Freeze drying of polyelectrolyte complex nanoparticles: effect of nanoparticle composition and cryoprotectant selection

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Abstract
This work investigates the impact of nanoparticle (NP) composition and effectiveness of cryo-/lyo-protectants in a freeze drying process, which was employed to convert liquid dispersions of polyelectrolyte complex (PEC) NPs into completely redispersible powders. PEC NPs, with and without peptide, were produced by complex coacervation. The cryo-/lyo-protectants investigated were mannitol, trehalose (TRE) and poly(ethylene glycol) (PEG). The solid state of lyophilised powders was studied by thermal analysis and X-ray diffraction. Cytotoxicity studies were done by MTS assay and flow cytometry. The presence of a cryoprotectant was essential to achieve a successful powder reconstitution. The concentration of TRE was optimised for each type of PEC NPs. Protamine- and hyaluronate-based NPs reconstituted better than chitosan- and chondroitin sulphate-based NPs, respectively. PEG polymers were found to be more effective cryoprotectants than TRE and best results were achieved using co-freeze drying of NPs with TRE and PEG. These ternary NPs/TRE/PEG samples were crystalline, with expected better storage stability. PEG polymers were well tolerated by Caco-2 cells, with the exception of linear PEG 10 kDa. This work shows that, as regards the formulation design and maximising NP loading in the dried product, optimisation of the cryoprotectant type and content is needed as it is highly dependent not only on the type of polyelectrolyte pair in the PEC, but also the polyions ratio.

KEYWORDS: polyelectrolyte complex nanoparticle, freeze drying, hyaluronate, chitosan, chondroitin, protamine
1. Introduction

Nanoparticles (NPs) have been widely investigated for medical applications such as drug delivery systems and tissue engineering (Shi et al., 2010). A considerable effort is currently being directed towards developing NPs composed of naturally occurring polymers such as polysaccharides, in view of their advantages such as biodegradability, safety, low toxicity, satisfactory stability, abundant resources in nature and low processing costs (Liu et al., 2008). Natural polysaccharides are mainly polyelectrolytes, which present an ionic form in aqueous solutions. These polyelectrolytes can spontaneously associate in complexes and form NPs with substances that bear opposite charge via electrostatic interactions.

Polyelectrolyte complex (PEC) NPs can be obtained using complex coacervation, a simple method carried out under mild conditions involving mixing of diluted aqueous solutions of polyelectrolytes at room temperature. This approach at making PEC NPs can prevent destruction of the structure and property of biochemical drugs, if incorporated into NPs, including nucleic acids, peptides and proteins (Cegnar et al., 2011; Yang et al., 2015). Furthermore, complex coacervation does not involve the use of organic solvents and/or surfactants and thus is a very attractive approach at making a wide range of NPs for drug delivery applications.

However, NPs, as colloidal systems, are thermodynamically unstable and are susceptible to aggregation after periods of storage as a dispersion. Long term stability is an important challenge in the development of NP formulations, especially systems made of polyelectrolytes (Katas et al., 2013). Freeze drying is an attractive approach to achieve the long term stability of NPs formulations as it requires small sample volumes and is relatively easy to scale up (Boge et al., 2018). Lyophilised formulations provide easy handling including shipping and storage (Lu and Pikal, 2004). Moreover, as solid state dosage forms, such as tablets or capsules, remain the formulations of choice for oral drug delivery, the NPs that are prepared as aqueous dispersions must be transformed into dry powders to be further processed into solid state dosage forms (Wong et al., 2018). It is recognised that stress generated during freeze drying (freezing and water removal) may adversely impact the properties of NPs (Abdelwahed et al., 2006; Fang et al., 2009). Additionally, inadequate reconstitution of freeze dried NPs in the aqueous media represents a major obstacle because it can lead to the formation of large particle aggregates and the loss of beneficial colloidal properties of the nanosystem (Cegnar et al., 2011). Many studies published to date...
presented that, to effectively stabilise NPs during freeze drying as well as to ensure their adequate reconstitution, suitable excipients are required (de la Fuente et al., 2008; Fang et al., 2009). They typically include using a cryo- and/or lyo-protectant that protects the NPs during the freezing and/or drying stage of the process, respectively.

Although lyophilisation of various types of NPs have been studied (Abdelwahed et al., 2006), optimal conditions depend mainly on the nature of particle systems, thus have to be customised for each formulation (Cegnar et al., 2011). Freeze drying of polyelectrolyte complex NPs presents a particular challenge, as they are mainly stabilised by charge, thus susceptible to changes in the medium parameters (pH and ionic strength) (Cegnar et al., 2011), their shape/structure is less defined (Umerska et al., 2014b) as opposed to e.g. poly(lactic-co-glycolic) acid-based NPs and are surfactant-free formulations and surfactants such as polyvinyl alcohol can act as cryo-/lyo-protectants and facilitate redispersibility of the NPs (Umerska et al., 2018). For PEC NPs, freeze drying of chitosan-based NPs has been studied (Rampino et al., 2013), including alginate/chitosan (Cegnar et al., 2011) and hyaluronate-coated chitosan NPs (Veilleux et al., 2018), but not other types of PEC NPs. Thus, there is a literature gap as the main focus of research is often formulate NPs with a specific delivery target but not on subsequent transformation into the solid state for long-term stability. For instance, de la Fuente et al. (2008) freeze dried only one type of polyelectrolyte hyaluronate-chitosan-tripolyphosphate NPs (concentration range between 0.075 and 0.5 mg/ml) in the presence of either glucose or trehalose at a relatively high concentration of 5% w/v, but freeze drying was not the main subject of the study and no deep investigation into this subject was performed such as effective minimising of cryoprotectant concentration or influence of the NP composition.

The addition of a suitable lyo-/cryo-protective agent before freezing prevents NPs from aggregating. It is well established that sugars can be employed for this purpose (Boge et al., 2018; Cegnar et al., 2011; Rampino et al., 2013). Non-reducing compounds such as trehalose, mannitol and sucrose are preferred to avoid potential Maillard reaction of the excipient with protein, especially for protein-based nano-formulations (Anhorn et al., 2008). Those compounds have been shown to be effective in maintaining the properties of NPs after the lyophilisation process (Anhorn et al., 2008; Holzer et al., 2009). The advantages of trehalose over other sugars include lower hygroscopicity, the absence of internal hydrogen bonds and consequently more flexible formation of hydrogen bonds with NPs, a very low
chemical reactivity and, finally, a high glass transition temperature (Abdelwahed et al., 2006; Crowe et al., 1996; Hafner et al., 2011). Apart from trehalose, poly(ethylene glycol) (PEG) polymers have also been used as stabilisers in freeze drying of NPs including chitosan NPs (Rampino et al., 2013), however no systematic study on the influence of molecular weight or branching has been published thus far.

In this work, two polycations, chitosan (CHIT) and protamine (PROT), and two polyanions, hyaluronic acid (HA) and chondroitin sulphate (CHON), were examined as candidate components of PEC NPs. These polyelectrolytes possess numerous advantages as they are reasonably cheap, biocompatible, biodegradable, of natural origin, already well characterised and have history of use in pharmaceutical products. They have received a particular attention as carrier materials because they present interesting biological and/or pharmacological properties. CHON exhibits anti-inflammatory activity (Iovu et al., 2008) and is currently used as a chondroprotective drug together with glucosamine. HA also exhibits anti-inflammatory effects (Ryan et al., 2013) and binds to the CD44 receptor, which is overexpressed in a wide variety of cancer cells and therefore has been extensively studied as a therapeutic target (Platt and Szoka, 2008). Moreover, HA coating considerably reduces hemolysis and hemagglutination of chitosan polyplexes and thereby facilitates intravenous administration (Veilleux et al., 2018). CHIT has antibacterial activities (Raafat et al., 2008), while PROT is used as an antagonist for heparin (Jaques, 1973) and due to the presence of arginine it provides membrane translocation activities (Reynolds et al., 2005).

Considering the above, the goal of this work is to identify if and how the type of a lyo-/cryo- protective agent, its concentration and composition of NPs (type, starting concentration and content of polyions as well as the presence of a peptide) affect the ability of liquid NP dispersions to be freeze dried into powders that completely redisperse upon reconstitution. The solid state of lyophilised products was examined as the amorphous/crystalline character of the powder may determine the storage shelf-life, while the cytotoxic properties may limit the use of some of the excipients from wider applications.

