, Dublin 8, Ireland

M. Roesslein · P. Wick
Laboratory for Materials - Biology Interactions, Swiss
Federal Laboratories for Materials Research and Testing
(Empa), Lerchenfeldstrasse 5, 9014 St Gallen,
Switzerland

G. Suarez · J.-J. Sauvain · M. Riediker
Institute for Work and Health, Rte. de la Corniche 2,
1066 Epalinges-Lausanne, Switzerland

S. Capracotta
NanoSight USA, 1415 Washington Heights, Rm 6611,
Ann Arbor, MI 48109, USA

Z. Magdolenova · S. Manø · M. Dusinska
Health Effects Laboratory, Department of Environmental
Chemistry, Norwegian Institute for Air Research,
Instituttveien 18, P.O. Box 100, NO-2027 Kjeller, Norway

Introduction

Nanotechnology is rapidly developing new applications and advanced materials into many manufacturing areas from information technology, energy storage and harvesting, to radically new medical technologies. The projected market figure for nanotechnology incorporated in manufactured goods by 2020 is approximately 3,000 billion US dollars worldwide (Roco 2011). Such exponential global growth is, however, calling for responsible and quantitative evaluation of the development of manufacturing nanomaterials and its associated metrology. This is particularly true since nanomaterials have unique physical and chemical properties that are useful for various consumer and industrial applications, but these very same properties may give rise to unique biological reactivity. This has led to mounting concerns over the safety of nanomaterials and pressure to control the potential risks (Schrurs and Lison 2012). To ensure compliance with environmental protection guidelines (OECD 2009) nanoparticles (NPs) produced, either directly or indirectly, must be fully characterised (Hassellöv et al. 2008; Tiede et al. 2009). This is fundamental in all areas of research and industry.

Among the different properties which need full characterisation, the size of NPs and the quality and stability of their dispersion often have a profound effect on their interactions with organisms and the environment. It has been extensively reported that NP response to the surrounding environment is size dependent (Jiang et al. 2008; Tenzer et al. 2011) due to their large surface area that interacts with their

surrounding matrix, and this can influence their reactivity with toxicity targets in the cells (Lison and Huaux 2011; Tsao et al. 2011). This has been found to be particularly relevant when the NPs are used for targeted applications. For instance, gold NPs have been used in medical application as contrast agents or nanocarriers (Tong et al. 2009; Kim et al. 2010), polystyrene NPs are a good model for diagnostics and environmental applications due to their relatively well-defined size and low cost (De Jong and Borm 2008; Fritz et al. 1997), silica NPs as drug delivery carriers due to their size-dependent toxicity (Greish et al. 2012; Lin and Haynes 2010; Mohamed et al. 2012) and iron oxide nanoparticles (SPIONS) as therapeutics carriers (Prina-Mello et al. 2013). Therefore, when working with NPs, it becomes crucially important to fully characterise the NPs physico-chemical properties and their interaction with the surrounding matrix or environment (Montes-Burgos et al. 2010; Warheit 2008). This is particularly true when investigating the efficacy and biodistribution of NPs *in vivo* where it is extremely difficult to validate any mechanism of interactions or kinetics from the biodistribution data without having accurate particle size distributions (Gaumet et al. 2008). Therefore the dispersion state of the NPs in solution becomes a main parameter to be investigated which can be correlated to particle stability, and subsequent shelf life and efficacy (Hassellöv et al. 2008). Thus, the implications are not only limited to biological applications but also to environmental and ecological perspectives, as well as regulatory (Elsaesser and Howard 2012; Hanna et al. 2013).

L. Horev-Azaria · R. Korenstein
Department of Physiology and Pharmacology,
Sackler School of Medicine, Tel Aviv University,
69978 Tel Aviv, Israel

A. Dybowska
Department of Earth Sciences, Natural History Museum,
Cromwell Road, London SW7 5BD, UK

L. Cooke · S. Anguissola
Centre for Bio-Nano Interactions (CBNI), University
College Dublin, Belfield, Dublin 4, Ireland

A. Haase · A. Luch
Experimental Research, Department of Product Safety,
Bundesinstitut für Risikobewertung (BfR),
Max-Dohrn-Strasse 8-10, 10589 Berlin, Germany

S. Contal · A. C. Gutleb
Département Environnement et Agro-biotechnologies
(EVA), Centre de Recherche Public Gabriel Lippmann,
41, rue du Brill, 4422 Belvaux, Luxembourg

A. Vennemann · M. Wiemann
IBE R&D Institute for Lung Health gGmbH,
Mendelstrasse 11, 48149 Münster, Germany

In fact, it has been extensively reported by many studies that the nature of the media in which the NPs are dispersed is driving their response and interaction at a biological level (Lison and Huaux 2011; Schrurs and Lison 2012). Several reports have defined and characterised some aspects of this bio–nano interaction through association with the NP physiochemical properties (Nel et al. 2009); particle size being the starting aspect for aggregation consideration, formation of protein coronas (Casals et al. 2010; Cedervall et al. 2007) and subsequent shielding of targeting moieties resulting in loss of NP specificity (Salvati et al. 2013).

Several techniques have been developed for measuring particle size, shape and dispersity from a suspension of particles; electron microscopy (EM), dynamic light scattering (DLS), disc centrifugation, Coulter principle and NTA amongst others. Some of these are labour intensive and time demanding whereas others are cost effective and user friendly. The most frequently used and user friendly NP size characterisation technique is DLS (Filipe et al. 2010). This technique measures the fluctuations in scattered light intensity caused by NPs moving under Brownian motion (Friskén 2001). However, the analysis is weighed towards larger particle size, and as a result, the presence of NP aggregates will bias the particle size distribution, resulting in inaccurate size determination. The use of NTA, with a lower concentration detection limit compared to DLS, analysing NPs on a particle by particle basis, offers a new method for the visualisation and characterisation of NPs in suspension.

The QualityNano project is a European Union Framework Programme 7 funded infrastructure project for developing best practice and innovation in nanomaterial safety testing. One fundamental activity of the QualityNano project is the establishment of quality control and quality assurance conditions for nanomaterial safety and assessment. As technologies and methodologies develop in this area, and the number of end-users increases, the validity of the methodologies and standards needs to be continuously monitored and updated to the most stringent industrial requirements. Quality standards can only be achieved by enlarging statistical numbering and introducing multivariate analysis. This can be achieved by adopting a systematic approach to parametrically assess the interlaboratory, inter-batches and multi-users variation. NTA has only been commercialised since 2006 (Filipe et al. 2010) but

the technique has, in recent years, grown rapidly in its adoption and use, with over 600 systems and 800 third party papers, consolidating the technique across many areas of application, such as therapeutic NP characterisation, developing solutions for diagnostics (e.g., exosomes), drug delivery and cancer treatment, development of bioanalytical assays, vaccine characterisation and nanotoxicology. Therefore the adoption of an interlaboratory comparison (ILC) to validate the NTA technique, by assessment of panels of nanomaterials from within the QualityNano project, derives from the extensive knowledge accumulated and shared between the QualityNano partners and NanoSight Ltd., Amesbury, UK, the company that manufactures the NTA systems. NTA performance is therefore assessed by a structured ILC using defined samples measured by multiple laboratories in order to ascertain the competence of the laboratory, the quality of the standard operating procedures (SOPs) and the reproducibility of the technique. Furthermore, through the use of defined protocols for measurement of standardised samples, the ability of the different NTA systems under evaluation to produce reliable and reproducible results can be investigated (multi-user's variation). It is therefore important to highlight that the uncertainty in the validity and consistency of results could have important implications for the determination of the effects of NPs since different results could be generated when applying different starting dispersions, thus confusing the outcomes. During NP characterisation, using best practice in-house developed SOPs does not always guarantee consistency with other laboratories, as previously shown in Roebben's study for the DLS technique (Roebben et al. 2011). The recommendation for adopting an ILC study is therefore ideal for both developing and testing protocols, as demonstrated in the study presented here, which has been strongly supported within the QualityNano consortium by NanoSight. The authors in this manuscript adopted the ILC principles for assessment from Roebben and coworkers and transferred this to the NTA technique. For instance, this study adopted the use of National Institute of Standards and Technology (NIST) traceable NPs and the supply and modification of protocols following each analysis round. Reproducibility analysis was carried out using classical statistical analysis based around the arithmetic mean values using the ISO 5725-2 approach (ISO 1994) and robust statistical analysis centred around methods described in ISO 5725-5 (ISO 1998). The ILC was also assessed in

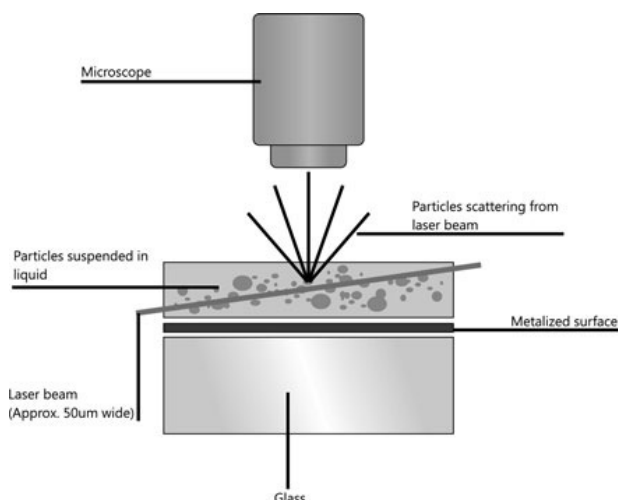


Fig. 1 Schematic of the optical configuration used in NTA

its inter-batch and multi-users variation carried out over four rounds using blind samples and well-defined SOPs to produce a protocol for NP size characterisation using NTA in order to reinforce the robustness of the NTA technique, its standardisation and effort towards defining regulatory guidelines.

NTA: technique principle and details

NTA utilises the properties of both light scattering and Brownian motion in order to obtain particle size distributions of samples in liquid suspension. A laser beam (of arbitrary wavelength but typically those available from laser diodes operating at 635, 532, 488, or 405 nm) is passed through a prism-edged glass flat within the sample chamber. The angle of incidence and refractive index of the glass flat are designed to be such that when the laser reaches the interface between the glass and the liquid sample layer above it the beam refracts, resulting in a compressed beam with a reduced profile and high power density. The particles in suspension in the path of this beam scatter light in such a manner that they can be easily visualised via a long working distance, 20× magnification microscope objective, fitted to an otherwise conventional optical microscope. Onto this is mounted either a charged coupled device (CCD), electron multiplied charged coupled device (EMCCD) or high-sensitivity complementary metal–oxide–semiconductor (CMOS) camera, operating at approximately 30 frames per second (fps), which captures a video file of the light scattered by particles moving under Brownian motion, within a field of view of approximately 100 µm × 80 µm × 10 µm (Fig. 1).

Within the field of view, particles are seen moving under Brownian motion, either directly by eye using the microscope oculars or via the camera. The proprietary NTA software records a video file (of typically 30–60 s duration) of the particles viewed and then simultaneously identifies and tracks the centre of each particle on a frame-by-frame basis. The image analysis software then determines the average distance moved by each particle in the x and y directions. This value allows the particle diffusion coefficient (D_t) to be determined, from which, if the sample temperature (T) and solvent viscosity (η) are known, then the sphere-equivalent hydrodynamic diameter (d) of the particles can be identified using the Stokes–Einstein equation (Eq. 1).

$$D_t = \frac{TK_B}{3\pi\eta d} \quad (1)$$

where K_B is Boltzmann's Constant.

Brownian motion occurs in three dimensions but NTA observes motion only in two dimensions. However, the fact that three-dimensional Brownian motion is tracked only in two dimensions is accounted for by the use of the following variation of the Stokes–Einstein equation (Eq. 2). It is possible, however, to determine D_t from measuring the mean squared displacement of a particle in one, two or three dimensions (Eq. 2, respectively).

$$\begin{aligned} \overline{(x^2)} &= \frac{2TK_B t}{3\pi\eta d} & \overline{(x, y)^2} &= \frac{4TK_B t}{3\pi\eta d} \\ \text{a} & & \text{b} & \\ \overline{(x, y, z)^2} &= \frac{2TK_B t}{\pi\eta d} & & \\ \text{c} & & & \end{aligned} \quad (2)$$

Thus, in the case where measurement of movement in two dimensions is made; Eq. 2b is employed.

One crucial advantage that NTA has over other measurement techniques is that it is not biased towards larger particles or aggregates. The software is based on the tracking of single particles, whereas typical DLS techniques place a strong bias on the largest particles present in the sample (Filipe et al. 2010). NTA therefore allows for the detection of secondary peaks, which may not be detectable or resolvable using other traditional measurements.

NTA: standardisation

Early steps towards standardisation of NTA have recently been demonstrated by an ASTM guidance

Table 1 History of development of NTA software showing list of parameters along with the number of user adjustable settings for each parameter

Part of analysis	Year NTA Version	2009 2.0	2010 2.1	2011 2.2	2012 2.3
Data collection	Capture time	215	215	5	5
	Shutter	1500	1500	1	1
	Camera gain	680	680	16	16
	Gamma	2	1	1	1
Data analysis	Brightness	186	186	1	1
	Image gain	1000	1000	1	1
	Blur	5	4	1	1
	Detection threshold	188	100	50	20
	Max blob size	3000	1	1	1
	Min track length	183	1	1	1
	Min expected particle size	9	9	4	1
	# Free user setting	11	8	4	3
	# Variations	3.8×10^{23}	1.5×10^{17}	1.6×10^4	1.6×10^3

paper published about the technique (ASTM 2012) providing an overview of the methodology to be followed for good practice and discussing aspects such as principles and limitations of the method, considerations of sampling, necessary concentration and interpretation of results, with particular reference to comparison with other techniques. An International Standardisation Community at ISO TC24 welcomed the submission of a new work item proposal on Particle Tracking Analysis (PTA)/NTA at their recent meeting (ISO 2013).

Standardisation of the NTA technique has previously been difficult to achieve due to the number of software options that a user has been required to choose in order to make a measurement. This was highlighted in an excellent paper comparing DLS and NTA (Filipe et al. 2010) which highlighted this issue of subjectivity of NTA results with user settings. Table 1 summarises the development of the software since that point, showing the progression of algorithm development allowing these settings to be effectively automated.

The ILC assessment design in this study employs the NTA2.3 software version which had three settings that users should set. These were: (1) capture time (unified for all laboratories and users by supplied SOP), (2) camera level and (3) detection threshold (guidance provided for 2 and 3 by the supplied SOP).

NTA: regulatory aspect

It is expected that the outcomes of ILC studies of the NTA technique could contribute to the defining of

regulatory policy and guidelines for the adoption of safe nanotechnology procedures in line with the goals of QualityNano, through the generation of SOPs for the accurate and reproducible size characterisation of NPs dispersed in water-based solvents.

Materials and methods

Test materials

NIST traceable polystyrene nanospheres, nominally 100 and 200 nm (1 % solids), were purchased from Thermo Fisher Scientific Inc., whilst gold NPs, nominally 60 and 80 nm (0.01 % solids), were purchased from BB International, Cardiff. Ham's F10 nutrient mix and Bovine Serum Albumin (BSA) were purchased from Sigma Aldrich Co. HPLC grade water was purchased from Rathburn Chemicals Ltd., Scotland. 0.02 µm Anotop 25 syringe filters (Whatman GmbH, Germany) were used to filter water and BSA samples prior to analysis.

Samples preparation

Samples were aliquoted from a single lot prior to the start of each round (either pre-diluted or neat), ensuring all participating labs received aliquots of the same materials. Rounds 1 and 2 were carried out on the same four monodisperse samples, dispersed in water, round 3 analysed three monodisperse samples, again dispersed in water. Round 4 included a bimodal

Table 2 Characteristics of nanoparticle samples used in the study

Particle composition	Source	Round robin	Nominal size (nm)	Initial concentration (% solids)	Diluent
Gold	BBI	1 and 2	30 ± 2 (TEM)	~0.01	H ₂ O
Carboxylated polystyrene	Invitrogen	1 and 2	100 ± 11 (TEM)	~0.1	H ₂ O
Aminated polystyrene	Polysciences	1 and 2	100	~0.1	H ₂ O
Silica	Polysciences	1 and 2	100	~0.1	H ₂ O
Polystyrene	Thermo Scientific	3	102 ± 3 (TEM)	~1	H ₂ O
Gold	BBI	3	60 ± 3 (TEM)	~0.01	H ₂ O
Gold	BBI	3	81 ± 4 (TEM)	~0.01	H ₂ O
Polystyrene	Thermo Scientific	4	102 ± 3 (TEM)	~1	H ₂ O, Ham's F10 Nutrient Mix, BSA
Polystyrene	Thermo Scientific	4	203 ± 5 (TEM)	~1	H ₂ O
Gold	BBI	4	81 ± 4 (TEM)	~0.01	H ₂ O

sample, and monodisperse nanospheres dispersed in biological media with and without the addition of BSA. The details of the composition and characteristics of the samples used in the present study are summarised in Table 2.

All samples were distributed from NanoSight. Samples for rounds 1 and 2 were prepared by University College Dublin, samples for rounds 3 and 4 were prepared by NanoSight. Protocols were all developed and distributed by NanoSight. Effort was taken to ensure samples were always analysed blind by participants, however, the solvent and the material were known, for MSDSs to be distributed.

Participants and systems

Twelve Laboratories from Europe and the USA participated in the four rounds of the ILC using instruments from NanoSight Ltd., Amesbury, UK, between September 2012 and April 2013 as per manuscript authorship. Laboratories were each allocated a unique participant number allowing results to remain anonymous. The participants in this comparison were not pre-screened in any way, some users having received no direct training on the system in their laboratory. The key characteristics of the group were that they were eager to ensure that they were using their systems correctly and to acquire reproducible results. Participants were given no additional training, although where issues/outlying results were identified, they were investigated thoroughly to establish the root

cause, the participants informed and the protocol appropriately improved (where possible).

Details of the NanoSight platforms, laser wavelengths and camera type used by each laboratory are given in Table 3. Laboratories reported results in the form of summary files (output by NTA) for each sample. Results were collated and analysed by NanoSight.

Protocol Development

Results were disseminated to all partners following rounds 2, 3 and 4. This allowed discussion with partners about how to proceed and allowed the group's

Table 3 NanoSight systems used for ILC by participant number

Lab code	Platform	Camera	Laser wavelength (nm)	Temperature control
1	LM10	sCMOS	532	Yes
2	NS500	EMCCD	532	Yes
3	NS500	sCMOS	405	Yes
4	NS500	sCMOS	405	Yes
5	LM20	CCD	635	No
6	LM10	CCD	635	No
7	LM10	CCD	635	No
8	LM20	CCD	635	No
9	NS500	sCMOS	405	Yes
10	LM20	CCD	635	No
11	LM10	EMCCD	405	Yes
12	LM10	sCMOS	405	No

expertise to be employed. Choice of samples for a round was only made following the collection and analysis of the results from the preceding round. This allowed an assessment of the progress, the choice of appropriate samples to challenge progress and time for development/improvement of the protocol.

ILC round 1

A preliminary ILC (round 1) was performed on 4 samples (nominally gold 30 nm, 100 nm carboxylated polystyrene, 100 nm aminated polystyrene and 100 nm silica), without any protocol provided. Participants analysed the samples according to their own 'in-house' protocols for using NTA. This highlighted a number of issues, such as the need to perform replicate measurements on each sample and a need to ensure sample dilution to an appropriate concentration for the NTA system, which were incorporated into the protocol for subsequent rounds.

ILC round 2

Round 2 analysed the same samples as round 1, but under a protocol developed to standardise methods between participating laboratories. The protocol covered sample handling and storage, sample preparation (standard dilutions for each sample), video capture (60 s duration) and data analysis and export. Each laboratory analysed each sample in triplicate to allow statistical analysis under repeatability conditions.

ILC round 3

The results and particle sizes from rounds 1 and 2 being known by the participants, three new samples were supplied for analysis in round 3 (nominally 100 nm polystyrene, 60 nm gold and 80 nm gold), along with an updated protocol, which was amended in light of the results for round 2. Samples were shipped with a temperature sensor which indicated if the contents were exposed to temperatures below 4 °C. Laboratories were also supplied with a range of disposables, including 0.02 µm syringe filters and HPLC grade water. Updates to the protocol included a system recalibration step (if required), software update to the newest version of NTA, vibration checks (to prevent mis-sizing), and recording six

replicate videos for each sample to improve statistical analysis.

ILC round 4

Unlike previous rounds, where all the samples were monodisperse nanospheres diluted in water, round 4 investigated both a bimodal sample (80 nm gold and 200 nm polystyrene) and monodisperse nanospheres (100 nm polystyrene) suspended in biological media (Ham's F10 nutrient mix) and biological media supplemented with BSA (Ham's F10 nutrient mix plus 5 mg/ml BSA). This allowed analysis of samples in more complex aqueous solutions than water. Samples were shipped with two temperature sensors, to indicate if the contents were exposed to temperatures below 4 °C or above 29 °C.

Results and discussion

Laboratories submitted reports containing modal sizes for each replicate of each sample run, along with the particle size distributions, with outlying results being discarded [consisting of 0 (of 48), 6 (of 48), 0 (of 36) and 3 (of 44) from rounds 1, 2, 3 and 4, respectively]. During each of the rounds where technical causes could be unambiguously identified or it was ascertained that a participant was not fully complying with the protocol, these results were discarded. This was (usually) assumed to be a weakness of the protocol, which was then modified to better ensure compliance in future ILC rounds.

Method reproducibility analysis was carried out using methods laid out in ISO 5725-2 (ISO 1994). The modal sizes obtained for each sample from rounds 2, 3 and 4 were analysed using both classical [average and standard deviation (SD) based] and robust [median and median absolute deviation based (MAD)] statistics as per ISO 5725-5 (ISO 1998). Robust statistics has the advantage that it is much less sensitive to outliers and so a median value better represents the centre of a distribution of results reported from different laboratories. In addition, the SD describing a second order effect is considerable more sensitive towards results that are further away from the mean value, and are therefore potential outliers, than towards all the results that are close to the mean value. MAD, as median absolute deviation, is less sensitive to large deviations at the extremes.

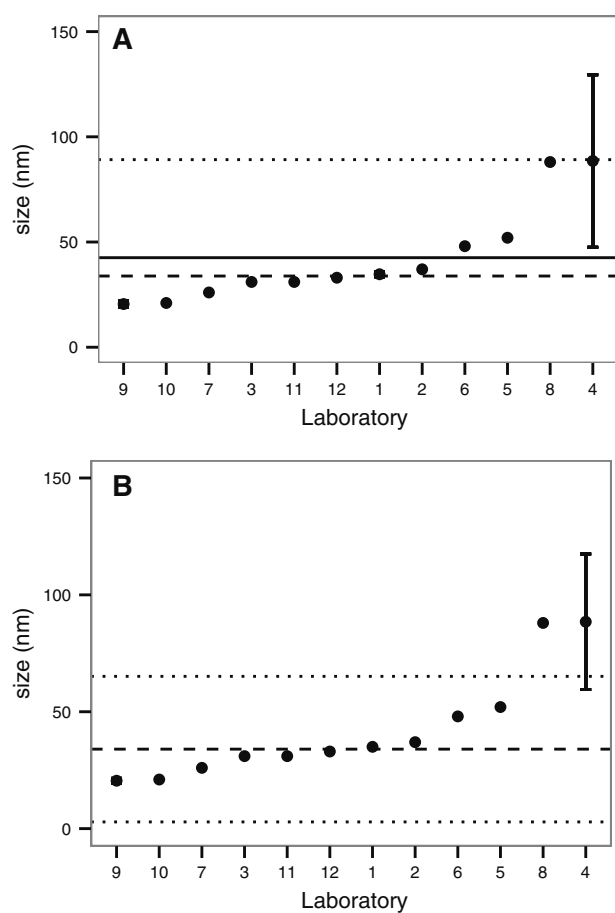


Fig. 2 Modal particle size for 30 nm gold particles from ILC round 1 showing results from each partner ordered by increasing modal value based on **a** classical (± 2 SD) and **b** robust (± 2 MAD) statistical analysis. The mean value (solid line), median (dashed line) and 95 % confidence intervals (dotted line) are also shown

Analysis of the results obtained from ILC round 1 shows the variation and lack of reproducibility of results from the 12 laboratories when no protocol was supplied. Figure 2 summarises the modal size results obtained during ILC round 1 on 30 nm gold nanospheres based on standard (a) and robust (b) statistical analysis. It appears clear from Fig. 2 that results from 2 of 12 laboratories do not match with the data of the other 10 partners. Therefore, in the case of classic statistical analysis the mean value is biased due to the presence of these outlier results (Fig. 2a) whereas most of the values are close to the median value. As expected, this discrepancy between mean and median values is considerably reduced by the robust statistical data treatment, which is typically more appropriate for such a study (Fig. 2b). The range of the 95 % confidence interval is also smaller. This poor reproducibility of an interlaboratory test, as indicated by a

coefficient of variation of 54.8 %, can easily be attributed to the absence of guidance in the ILC round 1 which included users who had had no training (either internal or external) in the technique or on their system. The lack of an adequately defined measurand in ILC round 1 resulted in an excessive uncertainty of the measurement, as predicted by the guide to the uncertainty in measurement (GUM) (JGCM 2008).

Between the last 3 rounds, the protocols were upgraded on the basis of issues encountered in the preceding round, covering all the aspects of the experimental process: sample shipping (temperature sensors), sample handling and preparation (e.g., dilution), sample storage, control of measurement parameters (e.g., video control, detection threshold), software update (e.g., vibration check) and analytical data treatment (e.g., number of replicates, statistic models).

