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1 **Chondroitin-based nanoplexes as peptide delivery systems – investigations into the self-**  
2 **assembly process, solid-state and extended release characteristics**

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16

17 **Abstract**

18 A new type of self-assembled polyelectrolyte complex nanocarrier composed of chondroitin  
19 (CHON) and protamine (PROT) was designed and the ability of the carriers to bind salmon  
20 calcitonin (sCT) was examined. The response of sCT-loaded CHON/PROT NPs to a change in  
21 the properties of the liquid medium, e.g. its pH, composition or ionic strength was studied and *in*  
22 *vitro* peptide release assessed. The biocompatibility of the NPs was evaluated in Caco-2 cells.  
23 CHON/PROT NPs were successfully obtained with properties that were dependent on the  
24 concentration of the polyelectrolytes and their mixing ratio. X-ray diffraction determined the  
25 amorphous nature of the negatively charged NPs, while those with the positive surface potential  
26 were semi-crystalline. sCT was efficiently associated with the nanocarriers (98-100%) and a  
27 notably high drug loading (13-38%) was achieved. The particles had negative zeta potential  
28 values and were homogeneously dispersed with sizes between 60 and 250 nm. CHON/PROT NPs  
29 released less than 10% of the total loaded peptide in the first hour of the *in vitro* release studies.  
30 The enthalpy of the decomposition exotherm correlated with the amount of sCT remaining in NPs  
31 after the release experiments. The composition of medium and its ionic strength were found to  
32 have a considerable influence on the release of sCT from CHON/PROT NPs. Complexation to  
33 CHON markedly reduced the toxic effects exerted by PROT and the NPs were compatible and  
34 well tolerated by Caco-2 cells.

35

36 KEYWORDS: chondroitin, protamine, calcitonin, nanoparticles, peptide delivery, polyelectrolyte  
37 complex, biocompatibility, toxicity, Caco-2 cells

38

39 **List of abbreviations:**

40 AB - acetate buffer

41 AE - association efficiency

42 ANOVA - one-way analysis of variance

43 Arg – arginine

44 ATR-FTIR - attenuated total reflectance Fourier transform infrared spectroscopy

45 CHON - chondroitin

46 COM1 - complex 1, composition: chondroitin/protamine mass mixing ratio=3.1, final chondroitin  
47 concentration=0.7 mg/ml

48 COM2 - complex 2, composition: chondroitin/protamine mass mixing ratio=3.1, final chondroitin  
49 concentration=1.4 mg/ml

50 COM3 - complex 3, composition: chondroitin/protamine mass mixing ratio=12.5, final  
51 chondroitin concentration=1.4 mg/ml

52 COM4 - complex 4, composition: chondroitin/protamine mass mixing ratio=3.1, final chondroitin  
53 concentration=2.1 mg/ml

54 COM5 - complex 5, composition: chondroitin/protamine mass mixing ratio=5, final chondroitin  
55 concentration=3.6 mg/ml

56 COM6 - complex 6, composition: chondroitin/protamine mass mixing ratio=0.2, final chondroitin  
57 concentration=0.16 mg/ml, positively charged nanoparticles

58 dH – enthalpy of process

59 DL - drug loading

60 DSC - differential scanning calorimetry

61 FBS - fetal bovine serum

62 HA - hyaluronic acid

63 HPLC – high performance liquid chromatography

64 kDa – kilodalton

- 65 kV – kilovolt
- 66 mA – milliampere
- 67 MEM - Eagle's Minimal Essential Medium
- 68 MMR - mass mixing ratio
- 69 MPS – mean particle size
- 70 MTS - 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
- 71 MWCO – molecular weight cut-off
- 72 NP – nanoparticle
- 73 PBS - phosphate-buffered saline
- 74 PDI - polydispersity index
- 75 PROT – protamine
- 76 PXRD - powder X-ray diffraction
- 77 sCT - salmon calcitonin
- 78 Tg - glass transition
- 79 TR – transmittance
- 80 ZP - zeta potential

81

## 82 **1. Introduction**

83 Considerable efforts have been dedicated towards incorporation of bioactive ingredients into  
84 nanoparticles (NPs) composed of biodegradable polymers (Hamidi et al., 2008). There are a  
85 considerable number of polymers and techniques that are used to produce NPs, which allows a  
86 broad differentiation of their internal and external structures as well as composition and biological  
87 properties. The choice of the nanoparticle manufacturing method is influenced by the solubility of  
88 the active compound to be associated/complexed with the NPs as well as the solubility, chemical  
89 structure, characteristic chemical groups, molecular weight and crystallinity/amorphicity of the  
90 polymer (des Rieux et al., 2006). The most commonly used polymers are polyesters (e.g.