2. Materials and methods

2.1. Materials

Hyaluronic acid sodium salt (HA) from Streptococcus equi sp. (sodium content 3.6% w/w, molecular weight app. 2900 kDa), chondroitin 4-sulphate sodium salt (CHON, sodium content 5.6% w/w, molecular weight 59 kDa), protamine sulphate (PROT, molecular weight
5.1 kDa), trehalose dihydrate and poly(ethylene glycol) (PEG) linear polymers with different molecular weights were purchased from Sigma-Aldrich (Ireland). Chitosan chloride high molecular weight (CHH, chloride content 15.5% w/w, degree of deacetylation 83%, molecular weight 110 kDa) was obtained from Novamatrix (Norway), while chitosan chloride low molecular weight (CHL, chloride content 13.6% w/w, degree of deacetylation 82%, molecular weight 42kDa) was acquired from Chitoceuticals (Germany). Four-arm branched PEG with a molecular weight of 10 kDa was sourced from JenKem Technology USA Inc. (Allen, USA). Salmon calcitonin (sCT) was obtained from PolyPeptide Laboratories (Sweden). APC annexin V and propidium iodide were purchased from BD Biosciences (USA) and CellTiter 96® Non-Radioactive Cell Proliferation Assay from Promega Corporation (USA). Other cell culture reagents were provided by Sigma Aldrich (Ireland). All other reagents, chemicals and solvents were of analytical grade.

2.2. Methods

2.2.1 Preparation of polyelectrolyte NPs

All NPs were synthesised using a surfactant- and solvent-free polyelectrolyte complexation in an aqueous media as previously described (Umerska et al., 2017, 2015, 2014b, 2012). Briefly, all solutions of polyions were made in deionised water. HA solutions were sonicated before mixing with a polycation solution for 2 hours at an amplitude of 80% (13 W) with the aid of a 130 Watt ultrasonic processor (SONICS VC130PB, Sonics and Materials Inc., USA) to obtain HA fragments with a molecular weight of app. 260 kDa. Chitosan-based NPs (CCH or CHL) were made using either 1 or 2 mg/ml solutions of the polycation. A volume of the chitosan solution was added to a predefined volume of the polyanion solution, HA or CHON at 1 or 2 mg/ml, using a one shot addition at room temperature under magnetic stirring. The stirring was maintained for 10 minutes to allow stabilisation of the system. Details of composition, including the total polyion concentration (TPC) and the polyanion to polycation mass mixing ratio (MMR) of the various systems made are summarised in Table 1.

PROT-based systems were made as follows. An aliquot of 4 ml of PROT aqueous solution with various concentrations, as outlined in Table 2, was added to 10 ml of a HA or CHON solution at room temperature under magnetic stirring. The stirring was maintained for 10 minutes to allow stabilisation of the system, as for the chitosan-based NPs.
To make salmon calcitonin-containing NPs, the peptide was dissolved at a concentration of 0.5 mg/ml in the HA or CHON solution and mixed with a solution of the polycation as described above.

Table 1 Composition and characteristics of chitosan-based NPs. The PS, PDI and ZP values were measured directly after manufacturing the NP systems, before cryoprotectant addition and freeze drying. HA – hyaluronic acid, CHON – chondroitin sulphate, CHH – chitosan high molecular weight, CHL – chitosan low molecular weight, PA – polycation, PC – polyanion, MMR - PA/PC mass mixing ratio, TPC – total polyelectrolyte concentration, sCT – salmon calcitonin, PS - hydrodynamic particle size, PDI – polydispersity index and ZP – zeta potential.

<table>
<thead>
<tr>
<th>NP system</th>
<th>PA</th>
<th>PC</th>
<th>MMR</th>
<th>TPC (mg/ml)</th>
<th>sCT conc. (mg/ml)</th>
<th>PS (nm)</th>
<th>PDI (mV)</th>
<th>ZP (mV)</th>
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<tr>
<td>HA/CHH_{1,1}</td>
<td>HA</td>
<td>CHH</td>
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<td>1</td>
<td>0</td>
<td>260</td>
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</tr>
<tr>
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<td>CHH</td>
<td>2.5</td>
<td>2</td>
<td>0</td>
<td>235</td>
<td>0.21</td>
<td>-42</td>
</tr>
<tr>
<td>HA/CHH_{5,1}</td>
<td>HA</td>
<td>CHH</td>
<td>5</td>
<td>1</td>
<td>0</td>
<td>218</td>
<td>0.21</td>
<td>-63</td>
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<tr>
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<td>255</td>
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<td>-87</td>
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<td>CHH</td>
<td>5</td>
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<td>0</td>
<td>161</td>
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<td>0.13</td>
<td>+35</td>
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<tr>
<td>CHON/CHL_{1,2}</td>
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<td>CHL</td>
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<td>2</td>
<td>0</td>
<td>128</td>
<td>0.12</td>
<td>+35</td>
</tr>
<tr>
<td>CHON/CHL_{2,5;1}</td>
<td>CHON</td>
<td>CHL</td>
<td>2.5</td>
<td>1</td>
<td>0</td>
<td>78</td>
<td>0.14</td>
<td>-46</td>
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<tr>
<td>CHON/CHL_{2,5;2}</td>
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<td>CHL</td>
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<td>2</td>
<td>0</td>
<td>100</td>
<td>0.12</td>
<td>-44</td>
</tr>
<tr>
<td>CHON/CHL_{5,1}</td>
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<td>CHL</td>
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<td>2</td>
<td>0</td>
<td>85</td>
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<tr>
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<td>CHL</td>
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<td>CHL</td>
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<td>1</td>
<td>0.5</td>
<td>95</td>
<td>0.22</td>
<td>-44</td>
</tr>
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Table 2 Composition and characteristics of PROT-based NPs. The PS, PDI and ZP values were measured directly after manufacturing the NP systems, before cryoprotectant addition and freeze drying. HA – hyaluronic acid, CHON – chondroitin sulphate, PROT – protamine, PA – polycation, PC – polyanion, MMR - PA/PC mass mixing ratio, TPC – total polyelectrolyte concentration, sCT – salmon calcitonin, PS - hydrodynamic particle size, PDI – polydispersity index and ZP – zeta potential.

<table>
<thead>
<tr>
<th>NP system</th>
<th>PA</th>
<th>PA conc. (mg/ml)</th>
<th>PC</th>
<th>PC conc. (mg/ml)</th>
<th>MMR</th>
<th>TPC (mg/ml)</th>
<th>sCT conc. (mg/ml)</th>
<th>PS (nm)</th>
<th>PDI (mV)</th>
<th>ZP (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HA/PROT_{1,1;low}</td>
<td>HA</td>
<td>0.714</td>
<td>PROT</td>
<td>0.343</td>
<td>2.1</td>
<td>1.06</td>
<td>0</td>
<td>159</td>
<td>0.11</td>
<td>-39</td>
</tr>
<tr>
<td>HA/PROT_{1,1;low}</td>
<td>HA</td>
<td>0.714</td>
<td>PROT</td>
<td>0.229</td>
<td>3.1</td>
<td>0.94</td>
<td>0</td>
<td>119</td>
<td>0.14</td>
<td>-51</td>
</tr>
<tr>
<td>HA/PROT_{6,3;low}</td>
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<td>PROT</td>
<td>0.114</td>
<td>6.3</td>
<td>0.83</td>
<td>0</td>
<td>87</td>
<td>0.20</td>
<td>-69</td>
</tr>
<tr>
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<td>0.714</td>
<td>PROT</td>
<td>0.114</td>
<td>6.3</td>
<td>0.83</td>
<td>0.5</td>
<td>140</td>
<td>0.20</td>
<td>-39</td>
</tr>
<tr>
<td>HA/PROT_{3,1;high}</td>
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<td>PROT</td>
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<td>0</td>
<td>159</td>
<td>0.14</td>
<td>-57</td>
</tr>
<tr>
<td>HA/PROT_{6,3;high}</td>
<td>HA</td>
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<td>PROT</td>
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<td>6.3</td>
<td>1.66</td>
<td>0</td>
<td>127</td>
<td>0.17</td>
<td>-94</td>
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<td>HA/PROT_{12,5;high}</td>
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<td>1.429</td>
<td>PROT</td>
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<td>12.5</td>
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<tr>
<td>CHON/PROT_{3,1;low}</td>
<td>CHON</td>
<td>0.714</td>
<td>PROT</td>
<td>0.343</td>
<td>2.1</td>
<td>1.06</td>
<td>0</td>
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<td>0.12</td>
<td>-49</td>
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<tr>
<td>CHON/PROT_{3,1;low}</td>
<td>CHON</td>
<td>0.714</td>
<td>PROT</td>
<td>0.229</td>
<td>3.1</td>
<td>0.94</td>
<td>0</td>
<td>78</td>
<td>0.15</td>
<td>-52</td>
</tr>
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</table>
2.2.2 Freeze drying and redispersibility of NPs

An appropriate amount of trehalose dihydrate (TRE), poly(ethylene glycol) (PEG, with molecular weight of 2,000 or 4,000 or 10,000 linear or 10,000 branched) or a mixture of TRE and PEG was added to NP dispersion and dissolved. The concentrations of TRE presented in this work refer to trehalose dihydrate. The ratios of anhydrous trehalose to NP in weight/weight ratios are presented in Figure 1.