The positive impacts that a well-established protocol and good compliance exert on the robustness of the NP size determination with NTA technique is further emphasised when we consider the calculated coefficient of variation as a comparative parameter between the different ILC rounds. The percentage coefficients of variation are summarised in Table 4 showing a

Table 4 Percentage coefficient of variation for each sample in the ILC

ILC Round	1 (% CV)	2 (% CV)	3 (% CV)	4 (% CV)
30 nm gold	54.8	10.5		
100 nm carboxylate polystyrene	33.3	9.3		
100 nm aminated polystyrene	31.2	15.9		
100 nm silica	34.5	10.0		
100 nm polystyrene			3.5	3.1
60 nm gold			5.1	
80 nm gold			4.1	
100 nm polystyrene + nutrient Mix				3.7
100 nm polystyrene + nutrient mix + BSA				4.7
80 nm gold (in bimodal sample)				5.5
200 nm polystyrene (in bimodal sample)				5.2
Average (% CV)	38.5	11.4	4.2	4.4
				3.8 ^a

^a Considering only monodisperse samples

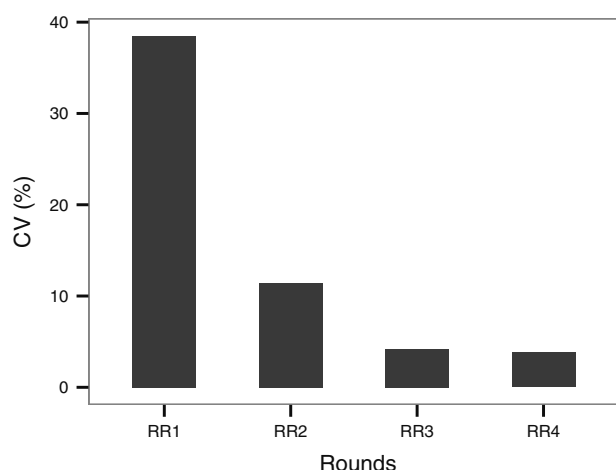


Fig. 3 Evolution of the average percentage coefficient of variation from ILC round 1 to ILC round 4. The percentage coefficient of variation is shown to decrease with each round due to improvements within the protocol

clear link between the improving reproducibility of measurements and successive rounds. The averaged percentage coefficient of variation calculated for monodisperse samples progressively decreases from ILC round 1 to round 4: 38.5, 11.4, 4.2 and 3.8 % (Fig. 3).

Once again, the combination of a powerful analytical technique with improved protocols gave rise to robust and reproducible determination of the modal size of both monodisperse and bimodal nanosphere samples prepared in water and complex biological matrices.

Standard and robust analyses were established with participant results from all ILC rounds. In the case of ILC round 4, the modal size results obtained on a nominal 100 nm polystyrene nanospheres based on classical and robust statistics are reported in Fig. 4a and b, respectively. In that round, the results obtained for 10 laboratories out of a total of 11 are within the 95 % confidence interval and so match with the mean and median values, for both classical and robust statistical analyses. The range of the 95 % confidence interval is dramatically decreased in comparison to the one obtained in ILC round 1. As mentioned earlier, the very low coefficient of variation calculated at ILC round 4 for monodisperse solution of polystyrene nanosphere (3.1 %) strongly emphasises the effect of a well-established SOP for highly reproducible and accurate NTA measurements.

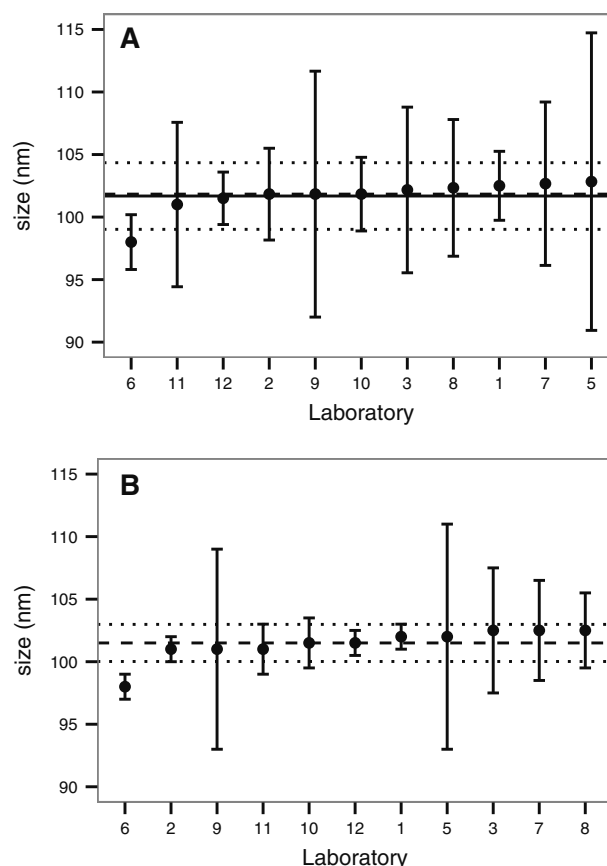


Fig. 4 Modal particle size for 100 nm polystyrene particles from ILC round 4, showing results from each partner ordered by increasing modal value based on **a** classical (± 2 SD) and **b** robust (± 2 MAD) statistical analysis. Mean (solid line), median (dashed line) and 95 % confidence intervals (dotted line) are also shown

By ILC round 4, the averaged modal particle size distribution resulting from the analysis of 11 laboratories (one laboratory failed to supply data) on monodisperse polystyrene nanospheres (100 nm) diluted in water clearly demonstrates the analytical reliability and power of the NTA technique, when supported by a well-defined SOP, as shown in Fig. 5. The average modal size and coefficient of variation corresponding to this set of measurements were calculated to be 101.7 nm and 3.12 %, respectively. Similarly, during the same round bimodal samples containing a mixture of nominally 80 nm gold (77–85 nm, TEM) and 200 nm polystyrene (203 $\pm$ 5 nm, TEM) nanospheres were also analysed and the modal particle size distributions obtained by 11 laboratories are depicted in Fig. 6. In that case, the calculated average modal sizes were

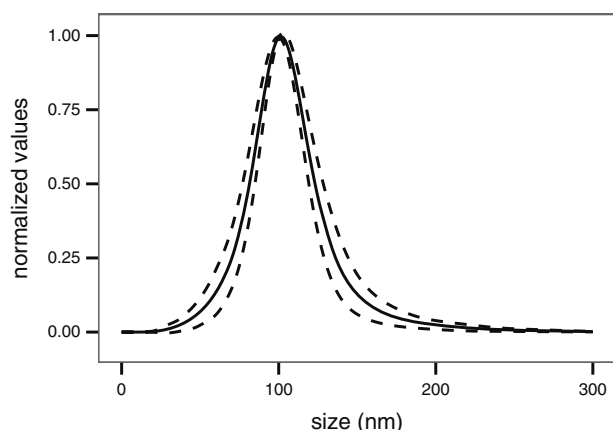


Fig. 5 Averaged particle size distribution of nominally 100 nm polystyrene nanospheres in water analysed by 11 laboratories in ILC round 4. Size distribution ± 1 SD is represented by the *two dashed lines*

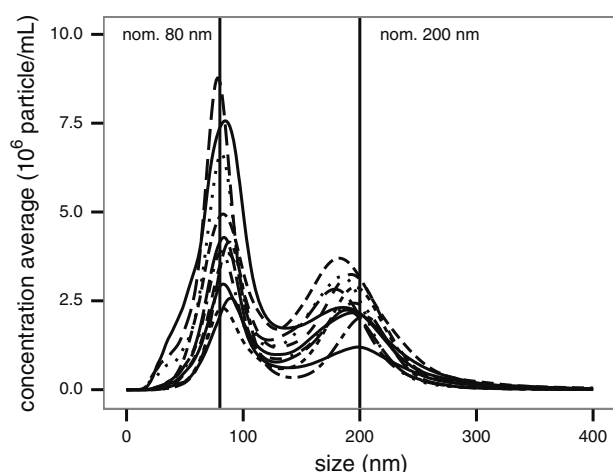


Fig. 6 Averaged particle size distributions of a bimodal mix of nominally 80 nm gold and nominally 200 nm polystyrene nanospheres in water analysed by 11 laboratories in ILC round 4

84.0 and 190.7 nm with corresponding coefficients of variations of 5.47 and 5.16 %, respectively. Both the relatively high NP size measurement accuracy and reproducibility confirm that NTA technique is also well adapted for the size analysis of bimodal samples.

Conclusion and outlook

Overall, it is clear that in the absence of shared guidelines on how to use a system and prepare samples, variability across laboratories can be large even for relatively monodispersed samples (ILC1). The definition of a shared protocol allowed improved

results to be obtained (ILC2), however, the ILC exercise clearly outlines that even a well-defined protocol (much more complete and detailed than what is usually described in the experimental section of published work) can still lead to a (~ 4 % CV) variability of outcomes. This has clear implications for the on-going debate on the definition of guidelines for reporting research in this field.

The ILC has shown that, with the guidance of a well-written protocol, users of NTA (even those with little or no training on the instrument) are able to obtain reproducible and accurate modal particle size results on a range of samples, both monodisperse and bimodal, on samples dispersed in water or biological media and on particles of different sizes and material composition. It is important to note here that the samples used in this study were all nominally spherical.

This study has shown that a well-designed and focused series of round robins can help identify relevant issues that have a significant effect on measurement results. It has been demonstrated to be an effective way of improving the understanding of the basics of measurement techniques such as NTA. This leads to a better specification of the measurand, and therefore to a more elaborate and more useful protocol, detailing important steps in the measurement procedure.

Future work will include additional rounds involving more complex mixtures and matrices and extension to other measurands that will help improve understanding of the effect of additional parameters that are relevant for these advanced measurement scenarios. With this, the current performance characteristics of NTA can be further determined. This may initiate new developments that remove the current limiting factors. All this would not have been possible without a soundly designed series of round robins. It is envisaged that the challenges to be faced to achieve similar outcomes for biological testing of NP–cell interactions could be even greater, because of the complexity of living organisms and difficulty in controlling such experiments to this level.

Further steps could also be the direct comparison of NTA measurements with other sizing techniques such as measurements based on DLS, which is known to have certain limitations, but is a more established technique.

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Original Article

Heparin conjugated quantum dots for *in vitro* imaging applications

Ciaran Manus Maguire, MSc^a, Omar Kazem Mahfoud, MSc^a, Tatsiana Rakovich, PhD^a,
Valerie Anne Gerard, PhD^b, Adriele Prina-Mello, PhD^{c,d,*}, Yurii Gun'ko, PhD^{b,d},
Yuri Volkov, PhD, MD^{a,d}

^aNanomedicine and Molecular Imaging Group, Department of Clinical Medicine, Institute for Molecular Medicine, St James' Hospital, Dublin 8, Ireland

^bNanomaterials Research Group, School of Chemistry, Trinity College Dublin, Dublin 2, Ireland

^cNanomedicine and Molecular Imaging Group, Department of Clinical Medicine, Institute for Molecular Medicine, St James' Hospital, Trinity College Dublin, Dublin, Ireland

^dCentre for Research on Adaptive Nanostructure and Nanodevices (CRANN), Trinity College Dublin, Ireland

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Abstract

In this work heparin-gelatine multi-layered cadmium telluride quantum dots (QD_{gel/hep}) were synthesised using a novel 'one-pot' method. The QDs produced were characterised using various spectroscopic and physiochemical techniques. Suitable QDs were then selected and compared to thioglycolic acid stabilised quantum dots (QD_{TGA}) and gelatine coated quantum dots (QD_{gel}) for utilisation in *in vitro* imaging experiments on live and fixed permeabilised THP-1, A549 and Caco-2 cell lines. Exposure of live THP-1 cells to QD_{gel/hep} resulted in localisation of the QDs to the nucleus of the cells. QD_{gel/hep} show affinity for the nuclear compartment of fixed permeabilised THP-1 and A549 cells but remain confined to cytoplasm of fixed permeabilised Caco-2 cells. It is postulated that heparin binding to the CD11b receptor facilitates the internalisation of the QDs into the nucleus of THP-1 cells. In addition, the heparin layer may reduce the unfavourable thrombogenic nature of quantum dots observed *in vivo*.

From the Clinical Editor: In this study, heparin conjugated quantum dots were found to have superior imaging properties compared to its native counterparts. The authors postulate that heparin binding to the CD11b receptor facilitates QD internalization to the nucleus, and the heparin layer may reduce the *in vivo* thrombogenic properties of quantum dots.

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Key words: Multi-layered; Heparin; Quantum dots; Imaging applications

Background

Semiconductor nanocrystals or quantum dots (QDs) are the focus of extensive research in the field of nanoscience due to their unique physical properties and a wide range of potential

applications in photonics, solar cells, biotechnology, medicine and imaging.

QDs have been shown to have numerous advantages over traditional organic dyes.¹ The quantum confinement effect in the quantum dots results in size dependent optical properties, namely tuneable UV-vis absorbance and photoluminescence spectra.² The size of the QDs can be controlled by increasing or decreasing the reflux time during synthesis.³ They also have longer fluorescence lifetimes, possess high photobleaching thresholds⁴ and have a long Stokes shift compared to organic dyes, which provides opportunities for use in confocal microscopy and Raman imaging. In addition, QDs have good stability in water and can be readily functionalised with various biological molecules such as antibodies⁵ or small molecules such as folic acid⁶ and naproxen.⁷

The highly luminescent properties of the quantum dots make them particularly useful for cell labelling and imaging studies. For biomedical applications, nanoparticles, in most cases, must be appropriately functionalised. The modification and functionalization

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*Corresponding author at: Lab 0.74, Nanomedicine and Molecular Imaging Group, Department of Clinical Medicine, Institute for Molecular Medicine, St James' Hospital, Dublin 8, Ireland.

E-mail address: prinamea@tcd.ie (A. Prina-Mello).

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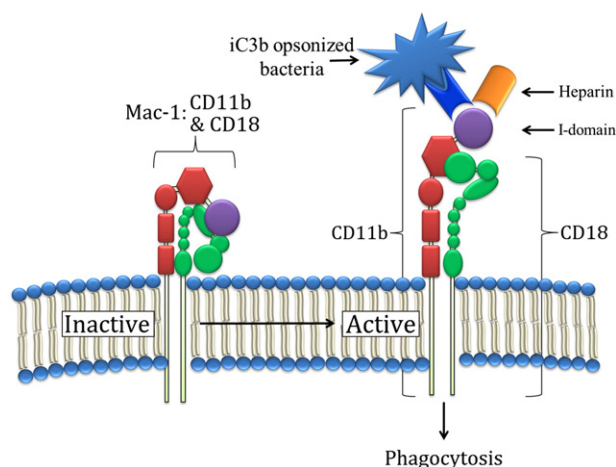


Figure 1. Heparin shares a binding site in the I-domain of MAC-1 with iC3b and as such binding of heparin to the CD11b receptor may induce phagocytosis.

of the QDs can provide advantages in the selective labelling of specific markers, with applications for the discrimination of multiple cells types, similar to the use of traditional fluorescent dyes in flow cytometry.⁸ The multilayering of material onto the surface of QDs, as shown in Figure 1, has implications in improved targeting and detection of features of interest.⁹ The use of biocompatible and surface-passivating coatings such as gelatine³ and poly-L-lysine¹⁰ provide an opportunity for using QDs as probes for imaging and targeting of cancer cells.

One of the most common cell types used in studies with nanomaterials are monocytes-derived macrophages. Monocyte cells originate in the bone marrow from a common myeloid progenitor and are released into the peripheral blood¹¹ followed by differentiation into macrophages in the tissue.¹² Macrophages are phagocytic cells that function in both the innate and adaptive immune pathways and play a crucial role in the clearance of infection, damaged, pathologically transformed or senescent cells and in the repair of tissue following inflammation.¹¹ Macrophages can be identified by the presence of specific proteins such as cluster of differentiation (CD) 14, CD11b and Macrophage-1 antigen (Mac-1). Mac-1 is a complement receptor which consists of non-covalently linked chains of CD11b and CD18.¹³ It is a pattern recognition receptor which is capable of binding many molecules found on the surface of bacteria, commonly proteins with an arginine-glycine-aspartate (RGD) sequence. Phagocytosis of the bacteria is caused by the binding of the Pathogen-Associated Molecular Pattern (PAMP) to the receptor.

One of the key ligands for Mac-1 is heparin, through its binding with CD11b.¹³ Heparin is an anticoagulant that is commonly used in biology and medicine.¹⁴ It is a polymer with a size range from 3–30 kDa, with chains in the 12–15 kDa size range most commonly used. The anti-coagulative nature of heparin is due to its binding to the inhibitory enzyme, antithrombin. Antithrombin inhibits pro-coagulant enzymes by forming a kinetically stable complex with them.¹⁵ Heparin accelerates this reaction, possibly through the alteration of the active site of antithrombin thereby increasing its activity. Most

notably, the alteration in the antithrombin active site on heparin binding increases its affinity for the pro-coagulant enzyme, Factor X_a.¹⁵ Inhibition studies using monoclonal antibodies against structural epitopes on MAC-1 has also showed that heparin interacts with at least one site in the I domain,¹³ the major ligand-binding site in integrins.¹⁶ Heparin can play a role in phagocytosis¹⁷ and in the stabilisation and transport of growth factors into the nucleus of cells.¹⁸ The inactivated complement component 3 fragment b (iC3b), which is involved in opsonisation and clearance of pathogens by macrophages, binds to the Mac-1 I-domain and initiates phagocytosis,¹⁹ but is inhibited by heparin as they share a binding site in the I-domain. As such, the binding of heparin to Mac-1 through CD11b may account for the initiation of phagocytosis (Figure 1).

In order to differentiate monocytes into macrophages, cells are commonly stimulated with granulocyte macrophage colony-stimulating factor (GM-CSF), Interleukin-4 (IL-4)²⁰ or Phorbol-12-myristate-13-acetate (PMA). The undifferentiated immortalised macrophage cell line, THP-1, is most commonly induced to differentiate with PMA, resulting in an altered phenotype. The undifferentiated monocyte cells do not express CD11b, whereas upon PMA activation the THP-1 cells have a higher appearance ($87 \pm 10\%$) of CD11b.²¹

In this study, heparin is incorporated on to the surface of gelatine coated cadmium telluride QDs (QD_{gel}) to form a heparin-gelatine-QD (QD_{gel/hep}) (Figure 2). It is anticipated that heparin will act as a stabilising and transport agent for the QD, allowing the QD to be internalised by the macrophage cell line, THP-1, and subsequent transport into the nucleus. An adherent epithelial colorectal adenocarcinoma cell line, Caco-2, and an adherent epithelial lung carcinoma cell line, A549, will be used as control cell lines as neither cell lines express the CD11b receptor. Caco-2 cells have also been noted for their poor ability to internalise nanoparticles^{6,22} and A549 cells are frequently used in cellular studies using nanoparticles^{23,24} and so provide an opportunity for comparison to previous work.

Methods

CdTe quantum dot synthesis

CdTe quantum dots were synthesized by a method based on that published by Gaponik.²⁵ All samples were stored at 4 °C to prevent decomposition or degradation of the products and preserve colloidal stability.

Characterisation of the QD samples is described in the supplementary materials.

CdTe-gelatine-heparin quantum dot synthesis

QD_{gel/hep} were prepared using a modified, previously published procedure.⁶ 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) activated heparin was prepared by dissolving heparin (51.00 mg) in EDC/4-dimethylaminopyridine (DMAP) solution (3×10^{-3} M). The activated heparin was then added to one half of the quantum dot solution to produce QD_{gel/hep}. Full experimental details are provided in the supplementary materials.

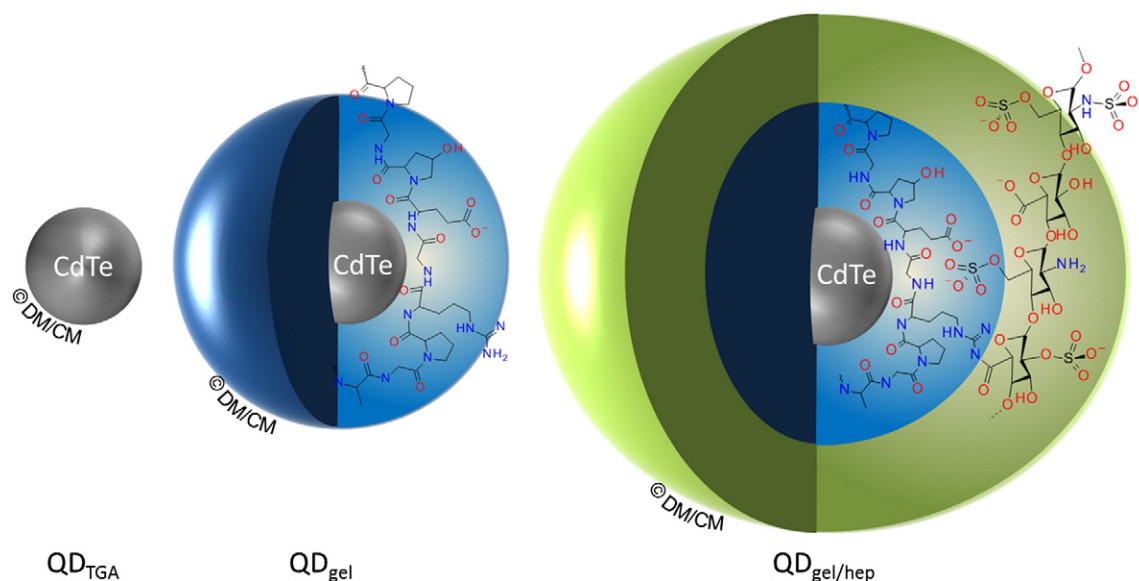


Figure 2. Formation of QD_{gel/hep} from QD_{gel}: Uncoated TGA stabilised cadmium telluride (CdTe) QDs (QD_{TGA}) are first functionalised with a layer of gelatine (QD_{gel}) to reduce toxicity and provide a means of further functionalisation. Heparin is then covalently bound to the surface of the QD_{gel} to form QD_{gel/hep} for biomedical applications.

All samples were stored at 4 °C to prevent decomposition or degradation of the products and preserve colloidal stability.

Characterisation of the QD samples is described in the supplementary materials.

In vitro biological testing

Live cell imaging

THP-1 cells (20,000 cells/well) were seeded in 8-well chambered slides (Lab-Tek, Nunc, Denmark), and differentiated by adding 100 ng/ml PMA for 72 hours. A549 and Caco-2 cells were seeded at a density of 15,000 cells/well and allowed to recover and adhere for 24 hours. All cells were then treated with 5 μM of the QD_{TGA}, QD_{gel} and QD_{gel/hep} solutions for 2 hours at room temperature. The cells were washed with PBS (Gibco, Biosciences) and the nuclei stained using Hoechst 33342 (1.25 μg/ml) (Sigma Aldrich) for 30 minutes at room temperature, washed with PBS and imaged using a Zeiss LSM 510 Meta Confocal Microscope (Carl Zeiss, Germany). Analysis was carried out using LSM Image Browser (Carl Zeiss, Germany) and ImageJ (Open Source).

Fixed cell imaging

THP-1, A549 and Caco-2 cells were plated in chamber slides as described above. All cell lines were then fixed using 3.7% paraformaldehyde (PFA), for 20 min at room temperature, and washed with PBS. All cells were then permeabilised using 0.5% Triton X-100 (Sigma Aldrich) for 15 min, washed, and incubated with 5 μM of QD_{TGA}, QD_{gel} and QD_{gel/hep} for 1 hour at room temperature. Cells were washed with PBS and the nuclei counterstained using Hoechst 33342 (1.25 μg/ml) at room temperature, washed with PBS, and imaged as described in the above paragraph.

Results

The characterisation of the QDs *via* various experimental techniques revealed an increase in QD hydrodynamic size (Table 1 and Figure 3), while the metallic core, as measured by TEM, remained constant, indicating the layering of organic material around the QD core. The increase in hydrodynamic diameter and the zeta potential of the QD_{gel/hep} compared to QD_{gel} alone indicates that heparin has been layered onto the surface, similar to previous work.²⁶ Based on the hydrodynamic diameter of the QD_{gel/hep} and the volume of a sphere, along with the density of heparin,²⁷ the mass of heparin loaded onto each QD could be calculated.

Assuming that heparin forms a uniform layer around the QD_{gel}, whose volume based on DLS results is 124.79 nm³, the volume of the heparin layer can be approximated as:

$$\text{Vol of Heparin layer} = \frac{4}{3} (\text{radius QD}_{\text{gel/hep}}^3) - \text{Volume of QD}_{\text{gel}}$$

yielding a layer of heparin with a volume of $8076.09 \pm 2.0 \text{ nm}^3$. Then based on the density of heparin, an approximation of the concentration of heparin can be made indicating that each QD_{gel/hep} structure will potentially have a layer of heparin with a concentration of $8.81 \times 10^{-9} \text{ ng}$ of heparin per QD.

Fourier Transform Infrared Spectroscopy (FTIR) was also carried out on the QD_{gel}, QD_{gel/hep} and on heparin alone. O-H, C = O and CH₂ stretching was identified at 3060, 1707 and 1424 cm⁻¹, respectively, for all samples. QD_{gel/hep} was noted as having more pronounced C-S stretching frequency at 909 cm⁻¹ compared to QD_{gel} and is attributed to the C-S stretching frequency found in heparin. The spectra are included in the supplementary materials (Figure S4).

Table 1

Summarised characterisation data for QD_{TGA}, QD_{gel} and QD_{gel/hep}. 100 QDs were sized for TEM analysis.