Aliquots of the formulation (4 ml) were dispensed into 15 ml Greiner plastic centrifuge tubes and immediately shock-frozen in liquid nitrogen (Abdelwahed et al., 2006; Molpeceres et al., 1997). Tubes with the frozen samples were placed in VirTis wide mouth filter seal glass flasks and attached to one of the ports of manifold of a benchtop VirTis 6K freeze dryer model EL (SP Scientific, USA). Vacuum of 29-31 mtorr was obtained by the use of an Edwards 5 RV5 rotary vane dual stage mechanical vacuum pump (Edwards, England). After 48 hours of freeze drying the tubes were removed, capped and stored at 4 °C if storage was required. Freeze drying was performed on at least three independent samples of each formulation.

Sample reconstitution was performed by adding 4 ml of water (or serum-free medium for cell culture experiments) to the whole content of powder in the 15 ml tube and manual, moderate shaking. No sonication or mechanically-assisted mixing was employed. Samples after reconstitution were analysed in triplicate.

2.2.3 Particle size and zeta potential analysis

The intensity-averaged mean particle size (particle size diameter) and the polydispersity index of the nanoparticles were determined by Dynamic Light Scattering (DLS) with the use of 173° backscatter detection and the electrophoretic mobility values measured by Laser Doppler Velocimetry (LDV) were converted to zeta potential by the Smoluchowski equation, as described before (Umerska et al., 2012). DLS and LDV measurements were done using a Zetasizer Nano series Nano-ZS ZEN3600 fitted with a 633 nm laser (Malvern Instruments Ltd., UK). Samples were placed directly into the folded
capillary cells (DTS1061) without dilutions. Each analysis was carried out at 25 °C with the equilibration time set to 5 minutes. The readings were carried out at least three times for each batch and the average values of at least three batches are presented. The results were adjusted taking into account viscosity of the continuous medium.

For the clarity of data presentation and discussion, hydrodynamic particle size (PS) ratios were calculated for all NP systems, defined as the ratio of the size after and before freeze drying. The redispersibility was considered as very good, when the particle size did not change more than 20% (i.e. the PS ratio was between 0.8-1.2). For comparison, Fang et al. (2009) considered the ratio of 0.7-1.3 (±30%) appropriate, indicating satisfactory reconstitution.

2.2.4 Separation of non-associated sCT and quantification of the peptide

Non-associated sCT was separated from nanoparticles by a combined ultrafiltration-centrifugation technique (Centriplus YM-50, MWCO of 50 kDa for HA-based NPs or Amicon Ultra-15, MWCO of 30 kDa for CHON-based NPs; Millipore, USA) using a validated technique as described before (Umerska et al., 2015, 2014b, 2014a). The quantity of total and non-associated sCT was measured by an isocratic HPLC method presented previously (Umerska et al., 2014b). Association efficiency (AE) and sCT loading (PL) were calculated with the use of the following equations:

\[ AE = \frac{(A - B)}{A} \times 100\% \quad \text{(Eqn. 1)} \]

where A is the total amount of sCT and B is the mass of non-associated sCT;

\[ PL = \frac{(A - B)}{C} \times 100\% \quad \text{(Eqn. 2)} \]

where C is the total weight of all the components of NPs.

2.2.5 Differential Scanning Calorimetry (DSC)

DSC experiments were conducted using a Mettler Toledo DSC 821e with a refrigerated cooling system (LabPlant RP-100, UK), according to the method presented by Umerska et al. (2015). Nitrogen was used as the purge gas. Hermetically sealed aluminium pans with three vent holes were used throughout the study and sample weights varied between 2 and 7 mg, depending on bulk density of the sample. DSC measurements were carried out at a heating/cooling rate of 10 °C/min.

2.2.6 Powder X-ray diffraction (PXRD)

Powder X-ray diffraction analysis was conducted using a Rigaku Miniflex II desktop X-ray diffractometer (Rigaku, Japan) operating at 30 kV and 15 mA and fitted with a Haskris
cooling unit. Ni-filtered Cu Kα radiation (λ=1.5408 Å) was used. Room temperature measurements were recorded for the range 5-40 2theta degrees at a step size of 0.05° per second. A low background silicon mount (Rigaku, Japan) was used to support the sample during measurements.

2.2.7 Cell culture studies

Human epithelial colorectal adenocarcinoma cells (Caco-2) were obtained from European Collection of Cell Cultures (Sigma-Aldrich, UK). Cells were cultured in 75 cm² cell culture flasks in Eagle’s Minimal Essential Medium (MEM), supplemented with 20% foetal bovine serum, penicillin (0.006 mg/ml), streptomycin (0.01 mg/ml), gentamicin (0.005 mg/ml), sodium bicarbonate (2.2 g/l), sodium pyruvate (0.11 g/l), pH 7.4 at 5% CO₂ and 37 °C humidified atmosphere (CO₂ incubator series 8000DH, ThermoScientific). Cells were supplied with fresh medium every second day and split after detaching with ethylenediaminetetraacetic acid (EDTA)-trypsin twice a week. For experimental purposes the passage number range was maintained between 20 and 30.

2.2.8 MTS assay

MTS assay was carried out according to the method presented previously (Umerska et al., 2015). Briefly, Caco-2 cells were seeded into flat-bottom 96-well plates in 100 µl of whole media (MEM supplemented with 20% FBS) at a density of 25,000 cells per well (cells were previously counted with the aid of Z1 Coulter Particle Counter, Beckman Coulter) and incubated at 37 °C for 24h. The medium was then replaced with 100 µl of the sample dispersed or dissolved in serum-free media. After 72 h of incubation, or 24 h when a serum-free medium at pH-5 was used, the supernatant was removed from the wells and replaced with serum-free media. A quantity of 20 µl of the MTS reagent prepared according to the manufacturer protocol was then added into each well; in case of positive control (0% viability) the media was replaced by 10% SDS solution in serum-free media 30 min before the addition of MTS reagent. After 4 hours the UV absorbance of the formazan product was measured spectrophotometrically (FLUOstar Optima microplate reader, BMG Labtech) at 492 nm. Positive control was treated as a blank and its absorbance was subtracted from each reading. The cells viability was expressed as the ratio of the absorbance reading of the cells treated with different samples and for negative control (cells treated with serum-free MEM), which was assumed to have 100% of the cells viability. IC₅₀ values (concentrations required to reduce the viability of cells by 50% as compared with the control cells) were
calculated by fitting the experimental points to the Hill equation (Goutelle et al., 2008). At least three different batches of samples were tested in cells with at least three different passage numbers.

2.2.9 Flow cytometry

The content of one 75 cm² flask of confluent Caco-2 cells was seeded into eight 25 cm² flasks, each containing Caco-2 cells suspended in 4 ml of MEM supplemented with 20% FBS. Cells were allowed to attach for 24-48 hours. The medium was then replaced with 3 ml of sample (sonicated HA). After 72 hours of incubation the supernatant was removed and cells were harvested with trypsin/EDTA. After neutralisation, the cells were combined with the previously removed supernatant and centrifuged (300 g, 5 minutes); the supernatant from centrifugation was discarded, and cells were washed with binding buffer (0.14M NaCl, 0.0025M CaCl₂ and 0.01M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), pH 7.4 adjusted with NaOH solution). 20 μl of the cells suspension were stained with 5μl of APC-Annexin V, 5 μl of propidium iodide and diluted with 70 μl of binding buffer and incubated in dark at room temperature for 15 minutes. Then the cell suspension was further diluted with binding buffer, transferred to a flat bottom 96-wells plate and applied to flow cytometric analysis. All analyses were performed by a BD FACSArray™ bioanalyser (Becton Dickinson, UK). The instrument was set up to measure the size (forward scatter), granularity (side scatter) and cell fluorescence. Antibody binding was measured by analysing individual cells for fluorescence. The mean fluorescence intensity was determined after correction for cell autofluorescence. Fluorescence histograms were obtained for 10000 individual events. Data were analysed using BD FACSArray™ system software and expressed as a percentage of control fluorescence in arbitrary units. At least three different batches of samples were tested in cells with at least three different passage numbers.

2.2.10 Statistical analysis

The data were analysed using one-way analysis of variance (Minitab software). The results are expressed as mean ± S.E.M. of at least three independent experiments. Tukey-Kramer multiple comparisons test was performed where appropriate. Statistical significance was considered when p<0.05.

3. Results and Discussion

3.1. Properties of polyelectrolyte complex NPs
Polyelectrolyte complex nanoparticles (PEC NPs) were prepared by a complex coacervation method based on previous reports (Umerska et al., 2017, 2015, 2014a, 2014b, 2012). Considering that a very wide range of particles were synthesised and presented in those reports, only formulations that yielded colloidally stable NPs as well as characterised by small size and large absolute value of zeta potential were selected for the current study and the PEC formation was conducted in diluted solutions to prevent aggregation caused by viscosity of the polyion solutions (Quiñones et al., 2018). Both, positively and negatively charged CHIT NPs were successfully obtained (Table 1). The main factor that determined the zeta potential was the polyanion to polycation mass mixing ratio (MMR) for both, HA/CHIT and CHON/CHIT NPs. The MMR also affected the size and size distribution of these PEC NPs. As it was impossible to obtain physically stable for at least 24 hours PROT NPs, only negatively charged PROT NPs were selected for the lyophilisation studies. The instability of positively charged PROT NPs was attributed to a large difference in molecular weight between PROT and polyanions (either HA or CHON) (Umerska et al., 2014b). Other parameters that affected the properties of obtained PEC NPs were the type of polymer used (HA versus CHON), polymer concentration and incorporation of the peptide.

3.2. Freeze drying and NP reconstitution

3.2.1. Freeze drying of NPs without a cryoprotectant

Given the fact that HA has been described as an efficient cryoprotectant for freeze drying of liposome formulations (Peer et al., 2003), first attempts at processing were made using native HA/CHH NPs (HA/CHH_{2.5:1} and HA/CHH_{5:1}) dispersions without addition of any protective agents. NPs processed in this way did not redisperse and in the reconstituted suspension aggregates as well as NPs with the average size of a 3-fold greater (HA/CHH_{2.5:1}) than the non-processed NPs were present. Similar to this outcome, unsuccessful trials to freeze dry HA-CHIT-tripolyphosphate NPs were made by de la Fuente et al. (2008), who attributed this failure to the presence of only a small amount of HA in the suspending medium after the formation of NPs.

The stability of PEC NPs in aqueous dispersions is mainly governed by electrostatic (charge) but also steric (due to the polymeric corona) stabilisation effects. Electrostatic stabilisation is the result of the presence of electrical double layers, which cause particle repulsion, and is determined by the magnitude of the surface or zeta potential, electrolyte concentration and valence (Tadros, 2010). As mentioned earlier, the formation of PEC NPs
must be conducted in diluted dispersions to prevent their aggregation. During the freezing step, as a result of ice formation, the liquid phase becomes more concentrated and the distance between the particles decrease, thus increasing interparticulate attraction leading to aggregation. Moreover, the polyelectrolytes are present as salts and the dispersions contain either chloride or sulphate anions from CHIT and PROT, respectively, and sodium cations from either CHON or HA. Although in the initial dispersion the concentration of inorganic counterions is low, the formation of concentrated cryo-solution during the freezing step can markedly increase their concentration, thereby reducing electrostatic repulsion forces (through charge screening).

3.2.2. Freeze drying of polyelectrolyte NPs with mannitol

Although Anhorn et al. (2008) demonstrated that mannitol can be an effective cryoprotectant for human serum albumin-based NPs, attempts to use mannitol (at 1, 2, 3 and 5% w/v) as a cryoprotectant for HA/CHH NPs (HA/CHH2.5:1) were unsuccessful. When HA/CHH NPs were lyophilised with mannitol, a substantial NP coagulation and/or aggregation into microparticles upon reconstitution was seen in all samples tested. Mannitol is a low molecular weight sugar alcohol, which forms a crystalline phase upon lyophilisation (Kim et al., 1998). It is probable that the growing crystals of mannitol induced mechanical stress and resulted in the reduction of space available for NPs. NPs in this nanoparticle-rich and poorly hydrated phase may more readily interact and form aggregates (Abdelwahed et al., 2006; Cegnar et al., 2011; Cesàro et al., 2008; Rampino et al., 2013). Therefore, to achieve an effective preservation of NP properties and successful reconstitution, it appears important that at least a fraction of the cryo-protectant remains molecularly disordered to stabilise the PEC NPs.

3.2.3. Freeze drying of polyelectrolyte NPs with trehalose

3.2.3.1. HA/chitosan NPs

As it was impossible to successfully redisperse the NPs when freeze dried on their own or with addition of mannitol, preliminary experiments were first conducted to test if trehalose (TRE) could be a viable cryoprotectant for HA/CHH NPs. HA/CHH2.5:0.75 and HA/CHH2.5:1 were freeze dried in the presence of different concentrations of TRE and the PS ratios are presented in Figure 1. When NPs (HA/CHH2.5:0.75) were lyophilised with 1% w/v TRE, the PS ratio did not change significantly in comparison to HA/CHH2.5:1 processed with no cryoprotectant and moderate particle aggregation was observed (Figure 1). An increase
in TRE concentration to 2% w/v resulted in a decrease in the PS ratio to 1.67±0.35 and 1.73±0.23 (for HA/CHH₂₅.₀.₇₅ and HA/CHH₂₅.₁, respectively) and, more importantly, only in a slight NP aggregation. A very good redispersibility was achieved when HA/CHH NPs were freeze dried at the TPC of 0.75 mg/ml with 3, 4 or 5% w/v of TRE, as the PS ratio was between 1.17±0.16 (3% w/v TRE) and 0.96±0.07 (5% w/v TRE) and aggregation was not seen. However, when the TPC was increased to 1 mg/ml, 3% w/v of TRE produced an acceptable redispersion and no aggregation was observed, but the particle size increased by 36% after freeze drying in comparison to the starting value.

The redispersibility of HA/CHH₂₅.₁ was considered to be very good when at least 4% w/v of TRE was used as the cryoprotectant. When the TPC was further increased to 2 mg/ml (HA/CHH₂₅.₂), a significant aggregation was observed and the particles did not fully

**Figure 1.** The particle size (PS) ratio (i.e. the ratio of PS after and before freeze drying) of HA/CHH NPs freeze dried with trehalose (TRE) at different concentrations. The NP TPC of 0.75 mg/ml was obtained by dilution of 1 mg/ml TPC nanodispersions with TRE solution. Upper axes present the equivalent TRE (as anhydrous sugar)/NPs dry mass ratio depending on the concentration of NP components. The green band indicates the PS ratio between 0.8 and 1.2, considered as very good redispersibility of processed NPs. TPC - total polyelectrolyte concentration, MMR - polyanion/polycation mass mixing ratio, sCT - salmon calcitonin.
redisperse even though the size of the particles remaining in non-aggregated fraction did not change when compared to the PS before lyophilisation (the PS ratio was 1.07±0.05). When 5% w/v TRE was employed, neither the change of the particle size, nor aggregation could be observed, therefore at 5% w/v TRE act as an effective cryoprotectant for HA/CHH\textsubscript{2.5;2}. Interestingly, when 11% w/v TRE was used in freeze drying, the particle size of HA/CHH\textsubscript{2.5;2} became smaller than before lyophilisation (the PS ratio was 0.89±0.02). However, this decrease in the particle size did not occur when the same sugar concentration was used to freeze dry HA/CHH\textsubscript{2.5;1}. The decrease in PS could be attributed to the compaction of the NP structure (de la Fuente et al., 2008).

An example how other properties of NPs (PDI and ZP), in addition to the PS, changed upon processing with a different concentration of TRE, is shown in Figure 2 for HA/CHH\textsubscript{2.5;0.75}. The changes in DPI values were similar to the changes in PS and the ratios of PS after and before freeze drying did not differ significantly from the equivalent ratios for PDI. The ZP ratios were seen unaffected by the processing and they remained in the 0.8-1.2 range. Therefore from now on, only changes in relations to the particle size will be discussed.

![Graph showing parameter ratios of particle size, polydispersity index, and zeta potential after and before processing of HA/CHH\textsubscript{2.5;0.75} freeze dried with different concentrations of TRE. The green band indicates the PS ratio between 0.8 and 1.2, considered as very good redispersibility of processed NPs.]

**Figure 2.** Parameter ratios of: particle size, polydispersity index and zeta potential after and before processing of HA/CHH\textsubscript{2.5;0.75} freeze dried with different concentrations of TRE. The green band indicates the PS ratio between 0.8 and 1.2, considered as very good redispersibility of processed NPs.
As shown previously (Umerska et al., 2012), the composition of NPs, especially the MMR of the polymers has a significant influence on their physical (i.e. PS, PDI and ZP) and biological properties. Therefore the next step in this study was to assess how the NP composition affected their redispersibility after freeze drying.

HA/CHH NPs with MMRs of 1 (containing more chitosan, positively charged) and 5 (containing more HA, negatively charged) were initially freeze dried at TPCs of 1 or 2 mg/ml with 4% w/v TRE and depending on if the powder redispersed, the concentration of TRE was further increased or decreased to determine the lowest concentration at which good redispersibility could be obtained.

Figure 1 illustrates the PS ratios before and after freeze drying of positively charged NPs (MMR = 1). When the TPC was 2 mg/ml (HA/CHH<sub>1;2</sub>), a significant aggregation was observed with 4% w/v TRE, but the PS ratio was satisfactory (0.93±0.06). Therefore the concentration of sugar was further increased and a successful reconstitution without aggregation could be seen at 5, 7 or 11% w/v of this cryoprotectant, with the PS ratios close to 1 for 5 and 7% w/v of TRE and a significant decrease in the particle size after freeze drying when 11% w/v of TRE was employed. On the other hand, the system with a TPC of 1 mg/ml (HA/CHH<sub>1;1</sub>) successfully reconstituted after freeze drying with 4% w/v TRE, with a low size ratio (1.09±0.06) and no aggregation observed. The redispersibility remained very good even when 1% w/v TRE was used for this system and when the concentration of TRE was further decreased to 0.5% w/v, a moderate aggregation occurred accompanied by an increase in the particle size by 36%. When the concentration of TRE was again decreased to 0.2 or 0.1% w/v, a significant aggregation and a 2.3-fold increase in the PS were observed.