NP	Max absorbance	Max emission	Quantum yield	Conc.	TEM Size (nm) [Std. Dev]	DLS Size (d. nm) [Std. Dev]	Zeta Potential (mV) [Std. Dev]
QD _{TGA}	558 nm	587 nm	35%	49.00 μ M	3.92 [1.04]	4.60 [0.815]	−37.40 [1.515]
QD _{gel}	569 nm	594 nm	36%	20.50 μ M	3.96 [0.94]	6.20 [1.965]	−39.10 [3.265]
QD _{gel/hep}	556 nm	593 nm	32%	19.00 μ M	3.94 [0.95]	25.03 [6.625]	−38.50 [0.895]

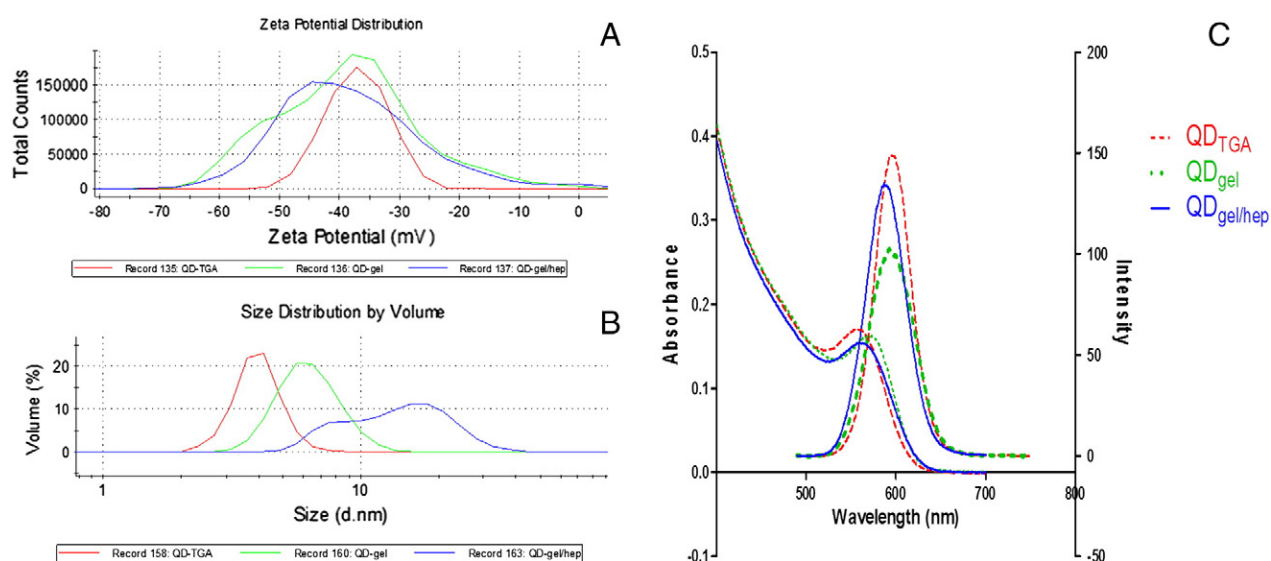


Figure 3. Averaged Zeta Potential (A), DLS (B), UV-vis and PL Spectra (C) of QD_{TGA}, QD_{gel} and QD_{gel/hep} illustrating the more negative zeta potential (A) and increase in hydrodynamic radius (B) of QD_{gel/hep} over QD_{gel} or QD_{TGA}. All three QDs have similar absorbance and emissions spectra (C). Line Colours: Red, QD_{TGA}; Green, QD_{gel}; Blue, QD_{gel/hep}.

Confocal imaging

The uptake of QD_{TGA}, QD_{gel} and QD_{gel/hep} by live and fixed THP-1 macrophages was investigated and compared to the uptake by A549 and Caco-2 cells using confocal microscopy (Table 2 and Figure 4). As shown below, the layer of heparin on the surface of the QD_{gel}, forming QD_{gel/hep} resulted in increased internalisation of the QDs into the cytoplasm and nucleus of the live THP-1 cells compared to QD_{gel} and QD_{TGA} alone (Figure 4, A–C). There was no detectable uptake of the QDs by live Caco-2 cells (Figure 4, G–I), with live A549 cells showing moderate uptake of QD_{gel} (Figure 4, E).

Upon permeabilisation of the fixed THP-1 cells, there was increased passive influx of the QDs. All QDs were visible in the nucleoli of the cells, with QD_{gel/hep} showing the greatest intensity in the nucleus compared to QD_{gel} (Figure 4, J–L). In the case of fixed permeabilised A549 cells, QD_{TGA} remained in the cytoplasm and bound strongly to the nuclear membranes. QD_{gel} and QD_{gel/hep} were highly localised in the nucleus and nucleoli (Figure 4, M–O). High levels of cytoplasmic staining were also visible. Fixed permeabilised Caco-2 cells had no detectable nuclear staining, with all QDs remaining in the cytoplasm (Figure 4, P–R).

Discussion

From the results of the characterisation of the quantum dots, it can be concluded that a gelatine/heparin bilayer was produced that encompassed the CdTe core. The quantum dots produced were of a quantum yield suitable for *in vitro* imaging,^{6,28} with distinct UV-vis and PL maxima (Figure 3). This approach to the formation of modifiable multi-layered QDs has enhanced opportunities for biological imaging and diagnostics applications due to the reduced toxicity,²⁹ improved colloidal stability of nanoparticles and potential for their use in personalised medicine.^{30,31} For the samples used for *in vitro* imaging, QD_{TGA} had a particle core size of 3.92 nm, QD_{gel} had a core size of 3.96 nm and QD_{gel/hep} had a core size of 3.94 nm, as measured using TEM (Supplementary Materials, Figure S1, S2, S3). DLS hydrodynamic size measurements of 4.60, 6.20 and 25.03 nm were recorded for QD_{TGA}, QD_{gel} and QD_{gel/hep}, respectively. The large increase in hydrodynamic radius for the heparin-quantum dots, relative to its core size suggests that a heparin layer was successfully formed on gelatine on the surface of the QDs. The zeta potential of the particles was recorded as −37.40 mV, −39.10 mV and −38.50 mV at pH 8 for QD_{TGA},

Table 2

Uptake and localisation of QD_{TGA}, QD_{gel} and QD_{gel/hep} in live and fixed permeabilised THP-1, A549 and Caco-2 cells.

Method	Cell	Compartment	QD _{TGA}	QD _{gel}	QD _{gel/hep}
Live	THP-1	Cytoplasm	**	-	***
		Nucleus	-	-	***
	A549	Cytoplasm	**	**	*
		Nucleus	-	-	-
	Caco-2	Cytoplasm	-	-	-
		Nucleus	-	-	-
Fixed Permeabilised	THP-1	Cytoplasm	**	*	**
		Nucleus	**	*	***
		Nucleoli	**	**	***
	A549	Cytoplasm	**	**	**
		Nucleus	-	**	**
		Nucleoli	-	**	**
	Caco-2	Cytoplasm	**	**	**
		Nucleus	-	-	-
		Nucleoli	-	-	-
		Nucleoli	-	-	-

-: No QDs detected, *: low concentration QDs detected, **: moderate concentration of QDs detected, ***: high concentration of QDs detected.

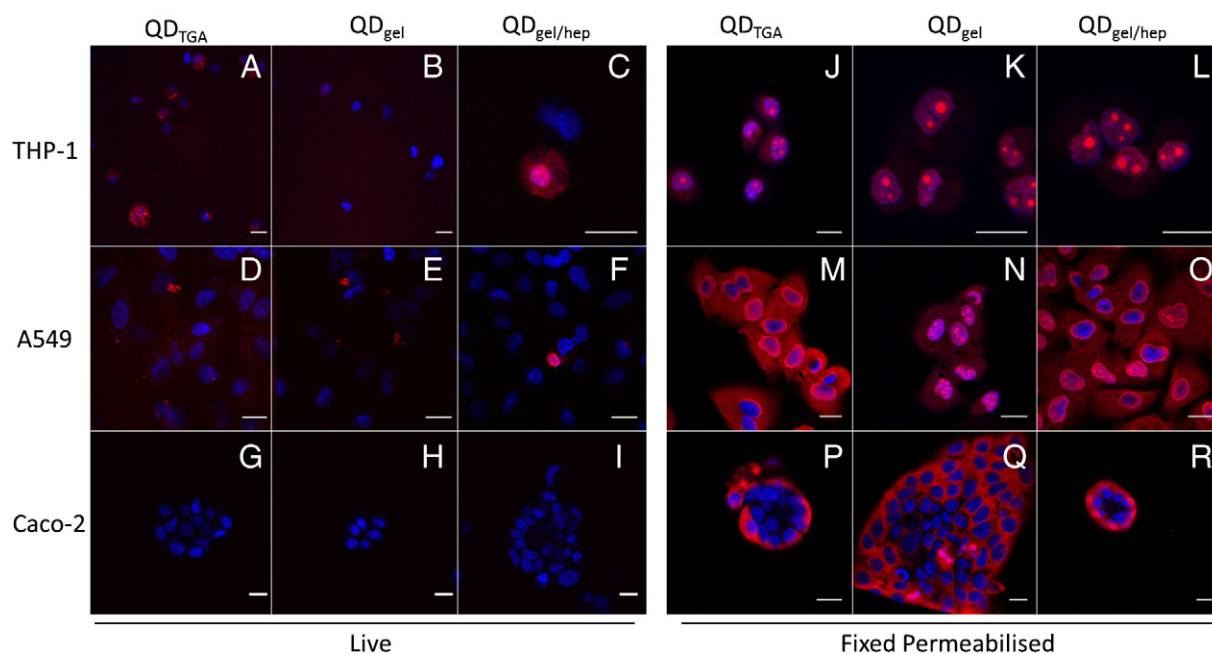


Figure 4. Confocal microscopy imaging of live and fixed permeabilised THP-1, A549 and Caco-2 cells using QD_{TGA}, QD_{gel} and QD_{gel/hep}. Scale bar = 20 μ m. All cells are treated with 5 μ M of QD solutions. Left panel: Live staining of THP-1, A549 and Caco-2 cells with QD_{TGA}, QD_{gel} and QD_{gel/hep}. Right panel: Fixed permeabilised THP-1, A549 and Caco-2 cells imaged using QD_{TGA}, QD_{gel} and QD_{gel/hep}.

QD_{gel} and QD_{gel/hep}, respectively. Due to the similar chemical structures of heparin and gelatine, it is expected that the zeta potential of both the gelatine-quantum dots and heparin-quantum dots would also be similar. The recorded zeta potential for the QD_{gel/hep} is consistent with that of other previously published results,²⁶ again suggesting that a heparin layer was successfully formed on the QDs.

Results from FTIR, although not conclusive, also suggest that heparin was conjugated to the gelatine layer of QD_{gel} to form QD_{gel/hep}. Gelatine and heparin share many functional groups, making chemical analysis more complex. Functional groups typically identified using FTIR include carbonyls, amides and

carboxylic acids, which are present in both gelatine and heparin. As such, direct comparison between the spectra is difficult. However, with this in mind, the C-S stretching frequency at 909 cm^{-1} visible in heparin is also detected in QD_{gel/hep} indicating the presence of heparin on the surface of the QD.

The results of the *in vitro* biological imaging of the quantum dots indicated that internalisation of QDs by live THP-1 cells was increased by the addition of heparin on to the surface of the QDs, compared to QD_{gel}. The QD_{gel/hep} was visible in the nucleus of the cells and also in the cytoplasm (Figure 4, C). No significant uptake of QD_{gel} by live THP-1 cells was detectable (Figure 4, B). QD_{TGA} were readily internalised by the THP-1

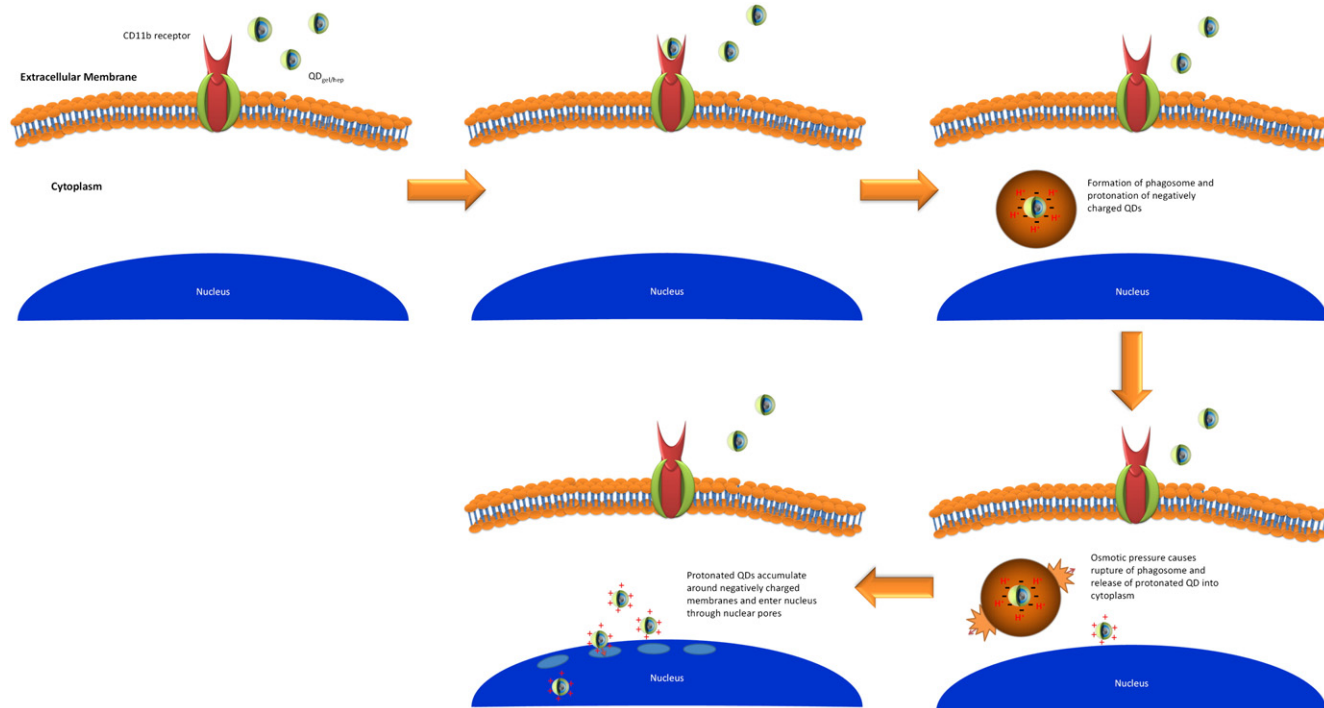


Figure 5. Schematic representation of internalisation of QDgel/hep through binding to CD11b. The QD-receptor complex becomes internalised into a phagosome, whereby the acidic environment results in the protonation of the QDs. A build up in osmotic pressure across the membrane results in swelling and rupture of the phagosome, leading to the release of the QD into the cytoplasm. The protonated QDs can then accumulate around negatively charged membranes and enter the nucleus through nuclear pores.

cells, but were predominantly confined to the cytoplasm (Figure 4, A). Only faint traces of QD_{TGA} were visible in the nucleus. This suggests that the heparin layer on the QDs increases the internalisation of QDs into the cytoplasm and the nucleus of the cell, possibly due to heparin binding to the CD11b receptor, from where it may be internalised into the nucleus (Figure 5), similar to how heparin assists in the stabilisation and transportation of lens epithelium-derived growth factor (LEDGF) into the nucleus of lens epithelial cells¹⁸ or the transport of oligodeoxynucleotides to the nucleus.³¹

Previous studies have shown that QDs are readily internalised by THP-1 cells, but depending on size and functional modification are restricted to the cytoplasm.^{3,32–34} From the results of this study, it can be seen that internalisation of red CdTe QDs into the nucleus of THP-1 cells can be achieved through functionalisation with heparin. Previously, only green emitting (2.10 nm core) CdTe QDs were localised within the nucleus of live THP-1 cells, while red emitting (3.40 nm core) CdTe QDs were restricted to the cytoplasm.³²

Permeabilisation of the fixed THP-1 cells with Triton X-100 dramatically increased the influx of the QDs. QDs were visible within the nucleoli, with QD_{gel/hep} having the highest level of uptake, followed by the QD_{gel} and QD_{TGA} (Figure 4, J–L). Cytoplasmic staining was also increased, compared to live imaging, again with the increased levels of QD_{gel/hep} being internalised, compared to the QD_{gel} and QD_{TGA} samples. Localisation of the quantum dots within the nucleoli has also been previously noted.³³ Fixation of THP-1 cells with PFA,

followed by permeabilisation with Triton X-100, results in high intensity localisation of quantum dots within the nucleoli.³² In particular, this is typical of negatively charged quantum dots targeting positively charged histone counterparts.³⁵

Internalisation of the QDs into the cytoplasm was suggested to be due to the acidic nature of the phagosome/endosome³⁶ typically around pH 5.^{37,38} In this environment, protons are transferred to the negatively charged particles, resulting in them exhibiting a positive zeta potential.³⁶ This in turn results in an accumulation of the quantum dots around negatively charged membranes.³⁵ In previous work it has been shown that the proton-sponge effect results from molecules on the surface of the quantum dots absorbing protons in acidic organelles, leading to an osmotic pressure build-up across the membranes.³⁹ This pressure can cause swelling and rupture of the endosomes/phagosomes, releasing the quantum dots into the cytoplasm.⁴⁰ It has also been reported that fixation method may play a role in the localisation of the QDs³³ in a similar manner in which fixation can influence the re-localisation of soluble proteins from the cytoplasm to the nucleus,⁴¹ as depicted in Figure 5. This suggests that the QDs used in this study may be binding to soluble proteins which have become localised to the nuclei of the cells following permeabilisation.^{32,42,43}

Internalisation of the QD samples in the live non-permeabilised A549 cells was very limited (Figure 4, D–F). The QD_{gel} sample was internalised to a higher degree compared to QD_{gel/hep} and QD_{TGA}. A549 cells are not phagocytic cells, and as such internalisation of QDs *via* this route is restricted.

Upon fixation and permeabilisation of the cells, internalisation of the QDs was enhanced further (Figure 4, M–O). All QD samples were visible in the cytoplasm of the cells, with the QD_{gel} and QD_{gel/hep} (Figure 4, N and O, respectively) being visible in the nucleoli also. The QD_{gel/hep} sample had the highest level of internalisation, followed by QD_{gel} and QD_{TGA}. QD_{TGA} bound strongly to the nuclear membrane of the cells (Figure 4, M), but were not internalised into the nucleus, possibly due to interaction with the negatively charged nuclear membrane of the A549 cells,³⁶ which is typically around -10.2 mV.^{36,44}

There was no detectable internalisation of the quantum dots in the live Caco-2 cells (Figure 4, G–I). Caco-2 cells have been noted to have poor ability to internalise nanomaterials²² and these results are consistent with previous studies,⁶ indicating their potential as a reference cell line for nanomaterial uptake experiments.

Permeabilisation of the fixed Caco-2 cells had the greatest effect and resulted in high concentrations of QDs being detected within the cytoplasm (Figure 4, P–R). In this instance, very low levels of QDs were detected within the nucleus, unlike with the fixed and permeabilised THP-1 and A549 cells.

As shown in the representative confocal micrographs, the formation of a gelatine/heparin bilayer on the QDs increases the internalisation of 3.9 nm QDs into non-permeabilised live THP-1 cells, compared to live non-permeabilised A549 and Caco-2 cells. There is a dramatic increase in the concentration of the heparin-quantum dots present within the nucleus of the live THP-1 compared to the other cell types. These results also suggest that red emitting 3.9 nm quantum dots, which are usually restricted to the cytoplasm, can be compartmentalised within the nucleus of the macrophage, through phagocytosis. This may be due to the heparin-quantum dots binding to the CD11b receptor, as hypothesised, and facilitating in the internalisation/phagocytosis of the quantum dots. One postulated mechanism in which this occurs is that binding of heparin to the CD11b receptor initiates phagocytosis, followed by transportation of the QD_{gel/hep} to the nucleus. This may be similar to the iC3b initiated phagocytosis of pathogens by Mac-1, through binding to the I-domain of CD11b,¹⁹ the same binding domain as heparin,¹³ and how heparin assists in the stabilisation and transportation of lens epithelium-derived growth factor (LEDGF) into the nucleus of lens epithelial cells¹⁸ or the transport of oligodeoxynucleotides to the nucleus.³⁰ Fixation of the cells using PFA resulted in increased influx of all of the QDs in to all cell and this is predominantly due to avoidance of cellular efflux mechanisms.^{45,46} The QD_{gel} and QD_{gel/hep} were frequently found in the cytoplasm, nucleus and nucleoli of the fixed permeabilised THP-1 and A549 cells, with the same QDs being restricted to the cytoplasm of Caco-2 cells.

Quantum dots have been shown to produce extremely negative effects *in vivo*, including pulmonary vascular thrombosis and rapid death at concentrations required for *in vivo* applications.^{28,47} It has been demonstrated in the study by Geys *et al.*⁴⁷ that highly negatively charged QDs activate the intrinsic or contact pathway in the coagulation cascade leading to massive thrombosis. Pre-treatment of the mice with heparin, however prevent this acute toxicity.⁴⁷ As such the heparin multi-layered QDs developed in this study could act to passivate the thrombogenic nature of negatively charged QDs thereby providing an opportunity for further investigation for *in vivo* applications.

In vitro platelet aggregation studies in human and murine platelet-rich plasma (PRP) shown by Geys *et al.*⁴⁷ confirmed the effect of carboxyl-QDs as dose dependent with maximal platelet aggregation starting at 540 pmol/mL carboxyl-QDs. Whereas at lower concentrations, they noted no response.

Contrarily to this previous study, work carried out by Samuel *et al.*⁴⁸ has led to contrasting results *in vitro* under static and dynamic shear stress conditions. In their work, they showed that negatively charged QDs are uptaken preferentially by HUVEC cells under shear stress of 0.5 dyne/cm². Furthermore in a recent paper by Samuel (under revision) it was shown that QDs activate human platelets by binding to platelet plasma membrane via up-regulation of GPIIb-IIIa and P-selectin receptors, and release of matrix metalloproteinase-(MMP)-2. These findings provide further clarity on the mechanism of functional response of platelets of ultra-small NPs under physiological experimental conditions as also highlighted in the work of Guidetti *et al.*⁴⁹ Interestingly, P-selectin levels were found to be not altered in the presence of heparin, therefore attenuating the size- charge- and dose-dependence highlighted in both reports.

In vitro cytotoxicity of both TGA capped and gelatine coated CdTe QDs has previously been carried out by our group.³ In the work by Byrne *et al.*,³ gelatine coated CdTe QDs were shown to be better tolerated and less toxic to THP-1 cells, compared to TGA capped QDs similar to QD_{TGA} used in this study. The gelatine coating of CdTe QDs, produced in the same manner as QD_{gel} used in this work, has also been shown to reduce the toxicity of QDs used in high concentrations over a longer incubation time.²⁹ Here, the authors used a concentration of 0.1 μ M of gelatine and TGA capped QDs with incubation times of 24 to 72 hours,²⁹ much longer than the incubation times presented in this work. This time and dose dependent toxicity of TGA and gelatine coated CdTe QDs has been also been shown in a neuroblastoma cells line, with gelatine coated QDs indicating a lower level of cytotoxicity.⁵⁰

The encapsulation of heparin into polymer based nanoparticles has also been shown to reduce the cytotoxicity of said nanoparticles⁵¹ and increases the blood circulation time of polymer and iron oxide based nanoparticles.⁵² These points, together with the ability of heparin to prevent platelet aggregation caused by QDs as indicated by Geys,⁴⁷ suggests that heparin functionalised QDs will have reduced cytotoxicity compared to their TGA stabilised counterparts. It is therefore envisioned that the multilayering of gelatine and heparin onto the surface of CdTe QDs to form the QD_{gel/hep} nanoparticles presented in this study, will result in increased biocompatibility and lower cytotoxicity.

Our study corroborates the potential use of multi-layered QDs for biological applications, through the synthesis of heparin modified gelatine QDs. The QDs produced were suitable for imaging application in both live and fixed cell lines. It has been shown that the layering of heparin on the surface of QD_{gel} allows for the internalisation of red emitting 3.9 nm CdTe QDs into the nucleus of live THP-1 cells and produces QDs that have high affinity for the nuclear compartments of fixed permeabilised THP-1 and A549 cells.

The results of this study leads to the opportunistic approach of using customised multi-layered QD for haemocompatible diagnostic imaging under physiologically relevant conditions. More experimental evidence is required to tease out the

biochemistry and mechanisms of interaction involved between QDs and the coagulation cascade.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.nano.2014.04.009>.

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Cellular uptake and biocompatibility of bismuth ferrite harmonic advanced nanoparticles

Davide Staedler^{a,1}, Solène Passemard, MSc, PhD^{a,1}, Thibaud Magouroux, MSc, PhD^b,
Andrii Rogov, MSc^b, Ciaran Manus Maguire, MSc^c, Bashir M. Mohamed, PhD^c,
Sebastian Schwung, MSc^d, Daniel Rytz, MSc, PhD^d, Thomas Jüstel, MS, PhD^e,
Stéphanie Hwu, MSc^b, Yannick Mugnier, MSc, PhD^f, Ronan Le Dantec, MSc, PhD^f,
Yuri Volkov, MD, PhD^{c,g}, Sandrine Gerber-Lemaire, MSc, PhD^a,
Adrielle Prina-Mello, MSc, PhD^{c,g}, Luigi Bonacina, MSc, PhD^{b,*}, Jean-Pierre Wolf, MSc, PhD^b

^aInstitute of Chemical Sciences and Engineering, EPFL, Batochime, 1015, Lausanne, Switzerland

^bGAP-Biophotonics, Université de Genève, 22 Chemin de Pinchat, 1211 Genève 4, Switzerland

^cNanomedicine Laboratory and Molecular Imaging Group, School of Medicine, Trinity Centre for Health Sciences, Trinity College, D8, Dublin, Ireland

^dFEE GmbH, Struthstrasse 2, 55743 Idar-Oberstein, Germany

^eFachbereich Chemieingenieurwesen, Fachhochschule Münster, Stegerwaldstrasse 39, 48565 Steinfurt, Germany

^fUniv. Savoie, SYMME, F-74000, Annecy, France

^gAMBER Centre and CRANN Institute, Trinity College, D2, Dublin, Ireland

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Abstract

Bismuth Ferrite (BFO) nanoparticles (BFO-NP) display interesting optical (nonlinear response) and magnetic properties which make them amenable for bio-oriented diagnostic applications as intra- and extra membrane contrast agents. Due to the relatively recent availability of this material in well dispersed nanometric form, its biocompatibility was not known to date. In this study, we present a thorough assessment of the effects of *in vitro* exposure of human adenocarcinoma (A549), lung squamous carcinoma (NCI-H520), and acute monocytic leukemia (THP-1) cell lines to uncoated and poly(ethylene glycol)-coated BFO-NP in the form of cytotoxicity, haemolytic response and biocompatibility. Our results support the attractiveness of the functional-BFO towards biomedical applications focused on advanced diagnostic imaging.

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Key words: Nanophotonic; Non-linear imaging; Bismuth ferrite; PEGylation; Biocompatibility

Background

Most of nanophotonics approaches (quantum dots, plasmonic nanoparticles (NP), up-conversion NP) for health applications present static optical properties (absorption bands, surface plasmon resonances) often in the UV-visible spectral region and do not fully allow for exploiting the tuning capabilities of new laser sources and their latest extensions in the infrared. To circumvent these limitations, a few research groups in the last years have introduced a new nanotechnology approach based on inorganic nanocrystals with non-centrosymmetric structures. Such nanomaterials present a very efficient nonlinear response, and can be easily imaged by their second harmonic generation

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*Corresponding author.

E-mail address: luigi.bonacina@unige.ch (L. Bonacina).

¹ These authors contributed equally to this work.

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(SHG) in multi-photon imaging platforms.^{1–6} Such harmonic NP (HNP) do not suffer from conventional optical limitations such as photobleaching and blinking allowing long-term monitoring of developing tissues.^{5,7} Several HNP have been recently synthesized and tested for biological applications.^{4–7} Particular care should be paid to assess the ability of these NP to reach intracellular targets without causing major interferences to the cell metabolism. In this context, this subcellular targeting becomes increasingly important as key parameters for the understating of complex events in living cells.^{8,9} In fact, the possibility to freely change detection wavelength can be exploited for subtle co-localization studies with organelle-specific dyes, as the signal from NP can be freely shifted to avoid overlap with fluorophores emission bands. However, one aspect that is not fully understood and¹⁰ remains uncertain is how nanomaterials interact with cellular interfaces such as cytoskeletal membranes since it is known that small alterations in their physicochemical properties can drastically influence the cells-NP interactions, especially the uptake mechanisms.^{9,11} Therefore, lead-NP-candidate identification process based on high throughput screening as decision-making process is a prerequisite for the validation of new SHG NP for bio-imaging applications. Here we present a study based on BiFeO₃ (bismuth ferrite, abbreviated as BFO) NP (BFO-NP), which were recently successfully introduced as photodynamic tools and imaging probes.¹² Nonetheless, such is the technological novelty of this new group of materials that there is still a knowledge gap that requires the scientific community attention towards the investigation of the interaction at the cellular and subcellular levels. The opportunity of closing this gap is presented by providing the first thorough investigation on the effects of BFO-NP in cellular metabolism and uptake mechanisms. Toxicity and biocompatibility were assessed by automated high content screening, recording cytotoxicity, lysosomal mass and cell permeability, in line with previously published works.^{13,14} Cellular uptake was investigated by co-localizing the NP with specific fluorophores for cell membranes and endosomes. Moreover, in this paper we present for the first time to our knowledge the most efficient protocol for the coating of these HNP with poly(ethylene glycol) (PEG) derivatives to promote colloidal stability and biocompatibility in biological media, and to allow post-functionalization with bioactive molecules.^{15,16} In this context, the biocompatibility, cellular uptake and intracellular localization of free and PEG coated BFO-NP were compared.

Methods

Preparation of a polydisperse suspension of BFO

The starting BFO suspension (2 mL, 62.5 wt % in ZrO₂ balls), provided by the company FEE (Germany) under a collaboration agreement, was diluted in EtOH (2 L) and ultra-sonicated for 12 h. After 10 days sedimentation, the upper portion of the polydisperse suspension (50 mL) was taken and mixed with oleic acid (4 mL). The volatiles were removed under vacuum and the residue was weighed and suspended in EtOH to obtain a stock solution at 3.6 mg/mL.

Coating of BFO-NP

BFO-NP (suspended in EtOH, 3.6 mg/mL, 583 μ L) were diluted in EtOH:toluene:25% aqueous ammonia (0.50:0.50:0.32 mL) and ultra-sonicated for 30 min. PEG oligomers **1** and **2**¹⁴ (1:1 ratio, 100 mg) were added and the suspension ultra-sonicated at 40 °C for 16 hr. The suspension was reduced to a small volume and distributed in plastic tubes for dilution with a mixture of dichloromethane (DCM):EtOH:water (1:1:1, 1 mL). After centrifugation (10 min, 13 000 rpm), the aqueous layer, containing the excess of unreacted polymers, was removed and a mixture of EtOH:water (1:1, 0.5 mL) was added to each plastic tube. The procedure was repeated 5 times to obtain a pure suspension of coated BFO-NP in DCM. After evaporation of DCM and addition of EtOH, the BFO-NP concentration was calculated by measuring the turbidity of the solution by spectrometry at 600 nm (Synergy HT) and by comparing the values with a standard-curve prepared using the stock solution at 3.6 mg/mL.

Characterization of uncoated and coated BFO-NP

Advanced physico-chemical characterization of uncoated and coated BFO-NP was recently performed and reported. In the present work, BFO-NP were characterized by dynamic light scattering (DLS) using a Zetasizer NanoZ (Malvern) for determination of mean hydrodynamic volume and zeta potential. Suspension of uncoated or coated BFO-NP (20 μ L) was diluted in 1 mL of distilled water. Acetic acid (100 μ L) was added and the resulting suspensions were ultra-sonicated for 30 min and analysed by DLS.

Nanoparticles characterization in biological media

The physico-chemical characterization of the NP was carried out by nanoparticle tracking analysis (NTA, Nanosight NS500). BFO-NP at 25 μ g/mL were vortexed for 5 s to disperse the particles and then diluted at 1 μ g/mL in different solutions (0.22 μ m filtered): diethylpyrocabonate (DEPC) water, Dulbecco's Modified Eagle Medium (DMEM), Roswell Park Memorial Institute (RPMI) and Ham's F-12 K (Kaighn's) Medium (F12K) culture media, and their supplemented form with 10% fetal bovine serum (FBS). Three culture media were chosen since commonly utilised for the in vitro culture cell models selected. The different dispersions were then analyzed via NTA for the physico-chemical characterization measurement of hydrodynamic radius and polydispersity index (PDI) at room temperature of BFO-NP. All measurements were carried out three times at physiologically relevant pH (pH = 7.4) and means and standard deviations (SD) were calculated. Quality assurance over the measurements carried out was guaranteed by the adoption of Quality Nano (QNano, FP7 project) standard operating procedures (SOPs), which have been developed as part of large inter laboratory comparative study focused on nanoparticle physico-chemical characterization.¹⁷

Cell model and culturing conditions

Human lung-derived A549 and NCI-H520 cancer cell lines and human monocytic THP-1 cell line are available from ATCC

(American Tissue Culture Collection, Manassas, VA, USA). A549 were grown in DMEM medium containing 4.5 g/L glucose, 10% FBS and penicillin/streptomycin (PS) in a 37 °C incubator with 5% CO₂ at 95% humidity. NCI-H520 and THP-1 were grown in complete Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% FCS and PS in a 37 °C incubator with 5% CO₂ at 95% humidity. For differentiation into macrophages, THP-1 cells were plated at a density of 20'000 cells/cm² in RPMI 1640 supplemented with 10% FBS and 100 ng/mL phorbol 12-myristate 13-acetate (PMA, Sigma-Aldrich) for 72 h. Differentiated THP-1 cells adhered to the bottom of the wells.

Fluorescent staining for cellular imaging

The cells (20'000 cells/cm²) were grown for 48 h or 72 h for activated PMA THP-1 cells, in a 24 well plate containing one rod-shaped microscope slide (BD Falcon). After this time cells were exposed to BFO-NP at 25 µg/mL or to vehicle (ethanol) at indicated time. For endosomes imaging the cell layers were exposed for 2 h to 15 µg/mL of the fluorescent probe FM1-43FX (Invitrogen). After incubation, the cells were fixed in 4% formaldehyde in PBS for 30 min, subsequently washed once in 0.1% Triton X-100 in PBS and twice in PBS, then maintained in formaldehyde. For membrane staining, cells were fixed and then exposed to 0.1 µg/mL Nile Red (Invitrogen) in PBS for 5 min, then rinsed twice in PBS and maintained in formaldehyde. Cell nuclei were stained with 4',6'-diamidino-2-phenylindole (DAPI).

Multiphoton laser scanning microscopy

Samples were observed using a Nikon multiphoton inverted microscope (A1R-MP) coupled with a Mai-Tai tunable Ti:sapphire oscillator from Spectra-Physics (100 fs, 80 MHz, 700–1000 nm). A Plan APO 40× WI N.A. 1.25 objective was used to focus the excitation laser and to epi-collect the SHG signal and dye markers fluorescence. Nanoparticles and fluorescent dyes (FM1-43FX and Nile Red) were excited at 790 nm and observed through tailored pairs of dichroic mirrors and interferometric filters (Semrock, FF01- 395/11-25 for SH, FF01-607/70-25 for fluorescence). Statistics were calculated by averaging measured values from samples between 40 and 170 cells per condition for cell labelling and between 8 and 100 cells per condition for co-localization measurements. Cell labelling was computed by dividing the number of cells labelled by at least one nanoparticle to the total number of cells in the microscope field of view and expressed as % of total cells. Co-localization with endosomes was estimated by counting the number of nanoparticles co-localizing with the fluorescence signal from cell membrane dye FM1-43FX and dividing this value by the total number of nanoparticles present inside each cell. The result of this procedure is expressed as % of all particles internalized in each cell.

In vitro dose and exposure endpoint determination

Cytotoxicity on three-cell line models was investigated *in vitro* after 24 h and 72 h incubation with BFO-NP. Following standardization of the BFO preparation protocols, all were injected

into 96 well plates to a final volume of 200 µL/well. A549, NCI-H520 and activated PMA THP-1 cells were initially plated at a cell density of 10'000 cell/cm² and incubated with 1.0, 2.5, 5.0, 7.5 or 10 µg/mL of uncoated and coated BFO-NP for 24 h and 72 h in a 37 °C incubator with 5% CO₂ at 95% humidity. Experiments were repeated three times, using triplicate wells each time for each formulation tested. Positive and negative controls were also included into each experiment in order to quantify the extent of toxicity response induced by each particle. Three positive controls were Valinomycin (VAL, Fisher Scientific) (final concentration 120 µM) to measure changes in mitochondrial transmembrane potential, Tacrine (TAC, Sigma-Aldrich) (final concentration 100 µM) to measure changes in lysosomal mass/pH and Quantum Dots (CdSe) (final concentration at 1 µM) to measure nanoparticle-induced uptake.¹⁴ After 24 h and 72 h incubation, cells were washed in phosphate-buffered saline solution (PBS) at pH 7.4 and fixed in 3% paraformaldehyde (PFA). A multiparametric cytotoxicity assay was performed using the Cellomics® HCS reagent HitKit™ as per manufacturer's instructions (Thermo Fisher Scientific Inc.). For each experiment, each plate well was scanned and acquired in a stereology configuration of 6 randomly selected fields. In total, each endpoint data plot in the heatmaps represents the analysis of an average of 270,000 cells = 3 runs × 3 triplicates × 6 fields × 5000 cells (on average from t = 0 h). Images were acquired at 10x magnification using three detection channels with different excitation filters. These included a DAPI filter (channel 1), which detected blue fluorescence of the Cellomics® Hoechst 33342 probe indicating nuclear intensity at a wavelength of 461 nm; FITC filter (channel 2), which detected green fluorescence of the Cellomics® cell permeability probe indicating cell permeability at a wavelength of 509 nm and a TRITC filter (channel 3), which detected the lysosomal mass and pH changes of the Cellomics® LysoTracker probe with red fluorescence at a wavelength of 599 nm.

Statistical analysis

Response of each cell type to the coated and uncoated BFO-NP was analysed by 2-way ANOVA with Bonferroni post-test analysis. A *P*-value <0.05 was considered to be statistically significant. In this work we are comparing 4 cell parameters associated with the cytotoxicity response of 3 cell lines exposed to uncoated or coated BFO, at 5 doses, with 3 controls; therefore, the statistical value associated with our work carried out by High Content Screening and data mining is of significance. To visualize the data, KNIME (<http://KNIME.org>, 2.0.3) data exploration platform and the screening module HiTS (<http://code.google.com/p/hits>, 0.3.0) were used. Knime is a modular open-source data manipulation and visualization programme, as previously reported.^{14,18–20} All measured parameters were normalized using the per cent of the positive controls. Z score was used for scoring the normalized values. These scores were summarized using the mean function as follows Z score (x-mean)/SD, as from previous work.^{14,18–20} Heatmaps graphical illustration in a colorimetric gradient table format was adopted as the most suitable schematic representation to report on any statistical significance and variation from normalized controls based on their Z score value. Heatmap tables illustrate the range

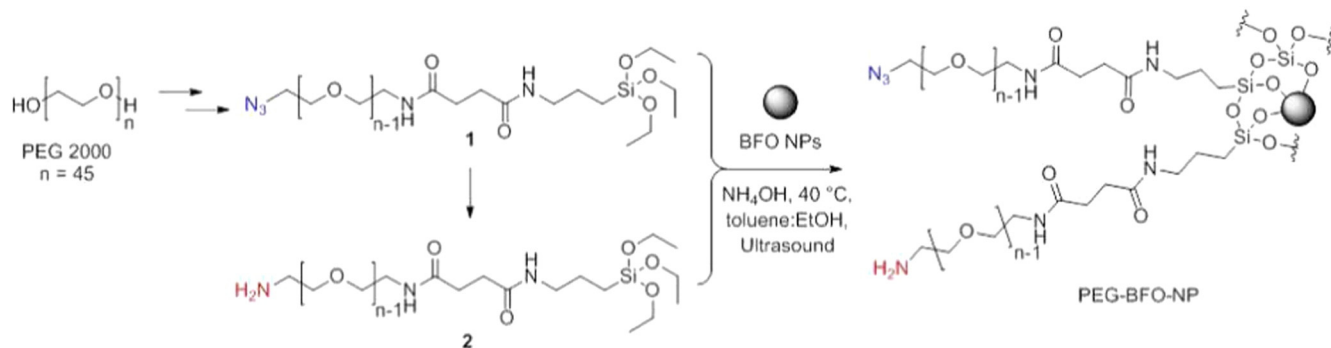


Figure 1. Synthesis of coated BFO-NP. PEG oligomer (1) and PEG oligomer (2) were synthesized from PEG 2000 (MW 2000 Da) as published,¹⁵ then used for the coating of BFO-NP.

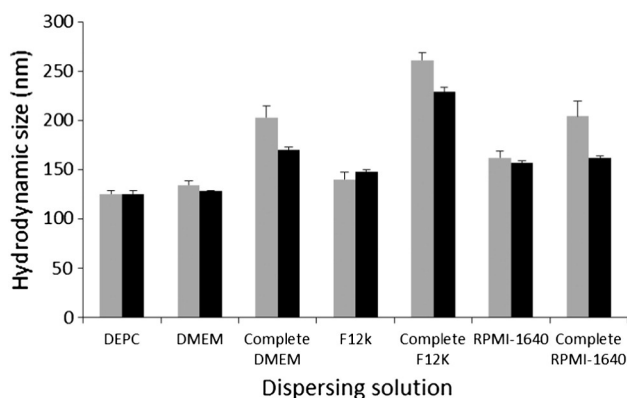


Figure 2. Nanoparticle tracking analysis of U-BFO-NP (light grey bars) and PEG-BFO-NP (dark grey bars) in biological media. Complete: media supplemented with 10% foetal bovine serum (FBS); DEPC: diethylpyr-carbonate; DMEM: Dulbecco's Modified Eagle Medium; F12k: Ham's F-12 K (Kaighn's) Medium; RPMI-1640: Roswell Park Memorial Institute 1640. Size distribution of BFO-NP after dispersion at 1 µg/mL in DEPC water solution (DEPC), DMEM medium (DMEM), DMEM medium supplemented with 10% FBS (complete DMEM), F12k medium (F12k), F12k medium supplemented with 10% FBS (complete F12k), RPMI 1640 medium (RPMI-1640) and RPMI 1640 medium supplemented with 10% FBS (complete RPMI-1640). All measurements were carried out three times at pH 7.4, then means and standard deviations (SD) were calculated.

of variation of each quantified parameter from the minimum (green) through the mean (yellow) to the maximum (red) according to the parameter under analysis.

Haemolysis assay

Fresh human blood in lithium heparin-containing tubes was obtained from leftovers of analytical blood with normal values. The plasma was removed by centrifugation for 10 min at 2500 rpm and the blood cells were washed three times with sterile isotonic PBS solution, then diluted 1:10 in PBS. Cell suspensions (300 µL) were added to 1200 µL of each solution containing NP or chemicals in human plasma or PBS at indicated concentrations. Nanopure water (1200 µL) was used as a positive control and human plasma or PBS (1200 µL) were used as negative controls. The mixtures were gently mixed then kept for 2 h at RT, centrifuged for 2 min at 4000 rpm and the

absorbance of the upper layers was measured at 540 nm in an absorbance multi-well plate reader (Synergy HT). The percentage of haemolysis of the samples was calculated by dividing the difference in absorbance between the samples and the negative control by the difference in absorbance between the positive and negative controls. Experiments were conducted in triplicate wells and repeated twice. Means $\pm$ SD were calculated.

Results

As previously stated, this work presents for the first time several important aspects relevant to the translation of BFP-NP into a novel biomedical imaging probe for advanced diagnostic screening.

Coating and characterization of BFO-NP

The presence of reactive hydroxyl groups at the surface of BFO-NP facilitates the surface coating chemistry. Modified poly(ethylene glycol) (PEG) 2000 (molecular weight of 2000 g/mol, 45 units) containing silane anchoring groups and reactive functionalities were synthesized as previously published¹⁵ and used to perform covalent coating via silane (Si) ligation (Figure 1).

A previously published study, by some of the authors, focused on the surface coating and post-functionalization of metal oxide NP such as iron oxide NP.¹⁵ In this work, BFO-NP were treated with an equimolar mixture of α -triethoxysilyl- ω -azido and α -triethoxysilyl- ω -amino PEG oligomers 1 and 2, prepared from linear PEG 2000, in the presence of aqueous ammonia.¹⁵ Ultra-sonication at 40 °C for 16 hours, followed by repetitive cycles of decantation/centrifugation into 1:1:1 DCM:EtOH:water resulted in coated BFO-NP (PEG-BFO-NP), which were suspended in EtOH for further characterization. Efficient coating was proved by FT-IR analysis (Supplementary Figure 1). Size and surface charge characteristics of uncoated BFO-NP (U-BFO-NP) and PEG-BFO-NP were measured using DLS and zeta potential techniques as previously described.¹⁵ Upon coating, the zeta potential value shifted from -29.0 ± 1.3 mV to -9.8 ± 0.3 mV and the mean hydrodynamic diameter decreased from 128.8 ± 11.2 nm to 96.1 ± 8.3 nm. The decrease in the hydrodynamic diameter of coated BFO-NP can be attributed to a hydro dispersion of the NP in the solvent as a result of a possible colloidal stabilisation.²¹

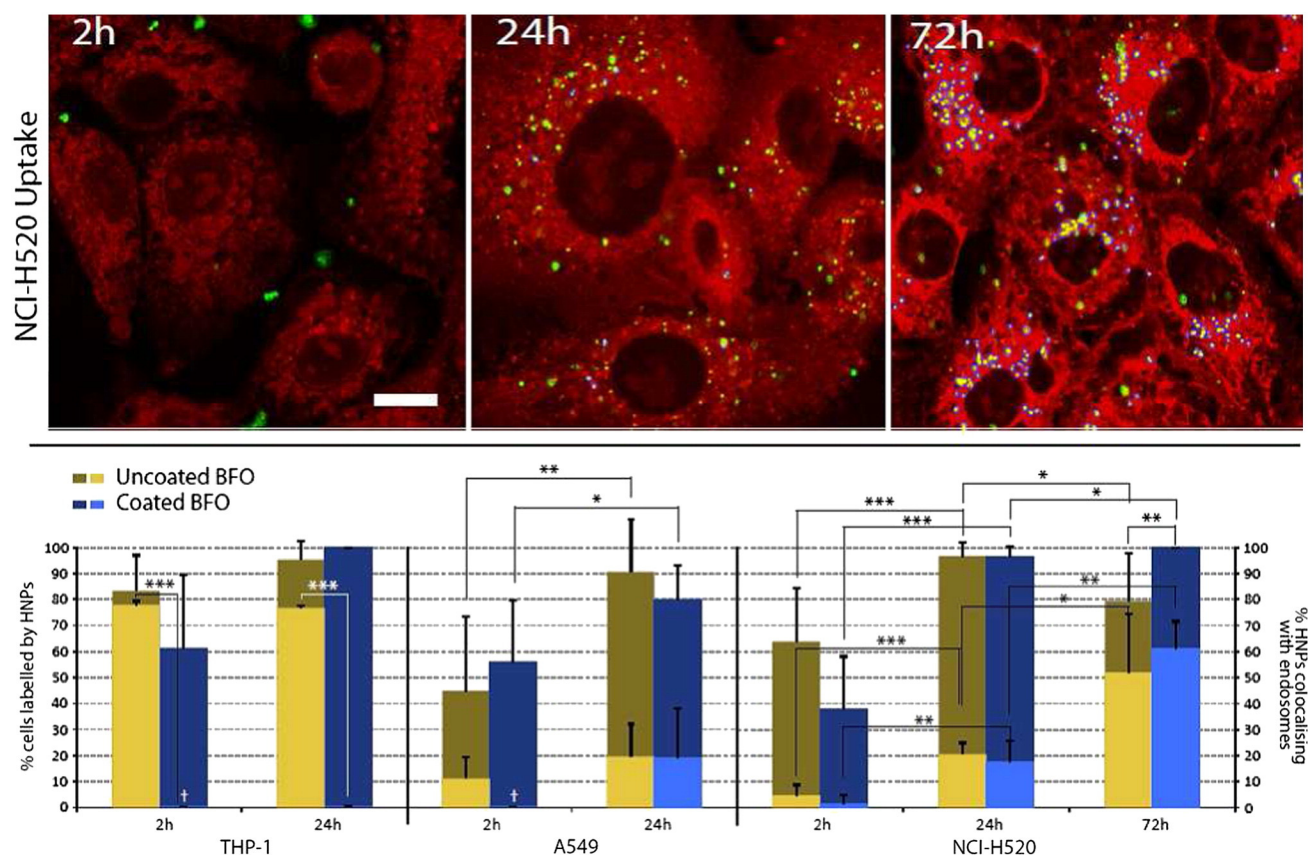


Figure 3. BFO-NP uptake in human-derived cell lines. First row: NCI-H520 cancer cells exposed to PEG-BFO-NP at 25 µg/mL for 2 h, 24 h and 72 h. Green: SHG signal of BFO-NP; yellow: co-localization between the probe for lipid membranes and the particles; blue circles: highlight of HNP co-localizing with FM1-43FX. Scale bar: 10 µm. Second row: % of labelled A549, NCI-H520 and THP-1 cells exposed to U-BFO-NP (brown bars) or PEG-BFO-NP (blue bars) at 25 µg/mL for 2 h, 24 h or 72 h (only NCI-H520). Statistical comparisons were done using a Student's *t* test: **P* < 0.05, ***P* < 0.01, ****P* < 0.001. †: no HNP co-localizing with endosomes were observed.

BFO-NP stability and characterization in biological media

For both uncoated and coated BFO-NP, extended physico-chemical characterization was carried out after ultracentrifugation and re-dispersion in ultrapure deionized water prior to incubation into relevant biological dispersing media. The two devised BFO-NP potential probes were also characterized by NTA, aiming at the identification of hydrodynamic radii, the colloidal and the aggregation stabilities at physiologically relevant conditions (Figure 2). Interestingly, U-BFO-NP formed aggregates in biological relevant media, particularly when the media were supplemented with serum. PEG-BFO-NP resulted in a decrease of aggregate formation, and thus a better stability of the suspension. This finding was further confirmed after 24 h incubation (Supplementary Figure 2). Through this process it was also possible to select the culture media to utilise for dispersion during the biological studies undertaken.