HA/CHH NPs with the MMR of 5 were satisfactory redispersible at both TPCs (1 and 2 mg/ml, systems HA/CHH<sub>5;1</sub> and HA/CHH<sub>5;2</sub>) when 4% w/v TRE was used. No aggregation was observed apart from HA/CHH<sub>5;2</sub> freeze dried with 0.1% w/v TRE (Figure 1). HA/CHH<sub>5;1</sub> had a very good redispersibility even after lyophilisation with only 0.1% w/v TRE. The properties of HA/CHH<sub>5;2</sub> were satisfactorily preserved at TRE concentration down to 0.5% w/v and at TRE 0.2% w/v a significant increase in the particle size after the process was observed (the PS ratio was 1.59±0.01). It can therefore be concluded that HA/CHH NPs with the MMR of 5 are characterised by better redispersibility than NPs with MMRs of 2.5 and 1. This may be explained by a markedly higher content of HA in these systems, supporting the finding of Peer et al. (2003) that HA can assist in efficient lyophilisation. Also, the corona in HA/CHH<sub>5</sub>
NPs is relatively larger than in the HA/CHH<sub>2,5</sub> system, the latter being composed predominantly of a dense core (Umerska et al., 2012). As the corona is characterised by a smaller density than the core, it is possible that the molecules of a cryoprotectant penetrate inside the corona in spaces between HA chains, therefore enabling better preservation of particle properties during exposure to stress associated with the freeze drying process.

The results of freeze drying of HA/CHH NPs with TRE presented here are in agreement with data obtained by de la Fuente et al. (2008), who did not observe significant changes in the particle size after lyophilisation of HA/CHIT/tripolyphosphate NPs with either 5% w/v TRE or 5% w/v glucose. However, it needs to be highlighted that the concentrations of NPs tested by de la Fuente et al. (2008) where lower (0.075-0.5 mg/ml) than those used in this work. Also, no optimisation study on the concentration of cryoprotectant, nor tests on the influence of NP composition on redispersibility were performed. As it is demonstrated here, optimisation of the concentration of cryoprotectant is of vital importance and the NP composition may have a significant influence on redispersibility, with NPs containing higher amount of HA content requiring less TRE for stabilisation. Some NP systems can be successfully redispersed even at the NP to cryoprotectant weight ratio of 1:1 (HA/CHH<sub>5;1</sub> with 0.1% w/v TRE).

It is generally accepted that TRE is a more effective cryo-/lyo-protectant than mannitol (Cegnar et al., 2011; Katas et al., 2013; Rampino et al., 2013). In contrast to mannitol, TRE was an amorphous excipient after the process and yielded the most suitable protective properties (Cegnar et al., 2011). Briefly, stabilisation of materials in sugar glasses has been explained by the formation of a glassy sugar matrix, which acts as a physical barrier between the particles and inhibits the diffusion on a relevant time scale (Allison et al., 2000; Hafner et al., 2011; Molina et al., 2004). Sugar molecules isolate individual particles in the unfrozen fraction, thereby preventing aggregation during freezing (Allison et al., 2000; Rampino et al., 2013). The sugar molecule interact with NPs via hydrogen bonding, maintaining them in the ‘pseudo-hydrated state’ during the dehydration step and replace water, thus providing protection from damage during dehydration and subsequent rehydration (Cegnar et al., 2011; Hirsjärvi et al., 2009; Rampino et al., 2013). Summarising the outcomes of studies on HA/CHH NPs, lower TRE concentrations were needed for formulations with lower NP concentrations in the native dispersions and higher HA/CHH MMRs (more negative ZP) to successfully preserve NP properties post-processing.
3.2.3.2. CHON- and PROT-based NPs

All HA/PROT NPs tested were successfully redispersed when freeze dried with 4% w/v TRE. The PS ratios after and before freeze drying were not significantly higher than 1.2 (Figure 3a) and no aggregation was observed in any of the samples tested. When TRE concentration was decreased to 1% w/v, no aggregation was observed, but a slight increase in the particle size by 27, 34 and 27% was seen for the following samples: HA/PROT\textsubscript{2.1;low}, HA/PROT\textsubscript{6.3;high} and HA/PROT\textsubscript{3.1;high}, respectively. Therefore, even at 1% w/v TRE, acceptable or very good redispersion was observed depending on the composition of the sample for all HA/PROT NPs tested. However, the results show that HA/PROT NPs redisperse better than HA/CHH NPs with an MMR of 2.5 and 1.
The redispersibility of CHON-based NPs was also examined in order to verify, if the polyanion (CHON versus HA) has any influence on reconstitution of NPs. As shown in Figure 3b, none of the formulations tested were successfully redispersed after lyophilisation with 1% w/v TRE. When the concentration of TRE was increased to 4% w/v, all formulations with the CHON/CHL MMR of 5 as well as CHON/CHL1:1 redispersed with a very good preservation of the particle size. In two formulations with a TPC of 2 mg/ml: CHON/CHL1:2 and CHON/CHL2:5:2, aggregation was observed and the particle size was significantly increased after the process. Freeze dried CHON/CHL2:5:1 satisfactorily redispersed after processing with 4% w/v TRE, but an increase in the particle size by 27% was observed.

The results obtained for CHON/CHL NPs confirm that the composition of the particles, especially the MMR of the polymers, and their concentration plays an important role affecting the ability of such NPs to be redispersed. Similarly to HA/CHH NPs, CHON/CHL NPs with a MMR of 2.5 were the most problematic in reconstitution studies and CHON/CHL NPs with a MMR of 5 were the easiest to fully reconstitute. Therefore, CHON/CHL NPs were characterised by worse redispersibility compared to HA-based NPs.

Finally, CHON/PROT NPs were freeze dried with 1 or 4% w/v TRE, and the results are shown in Figure 3c. Similarly to HA/PROT NPs, no aggregation was observed in any of the samples tested, and all samples satisfactorily redispersed even after processing with 1% w/v TRE.
In some samples an increase in the particle size by 30% could be observed, but they did not differ significantly (p>0.05) from the PS ratio of 1.2. Interestingly, some of the NPs freeze dried with 4% w/v TRE were significantly (p<0.05) larger than those lyophilised with 1% w/v TRE. The CHON/PROT\textsubscript{12.5;high} system as well as all NPs made using 0.7 mg/ml CHON decreased their size during freeze drying with 1% w/v TRE and the size ratios were smaller than 1. In conclusion, both HA/PROT and CHON/PROT NPs are characterised by very good redispersibility, which is considerably better than most of the chitosan-based formulations.

Differences in redispersibility of the NPs can possibly be explained by different properties of the polyelectrolytes used (molecular weight, charge sign, charge strength and charge density) and different NP structures. PROT has the highest charge density, CHON and CHIT have low charge density, while the charge density of HA is very low. Strong polyelectrolyte complexes are generally formed between polymers including anions and cations of strong acids or bases in their structure (Denuziere et al., 1996). In contrast, weak polyelectrolyte complexes are formed between weak acids and bases. CHIT contains primary amine groups that are weak bases and PROT contains guanidinium groups that are strong bases. HA has carboxylic groups (a weak acid), while CHON has both, sulphate (a strong acid) and carboxylic groups. Therefore it is expected that, considering the pairs of polyelectrolytes tested, CHIT forms with HA a weaker PEC than it does with CHON. Weaker binding between HA and CHIT compared with that between CHON and CHIT can influence the pore size and network complexity of particles and facilitate the penetration and interactions with TRE, thereby improving redispersibility of the HA/CHIT PEC NPs. It has been suggested that the PROT-based NPs do not show the core-corona structure (Umberska et al., 2015, 2014b), in contrast to the CHIT-based NPs. If the dense, tightly packed core is not formed in PROT-based NPs, it may facilitate the penetration of TRE into the NP structure and interactions between TRE and NPs. In summary, it can be concluded that the NPs can be ranked in terms of easiness of redispersibility as follows: HA/PROT > CHON/PROT > CHON/CHL and that HA/CHH NPs reconstitute better in comparison to PROT- and CHON-based NPs.