Interaction between BFO-NP and human derived cells

The interactions between U-BFO-NP and PEG-BFO-NP were then studied in three human-derived cell lines, one human adenocarcinoma cell line (A549) derived from alveolar epithelial type II cells, one lung squamous carcinoma cell line

(NCI-H520)⁶ and one human acute monocytic leukemia cell line (THP-1) as a model for macrophages.²² These experiments were aimed at observing the interaction between particles and cell membranes *in vitro* and to explore the uptake of particles in cells, particularly the endocytic pathways. Indeed, endocytosis represents one of the main internalization mechanisms of NP in cells,^{11,15,23} particularly in macrophages.^{23,24} For the quantification of particles associated with the plasma membranes and internalized into the cytoplasm, cells were exposed to BFO-NP, fixed and labelled with a fluorescent probe used to stain membranes and lipids.²⁵ Exposure time of 2 h and 24 h were chosen since considered representative minimum and maximum exposure time for diagnostic applications; whereas 72 h was identified as a critical time point for cytotoxicity response. HNP uptake by endocytosis was observed by co-localizing the SHG signal with a molecular probe specifically internalized in the endosomes.²⁶ It is worth pointing out that for the following uptake quantification we purposely adopted an extremely strong criterion (a cell is labelled if at least one particle is in contact with it). Even with this strict definition, we observed significant cell-dependent responses in the uptake between 2 h and 24 h, especially for macrophages. In fact, a consistent uptake by these latter cells was observed for the two diagnostically relevant

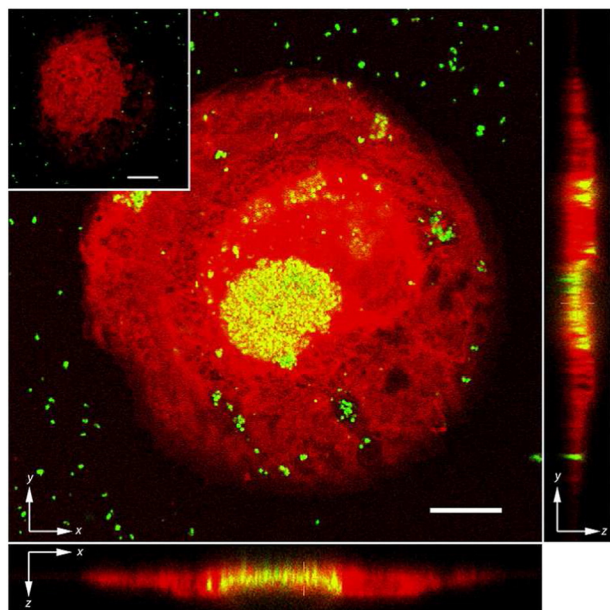


Figure 4. Uptake of uncoated BFO-NP by activated THP-1 cells. Two-photon microscopy image of an individual THP-1 cell exposed for 24 h at 25 µg/mL U-BFO-NPs. Red: FM1-43FX fluorescence. Green: SH emission by NPs. Three dimensional information are obtained by z-stacking of several images, the yz (right) and xz slice (bottom) views corresponds to cuts along the directions indicated by the yellow cross. Inset: Image of a THP-1 cell after 24 h exposure to 25 µg/mL of PEG-BFO-NP. Scale bars: 10 µm. One can see how the presence of PEG surface coating on NPs prevents NPs internalization by THP-1 cells.

exposure times (2 h and 24 h), whereas in human lung-derived NCI-H520 cancer cells the uptake of the HNP was still observable after 72 h incubation (as shown in Figure 3 low panel). Thus, from the results presented cell-dependent and time-dependent internalisation of the HNP into the three cell models adopted could be shown, as comprehensively presented between Figure 3 and the multiparametric heatmaps Figure 5. And more, it also emerges that consistency in the HNP internalization in human lung-derived cancer cells resulted to be time dependent (Figure 3). Indeed, HNP generally adhere to the cell membrane after 2 h and are then internalized when increasing exposure time. After 72 h exposure, BFO-NP formed aggregates in intracellular organelles, such as lysosome or endosomes. We did not observe a statistically significant difference in labelling between PEG-BFO-NP and U-BFO-NP after 2 h or 24 h exposure (Figure 3). Interestingly the interaction HNP with immune THP-1 cells was fairly rapid, as more than 80% of cells were found to have internalized at least one particle already after 2 h exposure. Such finding is in line with previous observations, since it is known that THP-1 phagocytic cells rapidly envelop and digest extraneous objects as initial response to infection or foreign body invasion.^{10,22,27} Conversely, the labelling of A549 and NCI-H520 cells showed a clear exposure dependent behaviour. After 72 h, the number of labelled cells was significantly higher in the presence of PEG-BFO-NP than with U-BFO-NP (Figure 3), suggesting the existence of an exocytosis mechanism for the uncoated BFO-NP

as already observed and reported for others metallic NP.^{28,29} In macrophages, the uptake of uncoated particles is mainly endocytic-dependent, whereas the PEGylation of BFO-NP completely reduced the endocytic-mediated uptake (Figure 3). This mechanism of particle internalization was confirmed by multiphoton laser scanning microscopy z-stack analysis of differentiated THP-1, which showed clusters of BFO-NP into the cytoplasm of cells having internalized uncoated BFO-NP after 24 h exposure (Figure 4) as opposed to the behaviour observed with coated BFO-NP, which showed a reduced uptake. In the lung-derived cancer cell lines only a weak fraction of HNP co-localized with the endosomes after 2 h and 24 h exposure, nonetheless this portion increased for the NCI-H520 cells after 72 h exposure to the particles.

Multiparametric cytotoxicity evaluation of BFO-NP

The cytotoxicity of uncoated and coated BFO-NP was investigated *in vitro* in order to address any cell-dependent and time-dependent toxicity aspects associated to the two human lung-derived NCI-H520 and A549 cancer cell lines and the macrophage-derived THP-1 cells. Multiparametric cytotoxicity was qualitatively and quantitatively measured after 24 h and 72 h exposure to increasing concentrations (1, 2.5, 5, 7.5 and 10 µg/mL) of HNP particles. Experiments were repeated three times, using triplicate wells each time for each formulation tested, as shown in Supplementary Table 1. Three key parameters of toxicity-attributed phenomena were analysed during this assay: cell number, lysosomal mass and pH, and cell membrane permeability, as previously reported.¹⁴ Indeed, it is known that some toxins can interfere with the cell's functionality by affecting the pH of organelles such as lysosomes and endosomes, or by causing an increase in the number of lysosomes present.^{14,30} Cell membrane permeability changes can be measured as enhancement of cell membrane damage and decreased cell viability as result of cell nuclear-staining counting reduction.^{14,31} Moreover, three positive controls were introduced in this analysis: CdSe quantum dots (QD), which are established toxic NP,¹⁴ Valinomycin (Vac), an inducer of energy-dependent mitochondrial swelling causing cell membrane permeability³² and Tacrine (Tac), a reversible cholinesterase inhibitor which causes increases in cellular lysosome content and lysosomal mass.³³ The rate of cell viability and proliferation was assessed by automated quantitative analysis of the nuclear count and cellular morphology; in parallel to that the fluorescent staining intensities reflecting cell permeability and lysosomal mass/pH changes were also quantified for each individual cell measured within each imaged field and then colorimetrically normalised against the respective controls (Figure 5). A multiparametric analysis of the cellular responses allowed the identification of the different toxicity levels between the two BFO-NP. In general, BFO-NP in concentration range of 1–10 µg/mL showed cell-dependent cytotoxicity on the cell models adopted. In agreement with previous results, THP-1 cells are the most affected cells between the three cell lines since these are the first line immune response to any foreign objects such as nanoparticles. For both PEG-BFO-NP and U-BFO-NP a time-dependent and dose-dependent toxicity was measured. However,

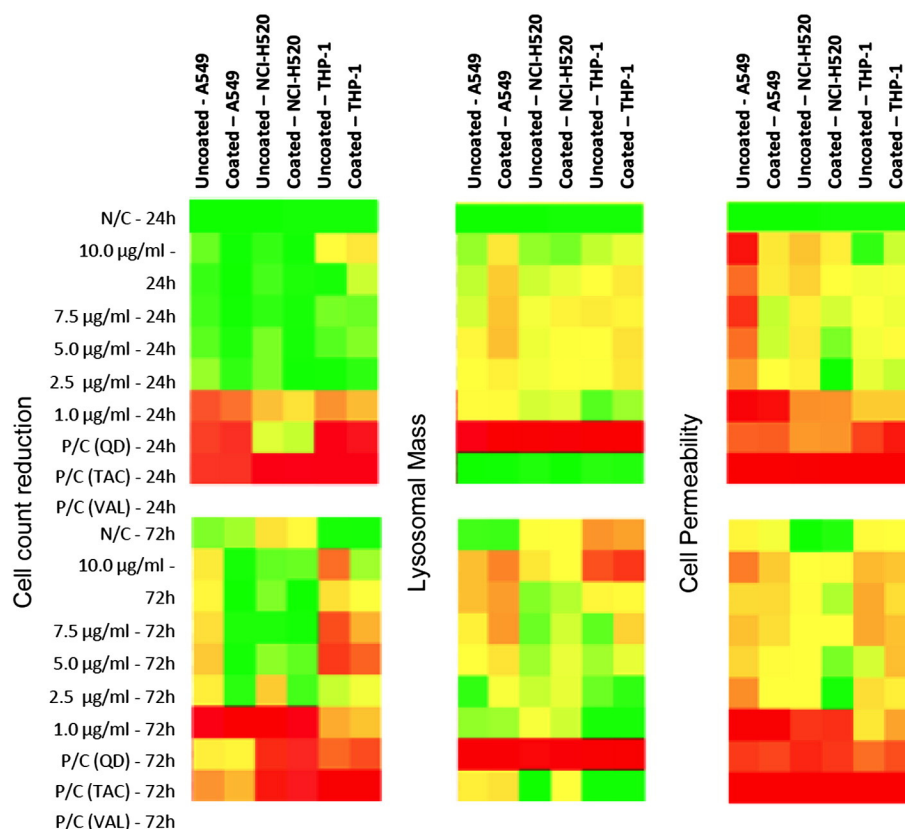


Figure 5. Heatmaps tables illustrating toxicity indicated parameters of BFO-NP in human-derived cell lines. Heatmaps were generated from the analysis of $n = 3$ experiments, each with triplicate wells for each of the parameters under investigation: cell count, lysosomal mass, cell permeability and nuclear area. Colorimetric gradient table spans from: Dark green: lower than 15% of maximum value measured; Bright green = 30%; Yellow = 50%; Bright orange: 60%; Dark orange = 75%; Red higher than 75% of maximum value. N/C untreated controls (negative) and P/C as positive controls such as QD: quantum dots, TAC: Tacrine, VAL: Valinomycin). Heatmap values are normalised using the per cent of the positive controls and, Z score was calculated as described in the statistical analysis section.

these effects were more pronounced when cells were exposed to uncoated particles. Among the parameters measured during the NP comparison, the cell permeability parameter showed the most pronounced difference, suggesting that BFO-NP, particularly uncoated particles, interfered mainly with the physiology of the plasma membrane, as also shown by the Vac response as positive control for cellular permeability. PEG-BFO-NP got stored in lysosomal compartments as from lysosomal concentration response, which was also comparable to the response to Tac used positive control for lysosomal response.

Haemolysis assay

After having assessed the colloidal stability of PEG-coated BFO-NP in biological media, the interactions with cell membranes were tested by assessing their haemolytic effect on human red blood cells (HRBC) according to established protocols.^{6,34} Haemolysis is defined as the destruction of red blood cells and it is regarded as a key parameter for the evaluation of NP biocompatibility.^{6,34,35} NP can exert haemolytic effect by electrostatic interactions with membrane proteins or by other NP-specific mechanisms such as the generation of reactive oxygen species (ROS),

causing irreversible damage to cells. The assessment of the haemolysis of uncoated BFO-NP showed a weak haemolytic potential (Figure 6), comparable to that of metallic NP observed in other bio-assays.^{35,36} Upon PEG coating, this potential was significantly reduced ($P < 0.001$, Figure 6). These statistically significant results proved that PEGylation of the metal oxide core contributed to the reduction of the interaction between cell membranes and particles surface.

Discussion

BFO-NP are a class of nanomaterials with unique optical and physical properties attracting the interest of researchers for several technological applications, in particular related to their multiferroics nature. We have shown recently their potential in the context of advanced non-linear optical imaging.³⁷ Recently BFO-NP were employed *in vitro* in a pilot study where they proved to be able to locally induce DNA damages by deep UV generation.¹² The cytotoxicity, haemolytic response and internalization mechanisms evidence here reported for coated BFO-NP suggested good biocompatibility and a great potential

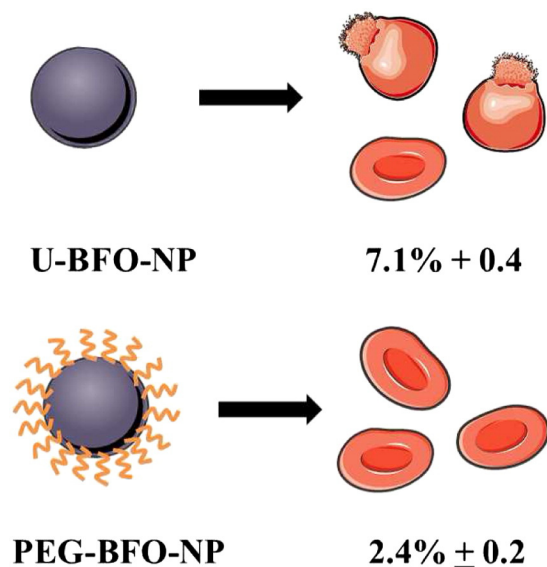


Figure 6. Haemolytic effect of U-BFO-NP and PEG-BFO-NP. HRBCs were exposed to NP (25 µg/mL) for 2 h. Results were expressed as % of haemolysis of NP-exposed HRBC compared to unexposed cells. Results are the means ± SD of triplicates of two independent experiments. Values of U-BFO-NP were compared to those of PEG-BFO-NP by a Student's *t* test ($P < 0.001$). This picture was done using Servier Medical Art images.

for biomedical imaging in diagnostic applications, e.g. flow cytometry.³⁸ Therefore, the aim of this work was, for the first time, to present the behaviour of these particles in their uncoated or PEGylated form, focusing on relevant exposure time point relevant to diagnostic applications. Such assessment comes timely after the first demonstrations of their interest for bio-imaging and selective photointeraction, as, due to their novelty, there is still a knowledge gap that requires the scientific community attention towards the investigation of their biological effect. NP must be coated with biocompatible polymers in order to stabilize the NP suspensions in biological media and to increase their biocompatibility.^{39,40} Coating with organic polymers also allows particles conjugation and functionalization with biologically-active ligands, such as targeting-specific ligands, therapeutic agents, peptides or antibodies.^{16,41} Here we present a method for the successful PEGylation of BFO-NP based on heterobifunctional PEG oligomers, for which a fast and convenient large scale synthesis protocol was recently published.¹⁶ PEG was selected as coating polymer due to its interesting properties for biomedical applications. Indeed, when compared to any other known polymer, PEG exhibits high hydrophilicity, low protein adsorption, low uptake by immune cells, and no toxicity properties.^{15,42,43} Our findings confirmed the important role of this kind of coating in terms of biocompatibility. Interestingly as presented here, PEG-BFO-NP are less toxic and their uptake in immune-responsive THP-1 cells is reduced. As a result of the multiparametric cytotoxicity evaluation, we hypothesize that BFO-NP toxicity is mainly mediated by electrostatic interactions with the surface of the particles and the cell membranes. The reduction of cytotoxicity observed upon coating is probably due to a steric barrier between

the surface of the coated particles and the lipid membrane of cells.^{15,16,42} PEG-BFO-NP were internalized in intracellular organelles and they remained located into the cells sensibly longer than U-BFO-NP. This opens interesting ways for biomedical applications, which require stable incorporation of HNP in cells. In the future, more detailed studies about BFO-NP co-localization in cells are needed in order to better understand the pathways involved in the internalization of these particles. Moreover, more detailed assessment of the immune response associated with these particles, such as a cytokine profile after exposure to the particles, could be interesting for the elucidation of their biological effects since they are immune-responding to foreign body.^{44–47} However, this work confirmed the biocompatibility of BFO-NP and their utility as nanoprobe for biological - in particular diagnostics - applications.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.nano.2014.12.018>.

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Leading opinion

A safe-by-design approach to the development of gold nanoboxes as carriers for internalization into cancer cells



Dania Movia^a, Valerie Gerard^{a,c}, Ciaran Manus Maguire^b, Namrata Jain^b, Alan P. Bell^d,
Valeria Nicolosi^{a,c,e}, Tiina O'Neill^f, Dimitri Scholz^f, Yurii Gun'ko^{a,c}, Yuri Volkov^{a,b},
Adriele Prina-Mello^{a,b,*}

^a Centre for Research on Adaptive Nanostructures and Nanodevices (CRANN), Trinity College Dublin, Dublin, Ireland

^b School of Medicine, Trinity College Dublin, Dublin, Ireland

^c School of Chemistry, Trinity College Dublin, Dublin, Ireland

^d Advanced Microscopy Laboratory, Trinity College Dublin, Dublin, Ireland

^e School of Physics, Trinity College Dublin, Dublin, Ireland

^f UCD Conway Institute of Biomolecular and Biomedical Research, University College Dublin, Belfield, D4 Dublin, Ireland

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ABSTRACT

Gold nanomaterials are currently raising a significant interest for human welfare in the field of clinical diagnosis, therapeutics for chronic pathologies, as well as of many other biomedical applications. In particular, gold nanomaterials are becoming a promising technology for developing novel approaches and treatments against widespread societal diseases such as cancer. In this study, we investigated the potential of proprietary gold nanoboxes (AuNBs) as carriers for their perspective translation into multifunctional, pre-clinical nano-enabled systems for personalized medicine approaches against lung cancer. A safe-by-design, tiered approach, with systematic tests conducted in the early phases on uncoated AuNBs and more focused testing on the coated, drug-loaded nanomaterial toward the end, was adopted. Our results showed that uncoated AuNBs could effectively penetrate into human lung adenocarcinoma (A549) cells when in simple (mono-cultures) or complex (co- and three-dimensional-cultures) *in vitro* microenvironments mimicking the alveolar region of human lungs. Uncoated AuNBs were biologically inert in A549 cells and demonstrated signs of biodegradability. Concurrently, preliminary data revealed that coated, drug-loaded AuNBs could efficiently deliver a chemotherapeutic agent to A549 cells, corroborating the hypothesis that AuNBs could be used in the future for the development of personalized nano-enabled systems for lung cancer treatment.

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1. Introduction

Gold nanomaterials have been used in biomedical applications since the discovery of gold colloids more than three centuries ago [1] and, as stated by Dreaden *et al.* in a recently published review [2], “a new ‘Golden Age’ of biomedical nanotechnology is upon us”. The recent, growing interest in gold nanomaterials arises from their unique combination of photophysical properties (such as large and tuneable light extinction, localized surface plasmon resonance (LSPR), surface-enhanced Raman scattering (SERS) and efficient

thermal ablation, to name a few), which can find exploitation in a wide range of diagnostic, biomedical and therapeutic applications.

To date, nanometer-sized gold materials are employed in the healthcare market as part of *in vitro* diagnostic devices [3] (e.g., First Response[®] pregnancy test – Carter-Wallace; Duopath[®] Verotoxins – Merck Millipore; ImmunoCAP[®] Rapid test – Phadia, Inc; and Verigene[®] platform – Nanospheres [4]) allowing the detection of biologically relevant molecules from complex fluids, such as blood plasma, with unprecedented limits of detection. In addition, the use of gold nanomaterials is currently under clinical evaluation for the treatment of solid tumours by laser-induced thermal ablation (e.g. the AuroLase[®] Therapy [5] by Auroshell[™] particles [6] – Nanospectra Biosciences, at present in phase I of clinical trials [4,7]) or by delivering anti-cancer agents (e.g. Aurimmune – CytImmune Sciences, currently in phase II of clinical trials [4,7]). Finally, gold nanomaterials (including nanoparticles, nanoclusters, nanorods,

* Corresponding author. Centre for Research on Adaptive Nanostructures and Nanodevices (CRANN), Trinity College Dublin, College Green, D2 Dublin, Ireland. Tel.: +353 1 8963259, +353 1 8963087; fax: +353 1 8963037.

E-mail address: prinamea@tcd.ie (A. Prina-Mello).

nanoshells and nanoporous surfaces) are employed in biomedical research [1,8] as *in vitro/in vivo* imaging tools [2,9], optical sensors [10,11], photothermal therapeutics [12] and drug delivery systems [2,13,14]. By exploiting the high affinity of gold surfaces for thiols, gold nanomaterials provide in fact optimal chemical reactivity conditions for functionalization of their surface with biologically active moieties (e.g. targeting and therapeutic molecules) [1]. In the near future, safely applicable (e.g. non-toxic) gold nanotechnology-products are expected to improve patients' treatment through a more adaptive and personalized approach to medicine [15], where a single construct can accumulate in the site of interest (targeting component) while allowing simultaneous diagnosis of the disease, tracking of the drug delivery (imaging component) and selective drug release to the target cells/tissue (therapy component). The personalized treatment approach through gold nanomaterials will therefore offer the opportunity to decrease the adverse side-effects of drugs, focussing the medical efforts at the target tissue/organ level [16].

The aim of our present experimental study was to evaluate the biological interactions of uncoated gold nanoboxes [17] (AuNBs) with *in vitro* models representative of the human alveolar barrier, thus defining their potential as candidates for perspective functionalization and translation into pre-clinical nano-enabled chemotherapeutic agents for the targeted and personalized treatment of lung cancer. AuNBs, which are hollow nanostructures in the shape of triangular prisms, were selected as nanoprisms are known to show a much higher degree of enhancement of the plasmon resonance energy than nanospheres [18], finding application, for example, in Surface Enhanced Fluorescence (SEF) and Surface Enhanced Raman Spectroscopy (SERS) and opening up the opportunity of using AuNBs as imaging component of a perspective nano-enabled personalized treatment against lung cancer. Worldwide, lung cancer is one of the leading causes of cancer-related mortality, with more than 157,000 deaths only in 2010 [19,20]. The survival rate of patients with lung cancer is poor, with less than 15% of patients surviving 5 years after diagnosis [21]. Lung cancer is also the third most common cancer in Ireland, accounting for 13.9% of cancers diagnosed in men and 9.4% in women during the 1994–2008 period [21]. The statistical data on the survival rate of lung cancer patients clearly evidence the current lack of effective treatments to cure lung cancer [22] and the pressing need of developing new therapeutic approaches, such as personalised nano-enabled treatments, against this disease.

In order to achieve our aim, a cross-disciplinary, three-tiered approach was used (Fig. 1). In Tier 1 the internalization of two types of uncoated AuNBs (differing from each other in size, ranging from 30 to 70 nm ca.) was assessed in *in vitro* mono-, co- and three-dimensional (3D)-culture models of human lung adenocarcinoma

(A549) cells, thus allowing an evaluation of their capability as carriers to enter into the cancer cells. The mechanism and time-dependence of cell internalization, the cytotoxicity and the biodegradation of uncoated AuNBs were also determined for assessing the safety of these nanostructures once they have entered the cells surrounding the targeted tumour tissue. Despite the fact that the Au nanoparticles with diameter above 2 nm are generally considered inert and non-toxic [23–25], dispersions of gold nanomaterials can in fact induce cytotoxicity [2,24]. Such toxicity can rise from residual chemicals of the synthesis process [23,26–28], free small molecules and metal ions present in the solution [29] or from the degradation of the gold core [23]. In Tier 2 and 3, through a safe-by-design approach [16], the long-term biocompatibility provided by a gelatine coating [30] was combined with the targeting of cancerous cells over-expressing folic acid (FA) receptors with the aim of assessing *in vitro* the ability of AuNBs to deliver a chemotherapeutic agent, namely Paclitaxel, following purpose-specific functionalization.

Our results demonstrated that uncoated AuNBs were internalised by A549 cells in mono-, co- and 3D-cultures. Although significant AuNBs internalization was achieved after 5–7 h exposure, AuNBs showed to be biologically safe in A549 cells up to 24 h. Concurrently, preliminary *in vitro* data suggested that AuNBs could indeed be a suitable carrier for the delivery of a chemotherapeutic agent following purpose-specific functionalization, thus ultimately advocating for further pre-clinical research on AuNBs as candidate for nano-enabled targeted treatment of lung cancer.

2. Materials and methods

Chemicals and solvents were purchased from commercial sources (Sigma–Aldrich, Fisher Scientific, Invitrogen and Calbiochem) and used as provided, unless otherwise specified in the manuscript.

For clarity purposes, uncoated AuNBs tested in Tier 1 will be referred to as AuNB₁ and AuNB₂, gelatine-coated AuNBs used in Tier 2 as AuNB₃, and, in Tier 3, gelatine-coated Paclitaxel-loaded AuNBs and gelatine-coated Paclitaxel-loaded AuNBs to which FA was conjugated will be indicated as AuNB₄ and AuNB₅, respectively (Fig. 2).

2.1. Synthesis of uncoated AuNBs (AuNB₁ and AuNB₂)

AuNBs in two different size ranges were synthesized according to previously published protocols [17,31]. A complete description of the synthetic procedure is reported in the Supporting Data.

2.2. Physico-chemical characterization of uncoated AuNBs

Table 1 reports the main properties of uncoated AuNBs (AuNB₁ and AuNB₂). Uncoated AuNBs were characterised by transmission electron microscopy (TEM), He-ion microscopy (HIM), Nanoparticles Tracking Analysis (NTA) and pH measurements, as described below.

2.2.1. Transmission electron microscopy (TEM) of AuNBs

The TEM specimens were prepared on 200-mesh Cu lacey carbon grids by drop-casting and were visualized under a FEI Titan Transmission Electron Microscope (FEI,

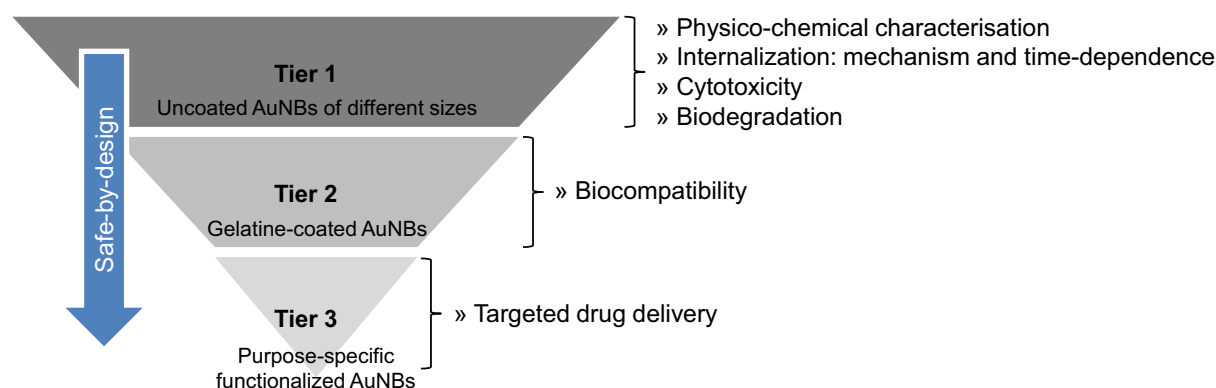


Fig. 1. Schematic of the safe-by-design, tiered approach used in this study to design a non-toxic nano-enabled carrier for therapeutic applications.