### 3.2.4 Freeze drying of sCT-containing NPs with trehalose

Having investigated freeze drying properties of HA/CHH carriers, sCT-loaded NPs were freeze dried in the presence of TRE to study the impact of the peptide presence on reconstitution and also the effect of processing on the peptide. The PS ratios are presented
in Figure 1 for HA/CHH₅:₁sCT and in Figure 3 for PROT- and CHON-based systems. In relation to HA/CHH₅:₁sCT, although no aggregation was observed when the TRE concentration used was in the range of 0.1-4%, a significant increase in the particle size after lyophilisation was seen when lower concentrations of TRE were used. The particle size increased by 36, 63 and 83% when 0.5, 0.2 and 0.1% w/v of the sugar was used in the process, respectively. Therefore the preservation of particle properties was not as good as for the equivalent system with no sCT. Thus, incorporation of sCT decreases the ability of HA/CHH NPs to be successfully redispersed. Nevertheless, it is noteworthy that even after increasing by 83% in size the, particles still remained small with sizes well below 400 nm. No impact of sCT loading was seen for the HA/PROT NPs. In contrast to HA-based NPs, incorporation of sCT into CHON-based NPs (system CHON/CHL₅:₁sCT) improved their redispersibility compared to the equivalent carrier systems with no peptide loaded (CHON/CHL₅:₁) (Figure 3). Despite the fact that the sCT presence worsened redispersibility of HA-based NPs and enhanced redispersibility of CHON-based NPs, HA/CHH/sCT NPs can be considered as easier to redisperse than CHON/CHL/sCT NPs, because after freeze drying with 1% w/v TRE their particle size was better maintained.

To confirm that no degradation of sCT occurred during the process, separation of non-associated sCT in the reconstituted sample (HA/CHH₅:₁sCT) by the previously described ultrafiltration-centrifugation technique (Umerska et al., 2014a) was conducted. The amount of peptide extracted from HA/CHH₅:₁sCT after lyophilisation was 98.90±1.57%, compared to 96.80±2.19% for the sample before freeze drying used here as control. The association efficiency (AE) was 98.92±0.28% and 98.94±0.27% before after processing, respectively, indicating no impact of lyophilisation on the integrity of sCT. Also, when trying to maximise the drug loading in the particles, the concentration of a cryoprotectant must be kept as low as possible. The sCT loading in samples (HA/CHH) lyophilised with 0.1, 0.2, 0.5 and 1% w/v TRE would be approximately 20, 14, 7.7 and 4.3% w/w, respectively, which can be considered as high. However, a compromise must be made between the preservation of properties of the particles and the drug loading.

In conclusion, the presence of sCT in the studied PEC NPs, with exception of the HA/PROT system, affected the properties of NPs post-processing, but in an inconsistent manner thus, if a cargo molecule is incorporated into PEC NPs it is likely that the optimum cryoprotectant concentration will be different to that used for empty NP carriers.
3.2.5. Freeze drying of HA/CHH NPs with PEG polymers

Apart from sugars, water soluble polymers could also be used as cryoprotectants. Lee et al. (2009) have shown that 2000 g/mol PEG successfully performed as a cryoprotectant for naproxen nano-suspensions. PEG chains in the crystal lattice are organised as lamellae and the proportion of the crystal form is dictated by the molecular weight of the polymer (Craig, 1995). As a consequence, PEG materials are semicrystalline and may exhibit a significant degree of disorder (Craig, 1995). Therefore, in this part of the study, HA/CHH\(_{2.5;1}\) with variable concentrations of different PEG polymers (2, 4 and 10 kDa linear PEG as well as 10 kDa four-arm branched PEG). HA/CHH\(_{2.5;1}\) was poorly redispersible when lyophilised with TRE (Figure 1).

Figure 4a shows the PS ratios after and before freeze drying of the systems with PEG polymers. When 0.5% w/v PEG was used, aggregation was observed during reconstitution, regardless of the type of PEG used. An increase in the polymer concentration to 1% w/v resulted in an acceptable redispersibility of NPs with no aggregation observed and PS ratios between 1.25±0.09 and 1.37±0.08 (for PEG 2 kDa and branched PEG 10 kDa, respectively). A further increase in PEG concentration from 2 to 5% w/v led to very good redispersibility of the systems with an increase in particle size after freeze drying smaller than 20%. Neither the molecular weight nor the structure of PEG molecules (linear versus branched) were found to have an impact on redispersibility of HA/CHH NPs. Also, PEG was found to be a more effective cryoprotectant than TRE, as only 1% w/v PEG was needed to obtain acceptable redispersibility of HA/CHH\(_{2.5;1}\) compared to 3% w/v TRE, where only marginal redispersion was seen. Very good preservation of the size of NPs was achieved at 2% w/v PEG compared to 4% w/v TRE.
Figure 4. The particle size (PS) ratio of HA/CHH$_{2.5;1}$ freeze dried with: a) different concentrations of PEG polymers and b) 2% w/v solutions containing different proportions of PEG-L 2 kDa and TRE. The green bands indicate the PS ratio between 0.8 and 1.2, considered as very good redispersibility of processed NPs.

3.2.6 Freeze drying of HA/CHH NPs with a mixture of trehalose and PEG

As PEG was shown to be a more effective cryoprotectant than TRE, attempts were made to decrease the concentration of the sugar required for the successful redispersion of HA/CHH$_{2.5;1}$ after freeze drying with a mixture of cryoprotectants: TRE and PEG. Also, it was of interest if a combination of these two excipients may have a synergistic effect. As it has been shown that viscosity of aqueous PEG solutions increases proportionally to an increasing molecular weight (Gonzalez-Tello et al., 1994), therefore PEG with the lowest molecular weight (i.e. 2 kDa) was selected for further co-lyophilisation studies with TRE. The
total concentration of cryoprotectants was kept at 2% w/v, and three combinations were tested: 3:1, 1:1 and 1:3 w/w of PEG/TRE.

As shown in Figure 4b, the PS ratio was below 1.2 for all three combinations. More importantly, aggregation was not observed. In relation to the synergistic effect of both cryoprotectants when used in a combination, this was clearly seen for the 1:1 and 1:3 w/w PEG/TRE mixtures, where the measured PS ratios were 1.19±0.05 and 1.155±0.04, while those calculated (theoretical) PS ratio values are 1.42 and 1.57, respectively. Therefore it can be concluded, that addition of PEG enabled to decrease the concentration of TRE required to successfully reconstitute freeze dried HA/CHH2.5;1 and that a synergistic effect of the excipient combination was observed.

3.3. Solid state analysis of the freeze dried products

3.3.1. Trehalose/NPs systems

The NPs (HA/CHH2.5;1) freeze dried without any cryoprotectant were PXRD amorphous (Figure 5) and no clear thermal events, with exception of a shallow peak of thermal decomposition after 200 °C, were observed in the DSC scans (Figure 6a).

Figure 5. Powder X-ray diffractograms of (from top to bottom): unprocessed TRE as trehalose dihydrate, freeze dried TRE (TRE FD), unprocessed PEG-L 2 kDa, freeze dried
HA/CHH$_{2.5;1}$ (referred to as NPs), NPs freeze dried in 1% w/v TRE (1% TRE+NPs FD), NPs freeze dried in 4% w/v TRE (4% TRE+NPs FD), NPs freeze dried in 1% w/v PEG-L 2 kDa (1% PEG-L 2kDa+NPs FD), NPs freeze dried in 5% w/v PEG-L 2 kDa (5% PEG-L 2kDa+NPs FD), NPs freeze dried in 2% w/v solution of 1:3 w/w PEG-L 2 kDa/TRE mixture ((1:3 w/w PEG/TRE) +NPs FD), NPs freeze dried in 2% w/v solution of 1:1 w/w PEG-L 2 kDa/TRE mixture ((1:1 w/w PEG/TRE) +NPs FD), NPs freeze dried in 2% w/v solution of 3:1 w/w PEG-L 2 kDa/TRE mixture ((3:1 w/w PEG/TRE) +NPs FD). Arrows indicate characteristic diffraction peaks of trehalose dihydrate and PEG-L 2 kDa.

\[\text{\textsuperscript{exo}}\]

\[\text{TRE FD}\]

\[\text{decomposition}\]

\[\text{NPs (HA/CHH$_{2.5;1}$) FD}\]

\[\text{4% w/w TRE+NPs FD}\]

\[\text{(FD TRE+ FD NPs) PM}\]

\[\text{dehydration}\]

\[\text{melting}\]

\[\text{TRE (as dihydrate)}\]

\[\text{Temperature (°C)}\]

\[\text{50 100 150 200 250 300}\]

\[\text{10 mW}\]

\[\text{2 mW}\]

\[\text{PEG-L 2 kDa, unprocessed}\]

\[\text{PEG-L 2 kDa FD}\]

\[\text{decomposition}\]

\[\text{2% w/w PEG-L 2kDa+NPs FD}\]

\[\text{(FD PEG-L 2kDa+ FD NPs) PM}\]

\[\text{Temperature (°C)}\]
Figure 6. Differential calorimetry thermograms of: a) unprocessed TRE as trehalose dihydrate, freeze dried TRE (TRE FD), freeze dried HA/CHH$_{2.5;1}$ (referred to as NPs), NPs freeze dried in 4% w/v TRE (4% w/w TRE+NPs FD) and a physical mixture of TRE and NPs freeze dried separately ((FD TRE+ FD NPs) PM); b) unprocessed PEG-L 2 kDa, freeze dried PEG-L 2 kDa (PEG-L 2 kDa FD), NPs freeze dried in 2% w/v PEG-L 2 kDa (2% w/w PEG-L 2kDa+NPs FD) and a physical mixture of PEG-L 2 kDa and NPs freeze dried separately ((FD PEG-L 2kDa+ FD NPs) PM); c) NPs freeze dried in 2% w/v solution of 3:1 w/w PEG-L 2 kDa/TRE mixture ((3:1 w/w PEG/TRE)+NPs FD), NPs freeze dried in 2% w/v solution of 1:1 w/w PEG-L 2 kDa/TRE mixture ((1:1 w/w PEG/TRE)+NPs FD), NPs freeze dried in 2% w/v solution of 1:3 w/w PEG-L 2 kDa/TRE mixture and a physical mixture of TRE ((1:3 w/w PEG/TRE)+NPs FD), PEG-L 2 kDa and NPs freeze dried separately ((FD PEG FD TRE+FD NPs) PM).