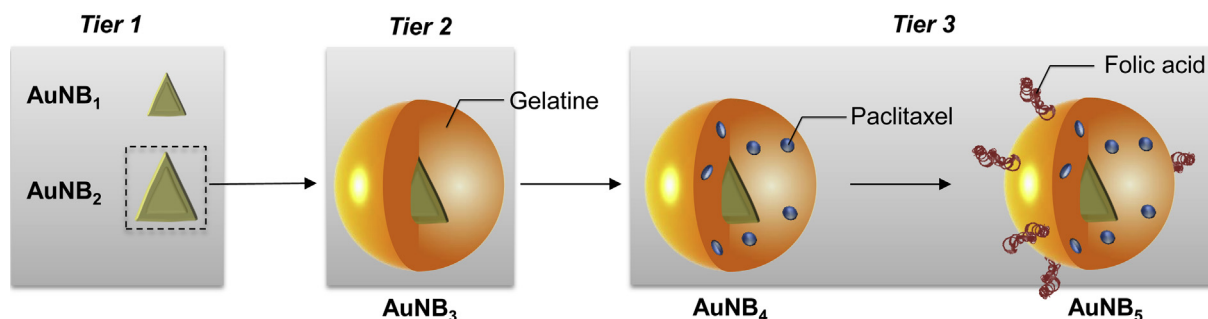


Fig. 2. Schematic of the structure of uncoated (AuNB₁ and AuNB₂) and functionalized (AuNB₃, AuNB₄ and AuNB₅) AuNBs.

Oregon, USA) operating at 300 kV. Size distributions graphs based on TEM images are reported in Fig. S1 in the Supplementary Data. The average size values (Table 1) were calculated on a sample population of 100 AuNBs.

2.2.2. He-ion microscopy (HIM)

AuNBs specimens were prepared on 200-mesh Cu lacey carbon grids or on silicon substrates by drop-casting, and imaged by a Zeiss Orion Plus He-ion microscope using an accelerating voltage of 30 kV. Samples were transferred into the chamber, which had undergone plasma clean overnight prior to loading samples, using a load lock. The working distance was 8 mm and a 10 μ m aperture. The probe current was between 0.5 and 1.5 pA. Images were acquired by collecting the secondary electrons emitted by the interaction between the He-ion beam and the specimen with an Everhart-Thornley detector. The image signal was acquired in a 32 or 64 line integration to each contributing line of the image.

2.2.3. Nanoparticle tracking analysis (NTA) and pH measurements

Stock solutions of AuNBs were vortexed for 5 s and then diluted in DEPC treated ultrapure DI water at final concentration of 1.3×10^{-11} M. The dispersions were analysed via the NTA NS500 system (NanoSight, Amesbury, UK), thus determining hydrodynamic radius and zeta potential. Measurements were carried out at pH = 7 and room temperature six depth positions, recording two videos of 60 s at depth 1 and two videos of 30 s at each subsequent position and applying a voltage of 24 V. The validation of the protocol used in this study to perform the NTA measurements has been previously published [32]. pH was measured by a Russell RL060P portable pH meter (Thermo Electron Corporation, USA) coupled with a Pellon junction microelectrode (VWR, Ireland). All pH measurements were carried out under the same environmental conditions.

Based on the higher stability of the AuNB₂ aqueous solution (zeta potential = -57.4 ± 8.2 mV) and therefore shelf-life and applicability as compared to AuNB₁ dispersion, AuNB₂ were then brought forward in Tier 2 and Tier 3 for a proof-of-principle study on targeted anti-cancer drug delivery.

2.3. Synthesis of functionalized AuNBs (AuNB₃, AuNB₄ and AuNB₅)

In order to provide preliminary evidence on the ability of AuNBs to act as carrier for anti-cancer treatments, AuNB₂ were first coated with gelatine (AuNB₃), then loaded with Paclitaxel (AuNB₄) and finally functionalized with FA (AuNB₅) (Fig. 2). A detailed description of the synthetic protocol, adapted from a previously published work [33], is reported in the Supporting Data. Gelatine was introduced to increase the long-term biocompatibility of AuNBs [30] and to allow the loading of the chemotherapeutic agent (Paclitaxel) onto AuNBs surface. Attachment of FA to the AuNBs surface had the scope of increasing the targeting specificity of our nanomaterial [34], producing a targeted nanotechnology-based system that delivers the drug specifically to the cancer cells reducing the interactions with healthy tissues [35].

Table 1

Physico-chemical properties of AuNBs. With exception of pH data, all values are reported as mean $\pm$ standard deviation.

	Stock solutions		Final experimental conditions ^a	
	TEM size (σ_{TEM} , nm)	pH (in water)	Hydrodynamic radius (in water, nm)	Zeta potential (mV)
AuNB ₁	37 ± 6	6.6	90 ± 57	-23.4 ± 8.3
AuNB ₂	62 ± 12	5.7	105 ± 38	-57.4 ± 8.2

^a Hydrodynamic radius and zeta potential were measured at pH = 7 and AuNBs concentration equal to 1.3×10^{-11} M.

2.4. Cell culture

Human alveolar epithelial adenocarcinomic cells (A549 cell line) and human monocytic leukaemia cells (THP-1 cell line) were obtained from the American Tissue Culture Collection (ATCC, USA). A549 cells were cultured in Hams F12K media (supplemented with 2 nM L-glutamine, 1% penicillin/streptomycin and 10% foetal bovine serum (FBS)), while THP-1 cells were cultured in RPMI 1640 media (supplemented with 2 nM L-glutamine, 1% penicillin/streptomycin and 10% FBS). Cells were incubated at 37 °C and 5% CO₂. Mycoplasma and phenotypic responses were regularly checked for contamination as part of the laboratory GLP. The passage number was restricted between five and twelve. At 80% confluence, A549 cells were detached from T75 flask substrate with TrypLE™ (Gibco, Invitrogen, Oregon, USA), centrifuged, counted using a Countess™ Automated Cell Counter (Invitrogen, Oregon, USA) and diluted in supplemented media at concentrations appropriate for each experimental protocol. When 60% confluence was reached, THP-1 cells were activated in a T25 flask with 30 ng/ml phorbol-12-myristate-13-acetate (PMA) (Sigma–Aldrich, USA) for 72 h to induce their differentiation into adherent macrophages and stop their natural proliferation. After 72 h THP-1 cells were washed and stained with 20 μ M Cell Tracker™ Green CMFDA (Invitrogen, Oregon, USA) for 45 min at 37 °C and 5% CO₂ to allow their identification in co-culture models. THP-1 cells were then washed with fresh supplemented media, detached from flask substrate with TrypLE™ (10 min, 37 °C), counted using a Countess™ Automated Cell Counter (Invitrogen, Oregon, USA) and diluted in supplemented media at concentrations appropriate for each experiment.

2.4.1. Mono-culture models

A549 and PMA-activated THP-1 cells were plated in 4-well Millicell® EZ Slide (Millipore™, MA, USA) at a concentration of 5000 and 1000 cells/well, respectively (final volume: 500 μ l/well). Cells were incubated for 24 h at 37 °C (5% CO₂) to allow cell attachment to the glass substrate. When cultured under inverted culture conditions, A549 cells mono-cultures grown on glass slides were placed on sterile glass-slide holders with the cells facing the bottom of the petri-dish and immersed into a dispersion of AuNBs in supplemented cell media.

2.4.2. Co-Culture models

To produce a co-culture representative of the cellular composition of alveoli in the human lungs, A549 and PMA-activated THP-1 cells were seeded in a ratio 9:1 [36]. The 9:1 ratio corresponds to the ratio between the total number of cells (230×10^9 cells) across the whole alveolar area, which are mainly epithelial cells, and the number of alveolar macrophages (22×10^9 cells) [37]. A549 cells (doubling time: 22 h) were plated in 4-well Millicell® EZ Slide at a concentration of 5000 cells/well and incubated for 24 h at 37 °C and 5% CO₂ to allow cell attachment. After 24 h, PMA-activated THP-1 cells were plated onto the A549 culture at a concentration of 1000 cells/well to achieve a 9:1 ratio (A549:THP-1 cells). Co-cultures were then incubated for 24 h at 37 °C and 5% CO₂ to allow THP-1 cells attachment. The adhesion of THP-1 cells on top of the epithelial cells was shown by confocal microscopy imaging (reported in Fig. S2 in the Supporting Data) at confirmation of the successful co-existence of the two cell types.

2.4.3. 3D-culture models

A549 cells were seeded onto layers (thickness around 1 mm) of Matrigel™ Basement Membrane Matrix or PuraMatrix™ Peptide Hydrogel (both from BD Biosciences, UK). Matrigel™ is a solubilized basement membrane preparation extracted from the Engelbreth-Holm-Swarm (EHS) mouse sarcoma, a tumour rich in extracellular matrix proteins; PuraMatrix™ is a synthetic matrix that is used to create defined 3D microenvironments for a variety of cell culture experiments. Briefly, Matrigel™ Basement Membrane Matrix was thawed by submerging the vial in ice in a 4 °C refrigerator overnight. The vial was then swirled to ensure that the matrix was evenly dispersed and, using cooled pipette tips and keeping the glass slides on ice, the Matrigel™ Basement Membrane Matrix was added to the surface of a 4-well Millicell® EZ slide following the Thick Gel Method described by the supplier. This

method is recommended by BD Biosciences to grow cells within a 3D matrix. Slides were incubated at 37 °C for 30 min prior cell seeding. For PuraMatrix™, the stock solution (1% w/v) was diluted to the working concentrations of 0.25% with sterile DI water. 250 µl of PuraMatrix™ dilutions were then added to the surface of a 4-well Millicell® EZ slide and gelation was promoted by carefully and slowly adding supplemented F12K medium to each well (500 µl/well). The glass slide was incubated at 37 °C for 1 h to complete the gelation of the hydrogel. After the hydrogel has assembled, the medium was changed twice over a period of 1 h to equilibrate the environment to physiological pH. A549 cells were carefully seeded at the top of the Matrigel™ or PuraMatrix™ layers at a concentration of 10⁶ cells/ml (500 µl/well). Cell cultures were grown for 4 d and cell medium was changed every 3 d.

2.5. Cellular internalization of uncoated AuNBs – qualitative analysis

The internalization of uncoated AuNBs into cells was assessed in *in vitro* mono-, co- and three-dimensional (3D)-cultures. Such *in vitro* models were exposed to AuNB₁ and AuNB₂ at a concentration of 1.3×10^{-11} M for 24 h. After 24 h exposure, three washings with phosphate-buffered saline (PBS) were carried out in order to completely remove the unbound and not-internalized AuNBs. Successful internalization of the nanomaterials was assessed by confocal microscopy and TEM.

2.5.1. Confocal microscopy

After fixation with 4% paraformaldehyde (PFA) for 10 min at room temperature, mono-, co- and 3D-cultures were stained with Hoechst 33342 for nuclei and rhodamine phalloidin (Invitrogen, Oregon, USA) for F-actin. The slides were incubated at room temperature for 1 h in the dark, rinsed with PBS and mounted in transparent mounting medium (VECTASHIELD, Vector Laboratories Inc., CA, USA) prior to confocal microscopy analysis by a ZEISS 510 Meta confocal microscope equipped with a Zeiss LSM 5 software (Carl Zeiss, Germany). AuNBs were imaged in reflectance mode at $\lambda_{\text{exc}} = 561$ nm. Qualitative confocal imaging was carried out by acquiring a series of z-stack images. Surface rendering of z-stack images was carried out by the open-source software BioluminaXD [38] in order to further elucidate the AuNBs internalization.

2.5.2. Transmission electron microscopy (TEM) of biological specimens

TEM images of ultrathin sections of A549 cells cultured as mono- and 3D-cultures and exposed to AuNB₂ were examined. After exposure to AuNB₂, A549 cells were fixed at room temperature in 2.5% glutaraldehyde (GA) in 0.1 M Sørensen's phosphate buffer (pH 7.4), rinsed with Sørensen's phosphate buffer and then post-fixed for 1 h in 1% osmium tetroxide in 0.1 M Sørensen's phosphate buffer. After dehydration at increasing concentrations of EtOH (from 30% up to 100%), the samples were immersed in an ethanol/Epon (1:1 vol/vol) mixture for 1 h before being transferred to pure Epon at 37 °C for 2 h. The polymerization was carried out at 60 °C for 24 h. For orientation purposes sections from each sample were cut at 1 µm, stained with toluidine blue, and examined by light microscopy (Nikon Eclipse TE300 epifluorescence microscope). From these survey sections areas of interest were identified and ultrathin sections of 80 nm, were obtained with a diamond knife using a Leica EM UC6 ultramicrotome (Leica, Germany). These sections were mounted on EM grids (300-mesh Cu grids) and stained with uranyl acetate and lead citrate before being examined with a TEM (FEI TECNAI Transmission Electron Microscope or FEI Titan Transmission Electron Microscope (FEI, Oregon, USA)).

2.5.3. Raman spectroscopy

The AuNBs internalized into A549 cells mono-cultures was also detected by Raman spectroscopy. A549 cells exposed to AuNB₁ and AuNB₂ as previously described were fixed with 2.5% GA in 0.1 M Sørensen's phosphate buffer (pH 7.3). Raman spectra were acquired using NTEGRA Spectra AFM-Raman microscope (NT-MDT, Russia) with a cobalt solid-state laser operating at a wavelength of 473 nm. All spectrums were recorded over the range of 2850–3800 cm⁻¹ for 10 s, at 100% laser power, using a 100 × oil immersion objective and averaged across 5 scans per sample. Obtained spectra were analysed with baseline correction, smoothing and normalization using Renishaw WIRE software (Renishaw, Gloucestershire, UK).

2.6. Cellular internalization of uncoated AuNBs – time-dependence

Absorbance spectroscopy was used to quantify the amount of uncoated AuNBs internalized into A549 cells overtime. AuNB₁ and AuNB₂ have specific absorption peaks in the UV/Vis region (at 560 nm and 700 nm, respectively) that do not overlap with those of cellular components (see Fig. S3 in the Supporting Data). A549 cells mono-cultures were formed in 96-well plates (5000 cells/well) and were exposed to AuNBs (1.3×10^{-11} M) for 0, 1, 3, 5 and 7 h. At each time-point, cells were washed carefully with PBS to eliminate any unbound, not-internalized AuNBs; fixation then followed by incubation with 4% PFA for 10 min. Absorption at 560 nm and 700 nm was recorded by an Epoch microplate reader (Biotek, USA), calibrated against untreated cells and corrected by subtracting the optical aberration of the 96-well plastic plate at 540 nm. The absorption at 560 nm and 700 nm of known concentrations of AuNBs was also recorded at the same time, generating the calibration curve and allowing the quantification of the internalized AuNBs.

2.7. Cellular internalization of uncoated AuNBs – mechanism

To determine if uncoated AuNBs were internalized into cells by active or passive transport routes, A549 mono-cultures were exposed to AuNB₁ and AuNB₂ (1.3×10^{-11} M) for 24 h in the presence of inhibitors of the cellular energy-dependent mechanisms of uptake, i.e. at 4 °C or in the presence of sodium azide (NaN₃). For qualitative analysis, A549 cells were stained with Hoechst 33342 for nuclei and rhodamine phalloidin (Invitrogen, Oregon, USA) for F-actin for 1 h at room temperature in the dark. After washing with PBS, specimens were analysed by confocal microscopy, as previously described. For quantitative analysis, the intracellular accumulation of AuNBs was quantified by absorption spectroscopy by an Epoch microplate reader, as aforementioned.

2.8. Cytotoxicity of uncoated AuNBs

High Content Screening and Analysis (HCSA) and Enzyme-Linked Immunosorbent Assay (ELISA) assays were used to quantify the cytotoxic responses and cytokine secretion of A549 mono-cultures when exposed to uncoated AuNBs at four concentrations (2.1×10^{-12} , 5.3×10^{-12} , 1.1×10^{-11} and 1.3×10^{-11} M) for 24 h. A549 cells were plated in 96-well plates (Nunc Inc., USA) at a concentration of 5000 cells/well.

2.8.1. High content screening and analysis (HCSA)

Following exposure to uncoated AuNBs, a multiparametric cytotoxicity assay was performed using HCS reagent HitKit™ as per manufacturer's instructions (Thermo Fisher Scientific Inc., USA). Briefly, this kit enables to measure cell viability, cell membrane permeability and lysosomal mass/pH, which are toxicity-linked cellular markers. The experimental layout for the automated microscopic analysis, based on the In Cell Analyzer 1000, was composed of untreated cells (negative control or N/T), cells treated with uncoated AuNBs and cells exposed to doxorubicin (positive control or P/T) at a dose (200 µM) above the drug's IC₅₀. Images were acquired in a stereology configuration of ten randomly selected fields at 10× objective lens magnification using three detection channels with different excitation filters. The rate of cell viability and proliferation were assessed by the automated analysis of the nuclear count and morphology (DAPI filter); in parallel the fluorescent staining intensities reflecting cell permeability (FITC filter) and lysosomal mass/pH changes (TRITC filter) were also quantified for each individual cell present in the examined microscopic fields (IN Cell Investigator, GE Healthcare, UK).

2.8.2. Cytokines secretion

The secretion of the pro-inflammatory cytokine Interleukin-6 (IL-6) from A549 cells exposed to uncoated AuNBs was quantified by ELISA (Human IL-6 DuoSet ELISA kit, R&D Systems, Minneapolis, USA), according to the manufacturer's manual. The assay was carried out in triplicate on cells supernatant solutions. The optical density of each well at 450 nm was determined by means of an Epoch microplate reader (Biotek, USA), calibrated against standards and corrected by subtracting the optical aberration of the 96-well plastic plate at 540 nm.

2.9. Biodegradation of uncoated AuNBs

In order to investigate whether uncoated AuNBs were degraded by A549 cells, AuNBs were retrieved from A549 mono-cultures after exposure. Briefly, A549 cells were washed with PBS after 24, 48 or 72 h exposure to AuNBs in order to completely remove unbound, not-internalized AuNBs. Cells were then trypsinized, centrifuged and resuspended in DI water, thus causing cell swelling and osmotic burst. Cell bursting was facilitated by cell membrane damage caused by placing the water-saturated cells suspensions at –20 °C. Multiple cycles of centrifugation/resuspension were carried out to clean the AuNBs suspensions from the residual biological material. Collected AuNBs samples were then imaged by TEM and HIM, as previously described in the physico-chemical characterization section. In addressing topics of the biological sciences, HIM offers various advantages over conventional scanning electron microscopy (SEM) approaches, such as the ability to image uncoated, non-conductive samples without the deposition of a metal (or other conductive) overcoat [39,40], which can indeed destroy, reduce and/or completely mask details of the specimen surface. In this study, we deployed the high resolution imaging capabilities of HIM to image the adsorption of biological material onto AuNBs surface and the changes in particles morphology with unsurpassed image quality and detail. The hydrodynamic radius of the collected AuNBs was measured by NTA, as previously described.

2.10. Biocompatibility of gelatine-coated AuNBs (AuNB₃)

Following 24 h and 72 h exposure of A549 mono-cultures to AuNB₃ at a working concentration of 10^{-11} M, Trypan Blue exclusion assay was carried out. The experimental design included a negative control (untreated cells), a positive control (cells treated with 70% methanol for 30 min at 37 °C) and cells treated with AuNB₂ (10^{-11} M) as internal control. Each treatment was carried out in triplicate and experiments were carried out in duplicate. Live and dead cells were quantified by a Countess™ cell counter (Invitrogen, UK). The percentage (%) of live cells was then calculated as for Equation (1):

$$\% \text{live cells} = \frac{\text{live cells}}{(\text{live cells} + \text{dead cells})} \times 100 \quad (1)$$

2.11. Functionalized AuNBs (AuNB₄ and AuNB₅) for drug delivery – proof-of-principle

Trypan Blue exclusion assay was carried out after exposing A549 mono-cultures to AuNB₄ and AuNB₅ (10^{-11} M) for 24 and 72 h. The experimental design included a negative control (untreated cells), a positive control (cells treated with 70% methanol for 30 min at 37 °C) and cells treated with molecular Paclitaxel (0.03 mM) as term of comparison. Experimental protocol and quantification of the percentage of live cells was performed as described in the previous section.

2.12. Statistical analysis

Two-way analysis of variance (ANOVA) followed by a Bonferroni post-test analysis was carried out (Prism, Graph-Pad Software Inc., USA). A *p* value <0.05

was considered statistically significant. Unless differently stated in the manuscript, all data are presented as mean values ($n_{\text{test}} = 3$) $\pm$ standard deviation and normalized to the negative control.

3. Results

The three-tiered approach adopted in this study consisted of systematic tests conducted in Tier 1 on uncoated AuNBs and of more focused, preliminary testing on the coated, drug-loaded nanomaterials in Tier 2 and 3. Such approach finds his routes in the concept of safe-by-design nanomaterials, where efforts are focused on characterizing the physical, chemical and biological properties of the core material, followed by “layering” as a method to produce safe nano-enabled theranostics [16].

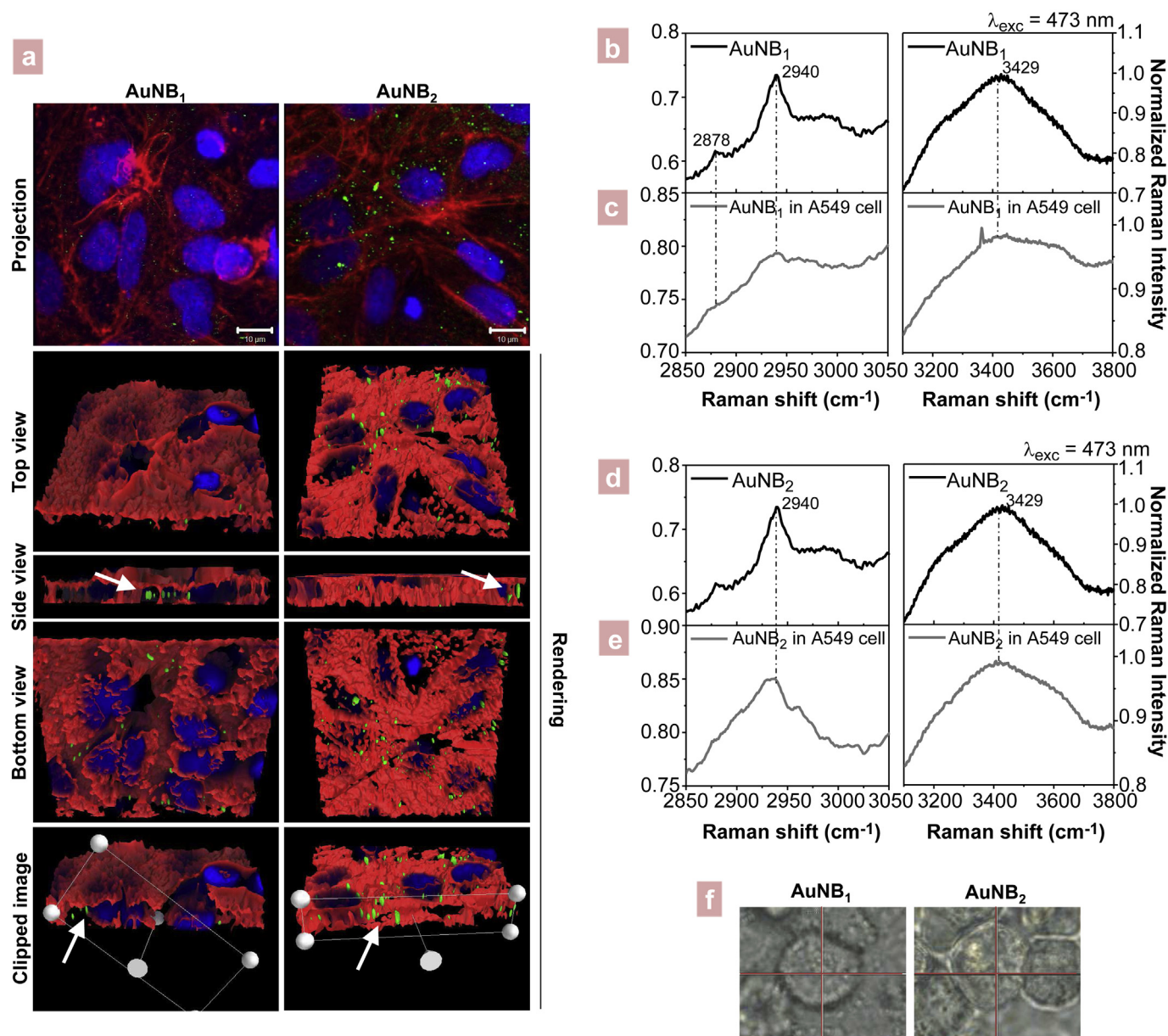


Fig. 3. Cellular internalization of uncoated AuNBs in mono-cultures of A549 cells after 24 h exposure. (a) Projections and rendered reconstructions of representative confocal micrographs of A549 cells exposed to AuNBs and stained with rhodamine phalloidin (F-actin, in red) and Hoechst 33342 (nuclei, in blue). AuNBs were imaged in confocal reflectance mode and are shown in green as pseudo-colour. The localization of AuNBs (indicated by arrows) in the cells cytoplasm is evident. Scale bars: 10 μ m (63 $\times$ objective lens). (b, d) Raman spectra ($\lambda_{\text{exc}} = 473$ nm) of (b) AuNB₁ and (d) AuNB₂. The characteristic Raman bands of AuNBs (2878, 2940 and 3429 cm^{-1}) are clearly shown. (c, e) Raman spectra ($\lambda_{\text{exc}} = 473$ nm) of (c) AuNB₁ and (e) AuNB₂ internalized into A549 cells. (f) Brightfield images of A549 cells exposed to uncoated AuNBs. The red lines intersection show the point at which the Raman spectra reported in images c and e were collected, clearly evidencing that AuNBs were detectable in the area corresponding to the cells cytoplasm.