PXRD studies showed that initially crystalline, unprocessed trehalose dihydrate became amorphous after lyophilisation of its aqueous solution (Figure 5). It can be seen that the presence of NPs did not affect the solid state properties of freeze dried TRE. As shown in Figure 6a dehydration of trehalose dihydrate starting material powder occurs at 97.7 °C and the anhydrous crystals melt at 211.5 °C, consistent with temperatures of 97 and 210 °C, respectively, reported by Ohtake et al. (2011). The thermogram of lyophilised TRE (Figure 6a) had a glass transition ($T_g$) of 119.3 °C, in agreement with the $T_g$ of 110-120 °C recorded by Ohtake et al. (2011). After freeze drying HA/CHH$_{2.5;1}$ with TRE, the $T_g$ with a similar midpoint (120.5 °C) was observed, confirming that the sugar was amorphous (Figure 6a). No crystallisation exotherm was recorded, showing that the presence of NPs did not destabilise
the structure of amorphous TRE and furthermore, no visible NP decomposition event (at
around 200 °C) was noted, in contrast to the equivalent physical mixture composed of
HA/CHH_{2.5;1} and TRE freeze dried separately, indicating improved thermal stability of NPs
lyophilised with TRE.

3.3.2. PEG/NPs systems

PXRD (Figure 5) and DSC (Figure 6b) show that unprocessed PEG-L 2 kDa was
crystalline. A narrow endotherm with an onset at 52.8°C and peak at 54.3°C was recorded
by DSC. After freeze drying PEG was crystalline, but the onset and peak of melting
endotherm appeared at lower temperatures (49.4 and 52.8 °C, respectively) and the
endotherm was broader compared to the unprocessed material. This may be a result of PEG
conformation change. PEG has a helical conformation consisting of seven chemical units and
two turns in a fibre identity period of 19.3 Å (Craig, 1995). On melting solid PEG, the helical
structure is lost and a liquid containing random coils is obtained. In aqueous solution the
system retains the helical configuration, but in a less ordered form (Craig, 1995). It is
possible that due to the rapid decrease in temperature, which takes place during freezing
aqueous solution of PEG with the use of liquid nitrogen before the lyophilisation process,
some chain conformation changes occur which affect the thermal behaviour of lyophilised
PEG.

The crystalline character of PEG was evident after freeze drying with HA/CHH_{2.5;1}. All
peaks present in the PXRD diffractograms of the PEG/NP systems were similar to PEG on its
own and no extra peaks were seen indicating that no new phase was formed (Figure 5). As
HA/CHH_{2.5;1} did not show any clear thermal events, the endotherms seen in the DSC
thermograms of all PEG/NP systems could be attributed to the melting of PEG (Figure 6b).
These endothermic peaks were broader than that of the unprocessed PEG and the onsets
occurred at lower temperatures however, as the onset temperature for these systems was
around 49.6-49.7 °C, this shows mostly the effect of processing on PEG and not interactions
NPs-polymer. Again, the thermal stability of NPs in the lyophilisates was improved, as
thermal decomposition of the physical mixture of separately freeze dried HA/CHH_{2.5;1} and
PEG starts at 120 °C (Figure 6b).

3.3.3. PEG/trehalose/NPs systems

PXRD (Figure 5) presented that lyophilisation of HA/CHH_{2.5;1} with all PEG-L 2 kDa/TRE
mixtures tested resulted in the formation of crystalline materials. The peaks characteristic of
PEG at approximately 19 and 23 2theta degrees were seen in all PEG/TRE/NPs diffractograms. TRE on its own is amorphous after lyophilisation, however, a clear presence of crystalline trehalose dihydrate was noticed in the freeze dried (1:1 w/w PEG/TRE)/NP system and these peaks were more pronounced for the freeze dried (1:3 w/w PEG/TRE)/NP sample. Hence the ratio of PEG to TRE dictates whether the sugar after freeze drying is amorphous or crystalline.

DSC scans (Figure 6c) show the thermal characteristics of PEG/TRE/NPs. In all thermograms an endotherm at approximately 50-55 °C is seen, which can be attributed to melting of PEG. A clear Tg in DSC traces of (1:1 w/w PEG/TRE)/NPs and (1:3 w/w PEG/TRE)/NPs is seen at approximately 120 °C, indicating of the presence of amorphous TRE (also evident from PXRD data, Figure 5), thus these materials are semi-crystalline. This Tg is comparable to that of pure, lyophilised TRE, thus suggesting no miscibility of PEG and TRE. Crystallisation and melting of TRE is visible in the thermograms of (1:1 w/w PEG/TRE)/NPs and (1:3 w/w PEG/TRE)/NP (Figure 6c). A crystallisation exotherm with an onset at 170-175 °C is immediately followed by an endotherm appearing at 205-208 °C for both samples. A small endotherm of TRE melting was also present in the physical mix (TRE and PEG were lyophilised separately), overlapping with an event of thermal degradation of sample.

The enthalpy of crystallisation of the (1:1 w/w PEG/TRE)/NPs system was 33 J/g, while that for the (1:3 w/w PEG/TRE)/NPs sample was only 11 J/g, consistent with PXRD showing greater crystallinity of TRE in (1:3 w/w PEG/TRE)/NPs. Interestingly, as no crystallisation was observed in freeze dried amorphous TRE, it appears that the sugar in (1:1 w/w PEG/TRE)/NPs and (1:3 w/w PEG/TRE)/NPs is present in an amorphous and also the crystalline, dihydrate form and the crystalline content is driving the amorphous phase to crystallise. The thermogram of (3:1 w/w PEG/TRE)/NPs showed primarily melting of the PEG component, followed by thermal decomposition starting at around 120 °C.

Overall, all PEG/TRE/NPs freeze dried mixtures were semi-crystalline with an evidence of crystalline PEG and trehalose dihydrate present, thus these powders can be considered as being more physically stable that lyophilised TRE/NPs.

3.4. Cytotoxicity of the freeze dried products

3.4.1 Trehalose-based samples

Cytotoxicity of TRE was examined, as it would be present in all the freeze dried NP samples tested. When using different concentration of the sugar alone, 4% w/v is able to
decrease the cell viability by 13%. TRE appeared to decrease the viability of Caco-2 cells only at concentrations higher than 2% w/v. It is probably due to the fact that the high concentration of sugar results in hyperosmolar solutions and thus may reduce cells viability unspecifically (Scherließ, 2011). The IC$_{50}$ of TRE was calculated to be 5.8% w/v.

Table 3 shows the cytotoxicity of HA/CHH NPs. At the concentration of 4% w/v, which is a concentration typically resulting in good redispersibility of practically all NPs investigated, TRE can be considered as non-toxic. Although significant, the decrease in cell viability caused by TRE is small (7% decrease in cell viability compared to control determined by flow cytometry and 13% by MTS assay). The toxicity (by MTS assay) of freeze dried formulations with the HA/CHH MMR of 1 were comparable to toxicity of pure TRE. On the other hand, the MTS assay showed that reconstituted NPs, previously processed with 4% w/v TRE, with the HA/CHH MMR of 5 (HA/CHH$_{5;2}$ and HA/CHH$_{5;1}$) were significantly less toxic than 4% w/v solution of TRE. The MTS results agreed with those from the flow cytometry assay. Lyophilised HA/CHH$_{5;1}$ and HA/CHH$_{5;2}$ had the amount of viable cells (Annexin V and PI negative) at 90.7±0.5 and 86.35±1.2%, respectively, thus the HA/CHH$_{5;1}$ was significantly less cytotoxic than the solution of trehalose on its own. Therefore, no toxic effects were observed after the incubation of cells with reconstituted NPs with the HA/CHH MMR of 5 moreover, the presence of HA/CHH$_{5;1}$ seemed to reduce significantly the cytotoxic effects of TRE. The HA/CHH MMR=1 systems (lyophilised with 4% w/v TRE) were the most toxic HA/CHH samples (Table 3). It was not possible to determine the cell viability by flow cytometry due to the presence of micro-sized aggregates, which caused analysis interference. Incorporation of sCT into HA/CHH NPs (system HA/CHH$_{5;1}$sCT) decreased the viability of cells only slightly (7% decrease by MTS and 9% by flow cytometry), but the difference was statistically significant when compared to equivalent NPs with no sCT (Table 3).