3.1. Tier 1

Uncoated AuNBs (AuNB₁ and AuNB₂) were synthesized by galvanic replacement reactions [17] [41], a method previously reported for the synthesis of bimetallic hollow nanostructures of a range of shapes and sizes. The AuNBs tested in Tier 1 were hollow nanoprisms with monodispersed sizes (height of the triangle: 37 ± 6 nm for AuNB₁ and 62 ± 12 nm for AuNB₂) and controlled thickness of the nanobox walls. The AuNBs aqueous dispersions produced were characterized by optimal colloidal stability (with zeta potential ranging from -20 to -60 mV ca.), thus allowing investigating the internalization of uncoated, non-functionalised AuNBs into the cells and any potential and/or unintended toxic effect that the gold core may induce as a consequence of cellular access.

3.1.1. Cellular internalization of uncoated AuNBs – qualitative analysis

In order to be one-step closer to *in vivo* complex scenarios, the effective internalization of uncoated AuNBs (AuNB₁ and AuNB₂)

was assessed in *in vitro* mono-, co- and 3D-culture models representative of the human alveolar barrier. The exposure time was fixed at 24 h, which can be considered a pharmacological relevant time-point if compared to the infusion schedules used in clinical practice for chemotherapeutic agents such as Paclitaxel, ranging between 3 h and 24 h [42].

In mono-culture models, the internalization of uncoated AuNBs was assessed in A549 (Figs. 3 and 4) and THP-1 (see Fig. S4 in the Supporting Data) cells, as well as in a murine model of phagocytic cells (J774 cell line) (as described in the additional experimental section reported in the Supporting Data). While human and murine macrophage-like (THP-1 and J774) cells were used to represent the resident phagocytic cells in the alveoli, the A549 cell line was chosen as a physiologically relevant *in vitro* model of the *in vivo* non-small cell lung cancer (NSCLC) [43], the most prevalent form of lung cancer originating from epithelial cells, and therefore as an optimal cellular target for personalized medicine approaches. In addition A549 cells, as immortalised cell line, are considered one of the closest cell model to mimic alveolar epithelial type II cells (e.g. presenting membrane-bound inclusions, which resemble

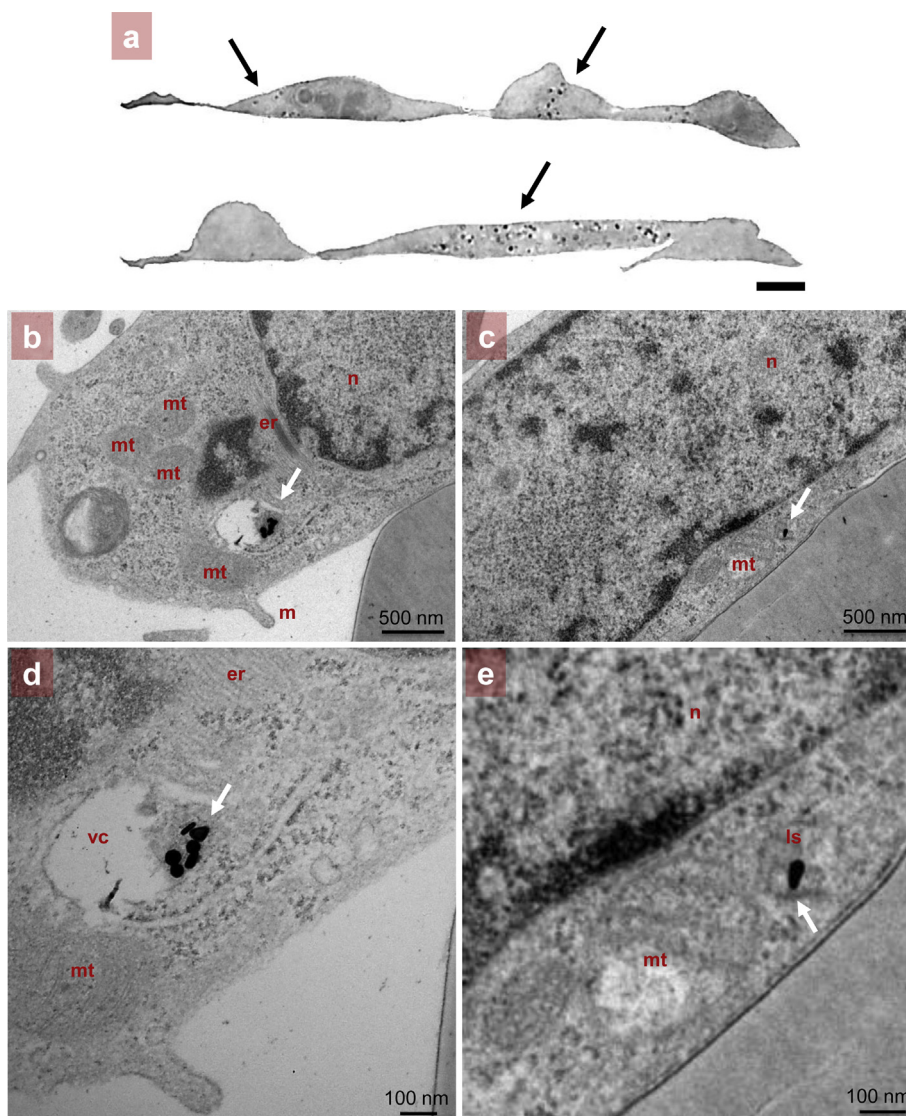


Fig. 4. Representative (a) light microscopy and (b–e) TEM images of ultrathin cross-sections (80 nm) of A549 cells exposed to AuNB₂ for 24 h. (a) The localisation of AuNB₂ (black dots indicated by arrows) into the cellular bodies is evident. Scale bar: 20 μm (100 × objective lens). (b–e) AuNB₂ (indicated by arrows) can be recognized by their geometrical shape and contrast as internalized in vacuoles and lysosomes. Images d and e are magnifications of images b and c, respectively. Abbreviations: er, endoplasmic reticulum; ls, lysosome; m: microvilli; mt: mitochondria; n: nucleus; vc: vacuole.

lamellar bodies of type II cells) [44] and, therefore, an appropriate epithelial alveolar cell model [45]. Finally, A549 cells have also proven to be a robust cell line/alveolar model for several previous nanomaterial-based studies [46–50].

Confocal microscopy images evidenced that uncoated AuNBs (imaged in reflectance mode) were effectively internalized into the A549, THP-1 and J774 cell mono-cultures (Fig. 3a, Fig. S4 and Fig. S6, respectively) and were found contained within the cells cytoplasm. Raman spectroscopy also confirmed that uncoated AuNBs were detectable in the areas corresponding to the A549 cells cytoplasm (Fig. 3c, e and 3f). In detail, the characteristic Raman signatures of AuNBs (bands at 2878, 2940 and 3429 cm^{-1}) (Fig. 3b and d) were detectable when A549 cells were exposed to AuNBs for 24 h (Fig. 3c and e). Such bands were unique of AuNBs and did not overlap with the Raman signals of the cell culture substrate (glass) or of the solutions in which cells were incubated and stored (Fig. S5 in the Supporting Data).

Further to this, light microscopy (Fig. 4a) and TEM (Fig. 4b–e) imaging of ultrathin cross-sections of A549 cells exposed to AuNB₂ and embedded in Epon resin showed that uncoated AuNBs (indicated by arrows in Fig. 4) were localized into the cellular bodies after 24 h. In detail, TEM images showed that AuNB₂ could be found stored in vacuoles and lysosomes of A549 cells (Fig. 4b–e), and suggested that AuNB₂ were trapped by cell membrane invagination (Fig. 4b and d).

Based on our experimental data showing that AuNB₁ and AuNB₂ were uptaken in macrophage-like cells (Fig. S4 and Fig. S6), we developed a co-culture system formed by A549 and THP-1 cells with the aim of mimicking the pivotal function of macrophages in the alveoli of the lung against inhaled particulate [51]; macrophages could therefore play a major role in the unexpected clearance of AuNBs before they reach the cellular target (i.e., the lung adenocarcinoma cells). Confocal microscopy analysis of A549/THP-1 co-cultures demonstrated that AuNB₁ and AuNB₂ were

internalized into A549 cells even when co-cultured with macrophage-like THP-1 cells (Fig. 5), and uncoated AuNBs could be detected in the cytoplasm of both cell types.

Finally, in 3D cell cultures, which were used to mimic more closely the native three-dimensional architecture and microenvironment of human lung adenocarcinomas [52], our results demonstrated that uncoated AuNBs were effectively internalized into A549 cells cultured onto hydrogels layers (Matrigel™ and PuraMatrix™). In particular, confocal analysis and TEM imaging showed that uncoated AuNBs were localised into the cytoplasm of cells grown onto PuraMatrix™ (Fig. 6a–b) or Matrigel™ (Fig. 6c). Interestingly, transmission electron micrographs evidenced that, in both 3D cell culture models, AuNBs were localised in lysosomes and characteristics cytoplasmic lamellar bodies (Fig. 6b and c).

3.1.2. Cellular internalization of uncoated AuNBs – time-dependence

Absorption measurements by a microplate reader were used with the purpose of defining the time needed to achieve significant internalization of uncoated AuNBs into A549 cell mono-cultures. In detail, the absorbance at 560 nm and 700 nm was measured to quantify the amount of internalized AuNB₁ and AuNB₂, respectively. Fig. 7a shows the concentration of AuNBs internalized into A549 cells at different time-points (0, 1, 3, 5 and 7 h). Significant internalization of AuNB₁ and AuNB₂ was achieved at 5 h and 7 h, respectively.

The influence of the *in vitro* environment on the time-dependent AuNBs internalization was also assessed. Confocal microscopy analysis of A549 cells cultured under inverted conditions and exposed to uncoated AuNBs showed that the internalization of these nanomaterials was nearly equal to zero after 24 h when A549 cells were cultured in these conditions (Fig. 7b). In agreement with previous studies on gold nanomaterials [53] and on other nanoparticles [2,54], the time-dependent cellular internalization of

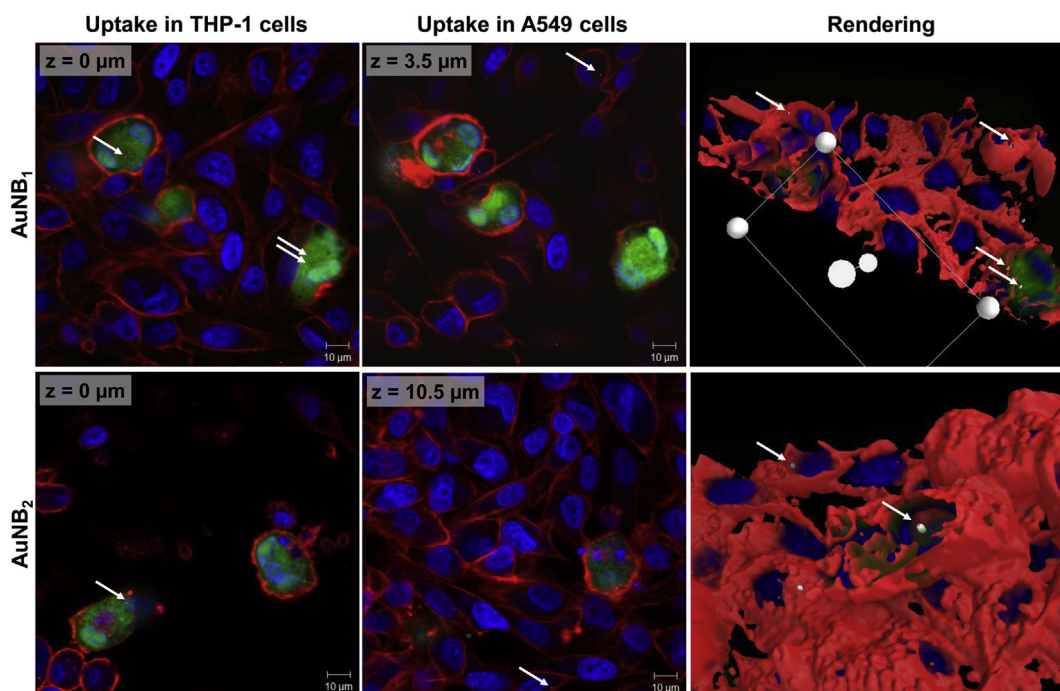


Fig. 5. Cellular internalization of uncoated AuNBs in co-cultures of A549 cells and macrophage-like THP-1 cells after 24 h exposure. Projections and rendered reconstructions of representative confocal microscopy images of AuNBs-treated co-cultures stained with Hoechst 33342 (nuclei, in blue) and rhodamine phalloidin (F-actin, in red). THP-1 cells were also stained with Cell Tracker™ Green CMFDA (in green). AuNBs were imaged in confocal reflectance mode and are shown in white as pseudo-colour. The localization of AuNBs in the cytoplasm of both cell types is highlighted by arrows. Scale bars: 10 μm (63 $\times$ objective lens).

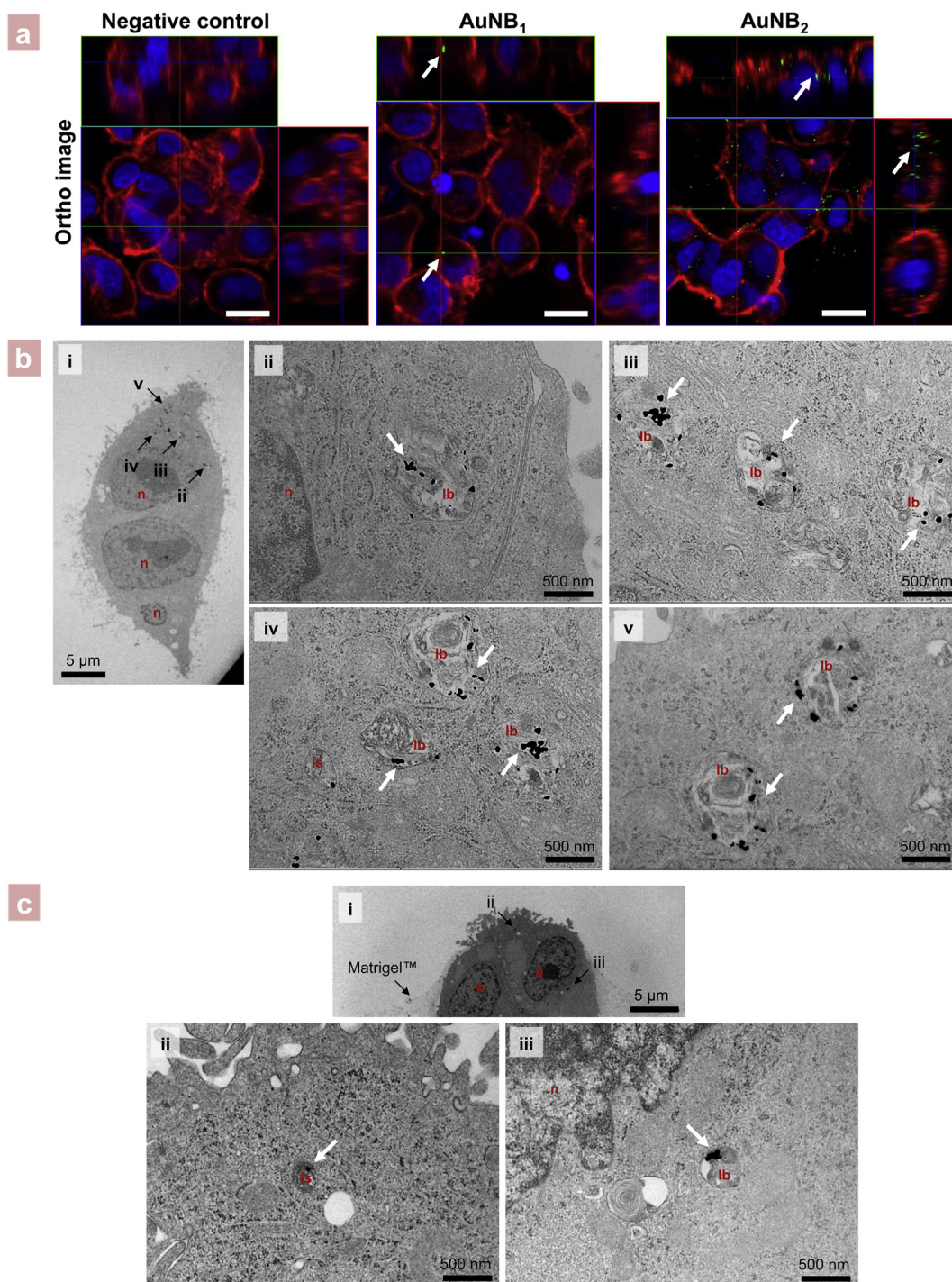


Fig. 6. Cellular internalization of uncoated AuNBs in 3D cell cultures grown onto (a, b) PuraMatrix™ (0.25%) or (c) Matrigel™. (a) Ortho-images of representative confocal micrographs of 3D cell cultures exposed to uncoated AuNBs and stained with rhodamine phalloidin (F-actin, in red) and Hoechst 33342 (nuclei, in blue). AuNBs were imaged in confocal reflectance mode and are shown in green as pseudo-colour. The localization of AuNBs (arrows) in the cells cytoplasm is evident. Scale bars: 10 µm (63 × objective lens). (b and c) TEM images of ultrathin cross-sections (80 nm) of 3D cell cultures grown onto (b) PuraMatrix™ (0.25%) or (c) Matrigel™ and exposed to AuNB₂ for 24 h AuNB₂ (indicated by white arrows) can be recognized by their geometrical shape and contrast as internalized in lysosomes and lamellar bodies. (b) Images ii–v are magnifications of image i. (c) Images ii and iii are magnifications of image i. Abbreviations: lb: lamellar bodies; ls: lysosome; n: nucleus.

uncoated AuNBs was therefore strongly influenced by their sedimentation rate.

3.1.3. Cellular internalization of uncoated AuNBs – mechanism

In order to define the mechanism of AuNBs internalization into lung adenocarcinoma cells, we investigated whether the cellular

uptake of the uncoated AuNBs was mediated by an energy-dependent mechanism. Thus, A549 cells were exposed to AuNB₁ and AuNB₂ at 4 °C or in the presence of 0.1% sodium azide (NaN₃) for 24 h, and the AuNBs internalization was quantified by absorption spectroscopy. Results were then compared to the 37 °C experiment. A549 cells incubated at 4 °C did not exhibit any

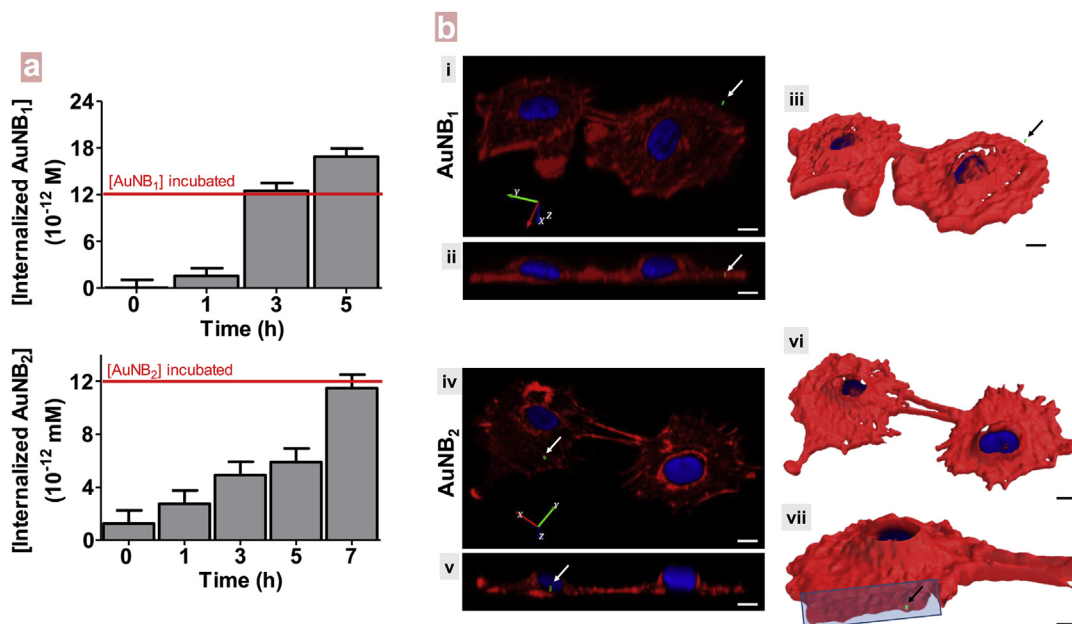


Fig. 7. (a) Internalization of uncoated AuNBs into A549 cells mono-cultures overtime. Concentration of AuNBs into cells after 0, 1, 3, 5 and 7 h, as quantified by absorption spectroscopy. Data are reported as mean $\pm$ standard deviation. (b) Internalization of (i–iii) AuNB₁ and (iv–vii) AuNB₂ into A549 cells cultured in an inverted configuration. Rendering of representative confocal images of AuNBs-treated cells, stained with rhodamine phalloidin (F-actin, in red) and Hoechst 33342 (nuclei, in blue). AuNBs (indicated by arrows) were imaged in confocal reflectance mode and are shown in green as pseudo-colour. (b: vii) Detail of image vi. The light-blue rectangle represents the clipping plane. The presence of a single uncoated AuNB inside the cell cytoplasm is highlighted. Scale bars: (i–vi) 10 μ m, (vii) 5 μ m (63 $\times$ objective lens).

reduction in the AuNBs uptake as compared to the equivalent incubation at 37 °C (Fig. 8a and b). Moreover, blocking the active transport mechanism of the A549 cells by 0.1% NaN₃ treatment impeded only the uptake of AuNB₁ (Fig. 8a), leaving unaffected the AuNB₂ internalization grade (Fig. 8b). One important aspect to consider when inhibiting endocytosis is to leave unaffected the F-actin cytoskeleton of the cell, since reorganization of the actin filaments can impact on cellular uptake processes [55], leading to multiple effects occurring simultaneously. In our study, the F-actin filaments maintained their overall morphology and distribution when A549 cells were treated with NaN₃ (Fig. 8c), in accordance with previously published studies [56].

The hydrodynamic radius of the internalized AuNBs (Fig. 8d) appeared significantly increased when retrieved from A549 cells exposed for 24 h, as compared to reference, as-synthesized AuNBs (0 h). This indicated protein opsonisation, e.g. the adsorption of proteins such as serum albumin (which is richly in the cell culture media in the form of FBS) onto the surfaces of uncoated AuNBs [57,58]. These data were strongly supported by HIM imaging (Fig. 8e), showing that AuNB₂ retrieved from A549 cells mono-cultures after 24 h incubation were coated in biological material. Protein opsonisation is strongly linked to endocytosis mechanisms [59], and it well correlated with our experimental data showing that AuNBs are internalized by A549 cells within 24 h.

3.1.4. Cytotoxicity of uncoated AuNBs

Cytotoxicity of uncoated AuNBs was measured by HCSA [47,48,60–62] on all the chosen concentrations (2.1×10^{-12} , 5.3×10^{-12} , 1.1×10^{-11} and 1.3×10^{-11} M) as fully described in the materials and methods section. Cell count, cell membrane permeability and lysosomal mass/pH were the cell parameters monitored (Fig. 9a). In addition, quantification of the secretion of the pro-inflammatory cytokine Interleukin-6 (IL-6) was carried out for each particle size and concentration (Fig. 9b and c). A549 cells did

not show any obvious reduction in cell count or any significant change in cell membrane permeability and lysosomal mass/pH when exposed to uncoated AuNBs for 24 h. It has been extensively proven that changes in the cellular membrane permeability indicate alterations of the physical condition of the cells, while a decrease or an increase of lysosomal mass/pH can be associated with an increased rate of cytotoxicity of the material tested. The secretion of IL-6 was also reduced when compared to the untreated (N/T) control, indicating that no inflammatory response was induced by exposure to uncoated AuNBs for 24 h.

3.1.5. Biodegradation of uncoated AuNBs

Evaluating the potential biochemically-induced changes of nanomaterials after administration can provide invaluable data (1) on the materials properties that are related to the observed cell responses or (2) on the potential expected responses following long-term exposure [63,64]. To assess whether uncoated AuNBs were subject to degradation following internalization into cells, AuNB₁ and AuNB₂ were retrieved from A549 cell mono-cultures by osmotic cell lysis after 24, 48 and 72 h internalization. TEM analysis showed significant changes in AuNBs morphology and wall thickness after 72 h internalization into cells, when compared to the reference, as-synthesized material (0 h) (Fig. 10a). Similarly, a significant reduction in the AuNBs size and changes in their morphology were evidenced after 72 h incubation with cells by HIM (Fig. 10b). This data was confirmed by the size distributions of AuNBs as calculated from TEM images (Fig. 10c), showing substantial reduction in AuNBs size after 72 h incubation.