### Table 3 Viability studies (MTS assay and flow cytometry) of Caco-2 cells treated for 72 hours with 4% w/v TRE solution or with reconstituted, HA-based NPs previously freeze dried with 4% w/v TRE. N/D – not determined; * - p < 0.05 vs. control, ** - p < 0.05 vs. TRE, *** - p < 0.05 vs. control and TRE

<table>
<thead>
<tr>
<th>Sample</th>
<th>% of viable cells by MTS assay</th>
<th>% of viable cells by flow cytometry</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum-free medium (control) – no TRE</td>
<td>100</td>
<td>91.3±2.7</td>
</tr>
<tr>
<td>Added</td>
<td>TRE</td>
<td>HA/CHH₅;1</td>
</tr>
<tr>
<td>-------</td>
<td>-----------</td>
<td>-----------</td>
</tr>
<tr>
<td></td>
<td>82.9±1.9*</td>
<td>96.6±5.3**</td>
</tr>
<tr>
<td></td>
<td>84.2±2.2*</td>
<td>90.7±0.5**</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>HA/PROT₆.₃;low</th>
<th>HA/PROT₆.₃;low</th>
<th>HA/PROT₃.₁;low</th>
<th>HA/PROT₂.₁;low</th>
<th>HA/PROT₁₂.₅;high</th>
<th>HA/PROT₆.₃;high</th>
<th>HA/PROT₃.₁;high</th>
</tr>
</thead>
<tbody>
<tr>
<td>68.8±14.35*</td>
<td>65.6±13.0*</td>
<td>60.8±9.6***</td>
<td>54.15±4.7***</td>
<td>67.6±4.1***</td>
<td>64.2±2.3***</td>
<td>46.8±6.2***</td>
</tr>
<tr>
<td>80.35±2.0*</td>
<td>81.3±2.0*</td>
<td>80.3±1.1*</td>
<td>77.9±1.05***</td>
<td>81.8±0.7*</td>
<td>78.35±1.6***</td>
<td>75.35±4.5***</td>
</tr>
</tbody>
</table>

Cytotoxicity of HA/PROT NPs freeze dried with TRE is presented in Table 3. The viability of Caco-2 cells was decreased markedly compared to control (serum-free medium). As noted earlier for PROT (Umerska et al., 2014b), the MTS assay was more sensitive than flow cytometry. The viability of cells decreased by approximately 10-15% compared to the medium in the apoptosis assay and a 30-50% decrease in the proliferation of the cells was observed by MTS assay. The lyophilised samples contained two ingredients which have been shown to decrease the amount of living cells: PROT (Umerska et al., 2014b) and high concentration of TRE. HA/PROT NPs freeze dried with TRE were markedly more toxic than TRE alone, however their toxicity does not differ significantly from PROT at the same concentration (Umerska et al., 2014b) and increases as the content of PROT in the formulation increases.

Table 4 shows the effects of freeze dried CHON/CHIT formulations had on Caco-2 cells. All formulations tested were well tolerated by the cell line. The cytotoxicity of freeze dried samples did not differ significantly from those of HA/CHH systems with equivalent composition. Also, similar effects were observed: incubation with formulations containing more CHON (higher MMRs) resulted in a higher % of viable cells and a significant increase in the number of living cells (especially visible for CHON/CHL₅;₁). Therefore, the presence of CHON/CHL NPs also protect Caco-2 cells from the negative effects of osmotic stress caused by TRE. In relation to lyophilised CHON/PROT NP formulations, the number of living cells decreased by 20-35%, depending on the composition of the sample (Table 4). In conclusion,
CHON/PROT NPs lyophilised with TRE were generally better tolerated than those composed of HA and PROT. Also, they were less toxic than PROT on its own.

Table 4 Viability (MTS assay) of Caco-2 cells by MTS assay treated for 72 hours with 4% w/v TRE solution or with reconstituted, CHON-based NPs, previously freeze dried with 4% w/v TRE. * - p < 0.05 vs. TRE

<table>
<thead>
<tr>
<th>Sample</th>
<th>% of viable cells by MTS assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>TRE</td>
<td>82.9±1.9</td>
</tr>
<tr>
<td>CHON/CHL₁:₁</td>
<td>79.6±8.3</td>
</tr>
<tr>
<td>CHON/CHL₁:₂</td>
<td>84.4±11.2</td>
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<tr>
<td>CHON/CHL₅:₁</td>
<td>98.4±7.9*</td>
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<tr>
<td>CHON/CHL₅:₂</td>
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<tr>
<td>CHON/CHL₅:₁sCT</td>
<td>86.0±8.7</td>
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<td>CHON/PROT₂:₁;low</td>
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<td>CHON/PROT₁₂:₅;high</td>
<td>75.3±5.4</td>
</tr>
</tbody>
</table>

3.4.1 PEG-based samples

Cytotoxicity of all PEG polymers (i.e. 2 kDa, 4 kDa, 10 kDa linear and 10 kDa branched PEG) was tested at the same concentration of 5% w/v by MTS assay and flow to compare their toxic effects. The results are shown in Figure 7. The PEG polymers, apart from 10 kDa linear PEG, were well tolerated by Caco-2 cells. MTS assay showed that the proliferation of cells was slightly increased after incubation with 2 and 4 kDa linear PEGs or 10 kDa branched PEG. A statistically significant decrease in the % of viable cells can be observed in the apoptosis assay for all these three polymer grades, however the viability was decreased by only approximately 5%. Interestingly, linear 10 kDa PEG decreased the viability of Caco-2 cells significantly (40.6±7.5% of viable cells remained by MTS assay).
Figure 7. Viability of Caco-2 cells after 72-hour exposure to solutions of different cryoprotectants at the concentration of 5% w/v. * - p < 0.05 vs control

As the linear 10 kDa PEG was the most toxic from all cryoprotectants examined, the cytotoxicity of this PEG was also tested at lower concentrations. At 3 or 4% w/v linear 10 kDa PEG remained toxic, however the detrimental effects were markedly reduced at 2% w/v and lower concentrations. The IC50 of 10 kDa linear PEG was 2.5% w/v, therefore it can be considered as more toxic than TRE.

4. Conclusion

A range of polyelectrolyte complex NPs were successfully obtained as solid state formulations with the employment of a lyophilisation process. The presence of a cryoprotectant was necessary to achieve a successful reconstitution and identified as a critical formulation parameter. Although freeze drying of PEC NPs was observed to be formulation specific, some general rules or guidelines on lyophilisation of such nanocarriers can be formulated based on the results obtained in this study:

1) NPs composed of a polycation and a polyanion that have a largely different molecular weight (here PROT-based NPs) are easier to redisperse than the NPs made of polymers with comparable molecular weight (here CHIT-based NPs).

2) NPs composed of weak polyelectrolytes with low charge density (HA) are easier to redisperse than NPs composed of strong polyelectrolytes with high charge density (CHON). Thus, NPs composed of weak polyelectrolytes are the preferred option if minimising cryoprotectant concentration is crucial to achieve satisfactory drug loading.
3) For a particular PEC pair the NPs that contain large stoichiometric excess of one of the polymers are preferred because they are easier to redisperse. The particles with lower crosslinking density are characterised by better redispersibility after freeze-drying because of easier penetration of cryoprotectant molecules. Ionised active ingredients may affect the redispersibility as they can modify crosslinking density.

4) If an active ingredient is incorporated into PEC NPs, it is likely that the minimal cryoprotectant concentration required for successful redispersion will be different to that used for empty NP carriers.

5) Low molecular weight compounds prone to quick crystallisation, such as mannitol, should not be used as cryoprotectants in freeze drying of PEC NPs. Compounds that form an amorphous state on lyophilisation, such as trehalose, are better candidates as cryoprotectants for freeze drying of PEC NPs.

6) Non-charged polymers capable of forming multiple hydrogen bonds with PEC NPs such as PEGs are more effective cryoprotectants than small molecular weight compounds. However, there is no benefit in increasing the molecular weight of PEG and/or branching and the linear 10 kDa PEG showed an increased toxicity.

7) Using a PEG/trehalose blend proved to be beneficial in terms of improved redispersibility.

5. Acknowledgements

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6. References


Ryan, S.M., McMorrow, J., Umerska, A., Patel, H.B., Kornerup, K.N., Tajber, L., Murphy, E.P.,