In light of the extensive and comprehensive work carried out in Tier 1 to assess the biocompatibility and suitability of uncoated AuNBs as carriers, a safe-by-design approach was applied and the functionalised, multi-layered AuNBs produced were subject to a proof-of-principle testing in Tier 2 and 3.

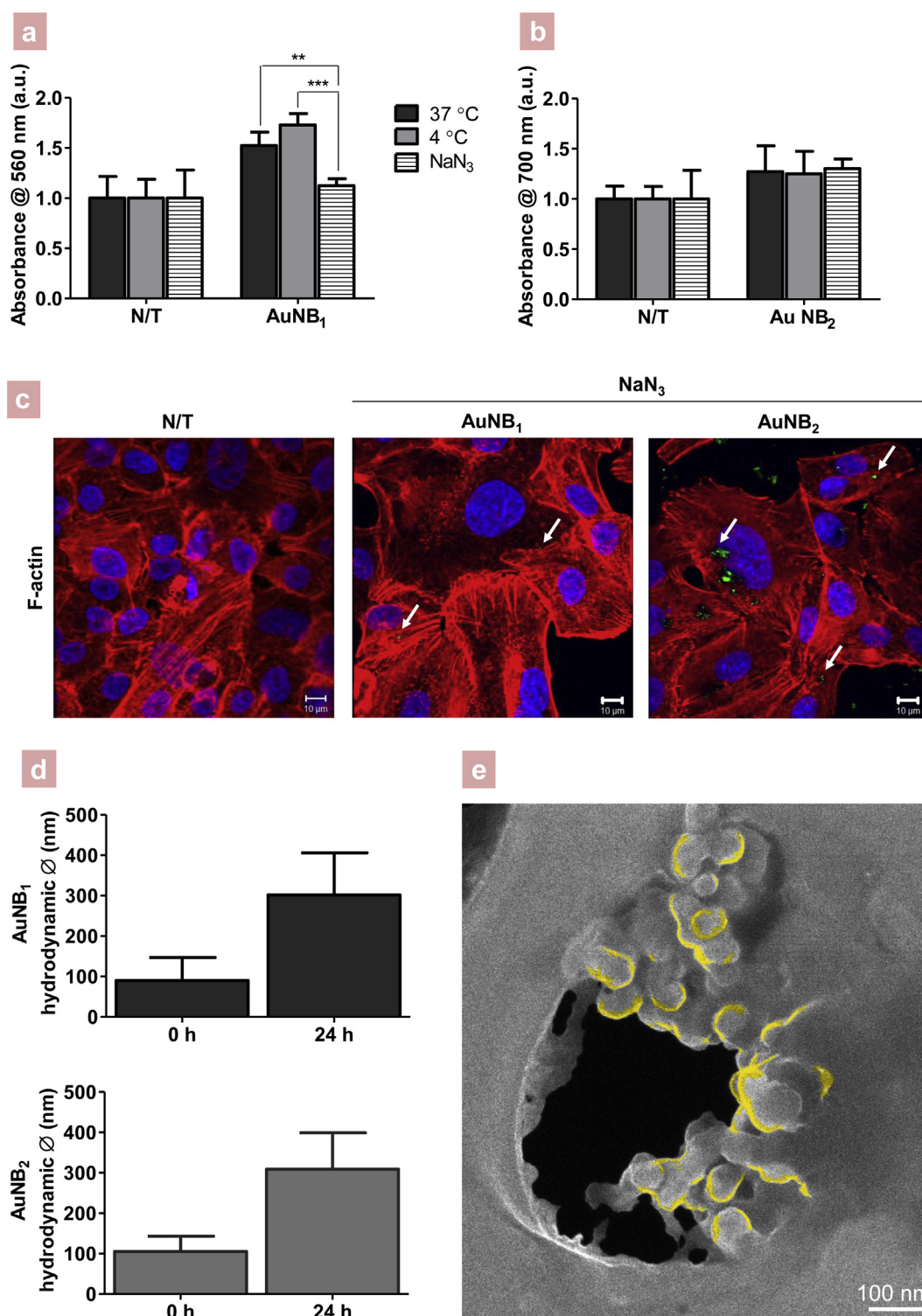


Fig. 8. (a–c) Inhibition of the internalization of uncoated AuNBs into A549 cells mono-cultures (a–b) Absorbance at (a) 560 nm and (b) 700 nm of untreated (N/T) and AuNBs-treated A549 cells. 560 nm and 700 nm are the $\lambda_{\max}$ of absorbance of AuNB₁ and AuNB₂, respectively. A549 cells mono-cultures were exposed to AuNBs at 37 °C (grey bars), 4 °C (light grey bars) or after pre-treatment with NaN₃ for 3 h (striped bars). Values are reported as mean $\pm$ standard deviation and are normalised on untreated controls. (c) Representative confocal images of A549 cells pre-treated with NaN₃ for 3 h. Cells were stained with rhodamine phalloidin (F-actin, in red) and Hoechst 33342 (nuclei, in blue). Uncoated AuNBs (indicated by arrows) were imaged in reflectance mode and are shown in green as pseudo-colour. Images clearly evidence that F-actin filaments maintain their overall structure as compared to an untreated (N/T) control and that uncoated AuNBs (arrows) were internalized by A549 cells even in the presence of NaN₃. (d) Changes in the hydrodynamic size ($\varnothing$) of uncoated AuNBs before (0 h) and after incubation with A549 cells for 24 h. Data are reported as mean $\pm$ standard deviation of NTA measurements. (e) Coloured HIM image of AuNB₂ (in yellow) after 24 h incubation with A549 cells. The AuNBs coating with biological material is evident. Scale bar: 100 nm.

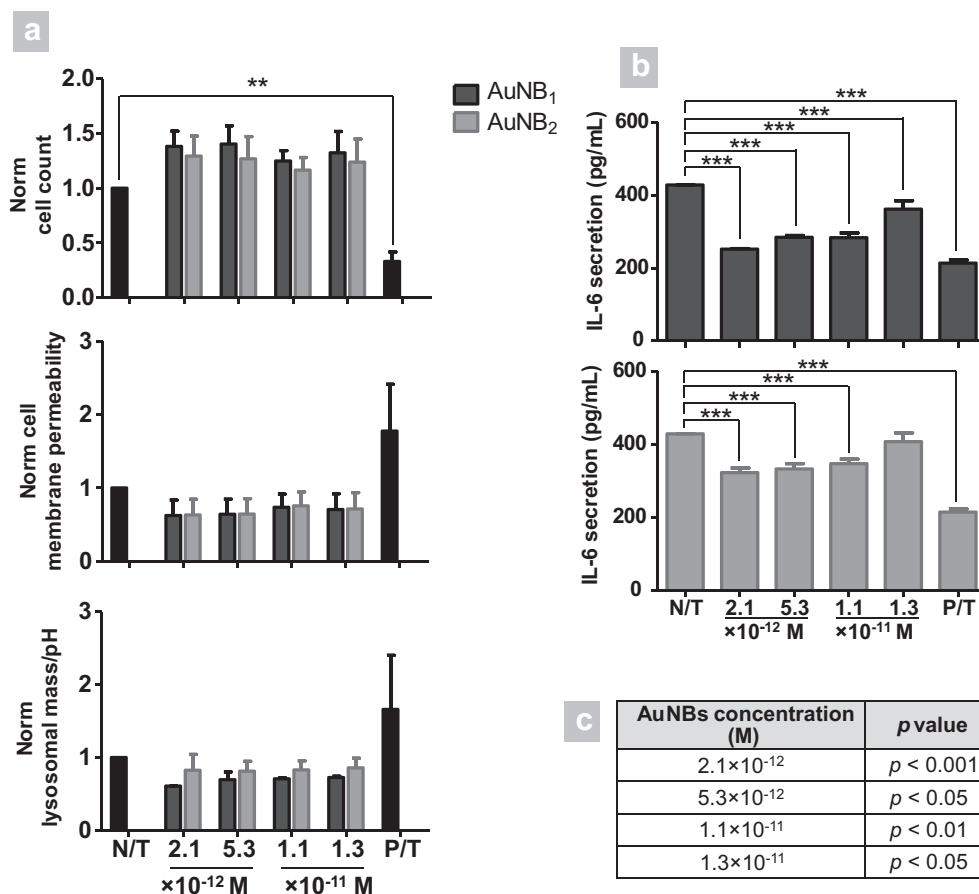


Fig. 9. Changes in the (a) cytotoxicity cell responses (cell count, cell membrane permeability and lysosomal mass/pH) and (b) IL-6 secretion from A549 cells exposed to AuNB₁ (black bars) or AuNB₂ (light grey bars) at four concentrations (2.1×10^{-12} , 5.3×10^{-12} , 1.1×10^{-11} and 1.3×10^{-11} M) for 24 h, as quantified by HCSA and ELISA respectively. Untreated and Doxorubicin-treated cells were used as negative (N/T) and positive (P/T) controls, respectively. The symbols (**) and (***) indicate significant changes ($p < 0.01$ and $p < 0.001$, respectively). Data are reported as mean $\pm$ standard deviation. (c) Statistical significance of the release of IL-6 in A549 cell cultures exposed to AuNB₂ as compared to the release of IL-6 in A549 cell cultures exposed to AuNB₁.

3.2. Tier 2

3.2.1. Biocompatibility of gelatine-coated AuNBs (AuNB₃)

Trypan Blue exclusion assay demonstrated that gelatin-coated AuNBs (AuNB₃) (Fig. 11a) did not cause any significant change in cell viability if incubated with A549 cells mono-cultures. Similarly, no significant reduction in cell viability was detectable when A549 cells were exposed to AuNB₂ for 24 h and 72 h (approximately 90% of live cells) (Fig. 11a), as previously shown by HCSA.

3.3. Tier 3

3.3.1. Functionalized AuNBs (AuNB₄ and AuNB₅) for drug delivery – proof-of-principle

Preliminary data showed that exposure to AuNB₄, AuNB₅ and molecular Paclitaxel (0.03 mM) caused a significant reduction in cell viability following 72 h treatment of A549 cells mono-cultures (Fig. 11b). Interestingly, folic acid-targeted AuNBs (AuNB₅) were more cytotoxic than the molecular drug formulation, showing a significant reduction in the percentage of live cells after 24 h and with less than 40% of live cells after 72 h exposure.

4. Discussion

A systematic qualitative and quantitative study, structured into a safe-by-design, three-tiered approach (Fig. 1), was carried out to

evaluate the biological interactions of AuNBs with *in vitro* cell models representative of the human alveolar region, thus defining their potential (or, conversely, their inefficiency) as candidates for perspective translation into pre-clinical nano-enabled chemotherapeutic agents for the targeted and personalized treatment of lung cancer. Our study included systematic tests on the internalization, cytotoxicity and biodegradation of uncoated AuNBs (Tier 1), followed by preliminary testing on the drug delivery efficacy of AuNBs that underwent purpose-specific functionalization (Tier 3).

Our results showed that uncoated AuNBs were efficiently internalized by lung adenocarcinoma (A549) cells (Figs. 3 and 4) within 5–7 h (Fig. 7a) and were characterized by short-term biocompatibility, causing no detectable cytotoxic or pro-inflammatory responses in A549 cells after 24 h exposure (as assessed by cell count, cell membrane integrity, lysosomal mass/pH and IL-6 secretion) (Fig. 9). Uncoated AuNBs were detected effectively in the area corresponding to A549 cells cytoplasm by confocal microscopy (Fig. 3a), TEM (Fig. 4) and Raman spectroscopy (Fig. 3b–f), the readout signals of which are unique and could be used as fingerprints to detect AuNBs in the complex cell culture environment. We found that the cellular internalization of uncoated AuNBs in A549 cells did not decrease at 4 °C compared to the equivalent exposure at 37 °C (Fig. 8). This standard experiment [48,65] suggested that the AuNBs internalization mechanism did not involve receptor-mediated endocytosis (RME) pathways. For gold nanoparticles the RME pathways (including caveolae-mediated,

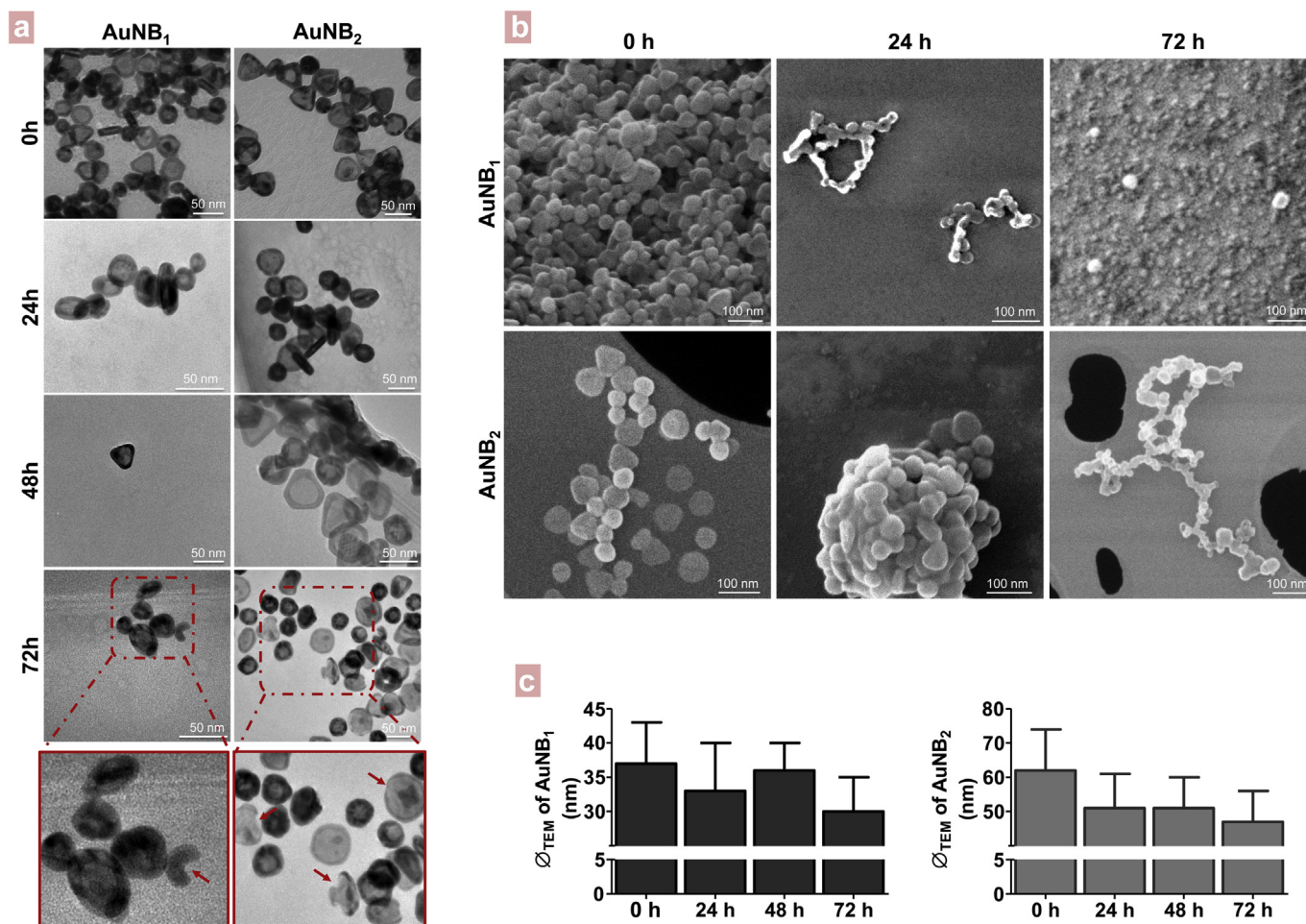


Fig. 10. (a) TEM images and (b) HIM images of flat-lying and edge-oriented uncoated AuNBs before (0 h) and after incubation with A549 cells for 24, 48 and 72 h. Arrows indicate distinct signs of AuNBs degradation. Scale bars: (a) 50 nm; (b) 100 nm. (c) Changes in the height ($\emptyset$) distribution of AuNB₁ (black bars) and AuNB₂ (grey bars) before (0 h) and after incubation with A549 cells for 24, 48 and 72 h as measured by TEM. Data are reported as mean $\pm$ standard deviation.

clathrin-mediated and caveolae/clathrin independent endocytosis have been proposed as the primary mechanism of cellular entry [23,66–68]. However, gold nanomaterials have also been reported to be uptaken by different pathways depending on the nanomaterial size and cell type tested [69,70]. Our results showed that the internalization of AuNB₁ was significantly reduced in A549 cells pre-treated with 0.1% NaN₃ (Fig. 8). This suggested that the AuNB₁ internalization mechanism occurs through an energy-dependent process. NaN₃ is widely used *in vivo* and *in vitro* to inhibit cytochrome-C oxidase, the last enzyme in the mitochondrial electron transport chain, producing a drop in intracellular ATP concentration [48,65]. An alternative, non-RME mechanism like macropinocytosis could be therefore responsible for AuNB₁ internalization. To support the evidence brought in this manuscript, Gao et al. [70] showed that nanoparticles within the size range of tens to hundreds of nanometres can enter cells *via* wrapping even in the absence of clathrin or caveolin coats. In a contrasting trend, 0.1% NaN₃ did not inhibit the uptake of AuNB₂ in A549 cells (Fig. 8), suggesting that AuNB₂ enter the cells by passive transport *via* cell membrane deformation and invagination [71] as a consequence of generic physical interactions with the cells, in an action similar to the cell-penetrating peptides [59]. Further confirmation of such mechanism is provided by TEM micrographs, revealing evidence of morphological changes in the cells structure such as cell membrane invagination (Fig. 5). The *in vitro* exposure conditions, such as

gravitational AuNBs sedimentation, also seemed to affect the time-dependent internalization of these nanomaterials (Fig. 7). Sedimentation of nanoparticles in cell culture is a crucial factor that should be considered when testing nanomaterials since it can affect their rate and extent of uptake *in vitro* [53], and ultimately the cell responses to the nanomaterial subject of investigation. Further research is needed to evaluate the internalization and cytotoxicity of uncoated AuNBs in A549 cells under dynamic conditions that mimic the administration of the nano-enabled drug carrier by intravenous therapy.

Despite uncoated AuNBs were also internalized into macrophage-like cell models (Fig. S4 and Fig. S6), AuNB₁ and AuNB₂ could be effectively delivered to adenocarcinoma cells in *in vitro* co-cultures of A549 and THP-1 cells (Fig. 5), suggesting that AuNBs could be used as carriers within the complex living alveolar tissue where resident phagocytic cells are present and have the main function of removing pathogens, senescent cells and external particles from the lungs [46,72,73]. Therefore, this result excluded that, in the alveolar region, macrophages could have a role in eliminating AuNBs before the nano-enabled treatment reaches the cancerogenic cellular target.

Similarly to the behaviour of other gold nanomaterials [74,75], uncoated AuNBs were successfully internalized also in 3D cell cultures mimicking the native three-dimensional architecture and microenvironment of tumours. Penetration of nanomaterials into

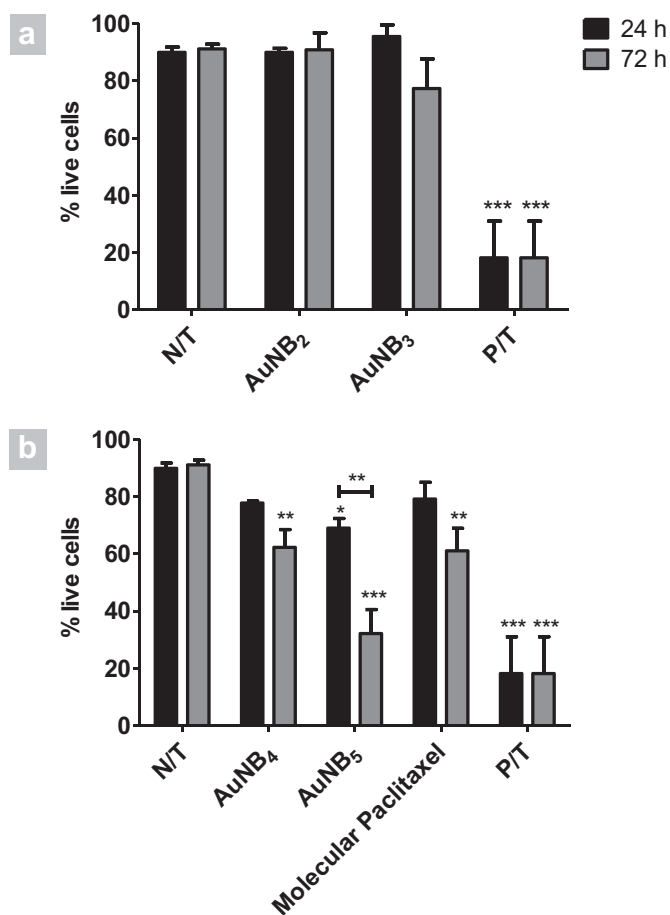


Fig. 11. Percentage (%) of live cells in A549 cells mono-cultures exposed to (a) uncoated (AuNB₂) and gelatine-coated (AuNB₃) AuNBs, or (b) functionalized (AuNB₄ and AuNB₅) AuNBs and molecular Paclitaxel, for 24 and 72 h. Untreated and methanol-treated cells were used as negative (N/T) and positive (P/T) controls, respectively. The symbols (*), (**), and (***) indicate significant changes: $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively.

the tumour environment and the tumour uptake are definitely key issues for exerting effective cancer therapy and they should not be neglected in nanomedicine studies. Since the penetration of nanomaterials in tissues is influenced by several physical factors of the particle itself [76] (e.g. particle size [77,78], hydrophobicity, hydrodynamic radius) and of the tissue microenvironment (e.g. the composition of the extracellular matrix [79]), 3D cell cultures offered a simple *ex vivo* tumour model for assessing the penetration of uncoated AuNBs of different size in a malignant tissue, and for evaluating their internalization in A549 cells in a more “*in vivo*-like” scenario. Our data indicated that uncoated AuNBs were able to reach the A549 cells by penetrating through Matrigel™ and PuraMatrix™ hydrogel layers, which ultimately reproduced the dense collagen matrix that surrounds the cancer cells *in vivo* and limits the delivery of drugs/nanomaterials to the target cells.

The final fate of uncoated AuNBs was also considered. Bio-persistence of nanomaterials raises many concerns regarding the safety of nanomedicine approaches, since it may lead to impaired cell function following chronic exposure to nanomaterials [80]. The TEM and HIM results presented in this study showed that AuNBs that were internalized by A549 cells had reduced size and changed morphology after 72 h incubation (Fig. 10). This evidence suggests that AuNBs could be biodegraded by A549 cells and supports the hypothesis that uncoated AuNBs are a promising candidate as

carriers for future personalised nano-enabled treatments. Further to this, TEM images showed that AuNB₂ internalized into A549 mono-cultures did accumulate into the lysosomes (Fig. 5c and e), which are the cellular organelles containing acid hydrolase, catabolic enzymes, thus suggesting that AuNBs degradation might happen in such cells compartment. The acidic environment of the lysosomes can in fact lead to acid etching of metallic nanoparticles, resulting in the generation of free ions from the nanoparticles surface and gradually decrease the nanoparticle core diameter [81]. However, despite biodegradation of uncoated AuNBs was evident after 72 h exposure, AuNB₂ did not show any detectable cytotoxicity at this exposure time (Fig. 11), suggesting that A549 cells were not affected by the free ions released by the biodegradation process.

Finally, our preliminary data showed that, while gelatin-coated AuNBs were not cytotoxic up to 72 h exposure (Fig. 11a), gelatin-coated AuNBs that were loaded with Paclitaxel (AuNB₄) and targeted with FA (AuNB₅) did cause a significant reduction in A549 cells viability after 72 h (Fig. 11b), suggesting that effective drug delivery and release to the cancer cells was achieved. Notably, exposure to AuNB₅ resulted in the greatest therapeutic effect as compared to AuNB₄ and molecular Paclitaxel, thus suggesting that FA played an important role in the efficient delivery of the nano-enabled treatment to lung cancer cells *via* interactions with FA receptors, which are over-expressed in NSCLC and can be used therefore as markers for the targeted treatment of lung cancer [82,83].

5. Conclusions

Creating an intelligent system to deliver therapeutic agents by taking advantage of targeted cell-type specificity, enhanced permeation and retention effect and reduced side-effects in physiologically healthy tissues has been the challenge of nanotechnology-based products in the last decade. In this study, we showed for the first time that uncoated AuNBs are biologically inert, can be subjected to biodegradation and can penetrate into lung cancer cells even in complex co- and 3D-culture microenvironments. Concurrently, our preliminary data on drug delivery ultimately validate our hypothesis that a safe-by-design functionalization of the AuNBs surface could produce in the future an AuNBs-based personalized medicine approach against lung cancer that delivers the drug specifically to the targeted cancer cells without affecting the surrounding healthy tissues.

Supporting data

Supplementary information is available on-line. Certain figures in this article are difficult to interpret in black and white. The full colour images can be found in the on-line version.

Authors' contribution

DM, YV and APM conceived this study, DM and APM designed the experiments and structured the paper, DM performed the biological experiments and analysed the data, carried out statistical analysis and drafted the paper. VG synthesized the AuNBs and performed their TEM characterization under YG guidance. CMM carried out NTA measurements, NJ carried out Raman spectroscopy experiments. AB carried out HIM and VN contributed to the design of the electron microscopy analysis. TT assisted and helped in the preparation of the biological specimens for TEM imaging and DS conceived the imaging of AuNBs in reflectance mode by confocal microscopy. YG contributed to reagents/materials/analysis tools. VG, CMM, NJ, AB, VN, TT, DS, YG, YV and APM revised the paper. APM finalised the paper.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.biomaterials.2013.12.057>.

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