91 poly(lactic acid) and poly(lactic-co-glycolic acid)), either alone or in combination with other  
92 polymers (des Rieux et al., 2006). However, the limitation of biodegradable water-insoluble  
93 polymers is that they are mostly hydrophobic, whereas nucleic acids, many peptides and proteins,  
94 which are recognised to have a great potential in therapeutics, are hydrophilic. This leads to  
95 difficulties for the drug to be efficiently encapsulated (Sundar et al., 2010). Hence, the preparation  
96 of NPs with the employment of more hydrophilic and naturally occurring polymers has been  
97 explored. Among polymeric NPs, those composed of polyelectrolytes (polyelectrolyte complex  
98 NPs or nanoplexes) attract particular attention e.g. because of their water soluble character  
99 (Hartig et al., 2007). Amongst cationic polymers used in the formation of nanoplexes, undoubtedly  
100 chitosan is the most extensively investigated (Boateng et al., 2014). Recently, other polycations  
101 have also been employed in the formation of nanoplexes, e.g. polyarginine (Oyarzun-Ampuyero  
102 et al., 2011) and protamine (Umerska et al., 2014a). Protamine is a naturally occurring and  
103 strongly charged cationic protein already used in formulations containing insulin (AHFS Drug  
104 Information, 1989). Protamine (PROT) is rich in arginine and displays a membrane translocation  
105 activity (Reynolds et al., 2005). PROT offers a long history of use and established biological  
106 effects and safety in humans (Reynolds et al., 2005). It has been demonstrated to form  
107 polyelectrolyte complexes with oligonucleotides (Junghans et al., 2000; González Ferreiro et al.,  
108 2001) and glycosaminoglycans: hyaluronic acid (HA) (Umerska et al., 2014a) and heparin (Mori  
109 et al., 2010).

110 The recently described HA/PROT NPs have been shown to successfully encapsulate salmon  
111 calcitonin (sCT) with the association efficiency up to 100% and the advantage of high peptide  
112 loading (10-40% w/w) (Umerska et al., 2014a). However, the release of sCT was relatively quick  
113 as most of the peptide associated with the particles was released within 2-4 hours due to the  
114 weak electrostatic interactions between the species forming the NPs. Thus the strengthening of  
115 intermolecular interactions may decrease the release rate of sCT. Chondroitin sulphate (CHON)  
116 can be considered as a suitable candidate to form polyelectrolyte complexes with PROT and also

117 with sCT. CHON have weak (carboxylate) and strong (sulphate) acid residues, in contrast to HA,  
118 which only has carboxylate groups. Moreover, the charge density in CHON molecules is higher  
119 than in HA (Denuziere et al., 1996). Therefore it is anticipated that the electrostatic interactions  
120 between CHON and PROT as well as CHON and a cationic sCT will be stronger compared to  
121 HA-based interactions. Due to its acidic nature CHON is able to produce ionic complexes with  
122 positively charged molecules. Indeed, similarly to HA, CHON has been shown to form  
123 polyelectrolyte complexes with chitosan (Denuziere et al., 1996, Place et al., 2014),  
124 trimethylchitosan (Place et al., 2014), lysozyme (van Damme et al., 1994) and polyethylenimine  
125 (Pathak et al., 2009).

126 CHON is an abundant glycosaminoglycan found in cartilage, bone and connective mammalian  
127 tissue. It exhibits a wide variety of biological functions and is currently used as an anti-  
128 inflammatory, chondroprotective and antirheumatic drug. CHON has been shown to be absorbed  
129 after oral administration in humans as a high molecular weight polysaccharide (Volpi, 2002). sCT,  
130 currently recommended for short term use in Paget's disease, acute bone loss due to sudden  
131 immobilisation and hypercalcaemia caused by cancer (EMA, 2014), has also been considered  
132 as a promising candidate to be used in osteoarthritis (Manicourt et al., 2005) and in combined  
133 therapy with alendronate in patients with rheumatoid arthritis (Ozoran et al., 2007). The biological  
134 and pharmacological properties of sCT are therefore complementary to those of CHON.

135 A combination of CHON with its anti-inflammatory and chondroprotective action and PROT, due  
136 to its membrane-translocating activity, may be interesting from the therapeutic point of view and  
137 such hybrid CHON/PROT NPs may have the potential to form carriers for the oral delivery of  
138 peptides, in particular sCT. Patient compliance was identified as one of the major issues of long-  
139 term therapies involving parenteral administration of peptides, hence developing such a delivery  
140 system is of significance (Lee and Sinko, 2000). The low bioavailability of sCT after oral  
141 administration has been attributed to proteolytic enzymatic degradation and low intrinsic intestinal  
142 membrane permeability (Lee and Sinko, 2000), however a correlation between enhancement of

143 sCT absorption and mucoadhesion in rats was found by Sakuma et al. (1999, 2002). The  
144 Sakuma's delivery system comprised NPs with hydrophilic, ionic polymeric chains attached to the  
145 NP surface and sCT incorporated in the NPs non-covalently. These NPs also protected the  
146 peptide against digestive enzymatic degradation *in vitro* and shielding sCT from pepsin and  
147 trypsin was also observed for polymeric HA/PROT NPs (Umerska et al., 2014a).

148 Considering the above and that no drug delivery system, especially in the nanoparticulate format,  
149 comprising CHON and PROT has been reported to date, the aims of the current work were to  
150 investigate the conditions of such carrier formation by adopting the previously presented  
151 manufacturing process (Umerska et al., 2012; Umerska et al., 2014a, Umerska et al., 2014b), to  
152 evaluate the conditions of NP formation and their properties as well as to explore the ability of  
153 CHON/PROT NPs to bind and release sCT. Bearing in mind that CHON/PROT NPs are  
154 polyelectrolyte complex NPs, their potential as extended/controlled drug release systems was  
155 also studied and evaluation of suitability of solid-state techniques, as methods supporting the  
156 peptide release studies, was performed.

## 157 **2 Materials and methods**

### 158 **2.1 Materials**

159 Chondroitin 4-sulfate sodium salt (CHON) and protamine sulphate (PROT, molecular weight of  
160 5.1 kDa; manufacturer's data) were purchased from Sigma (Ireland). Salmon calcitonin (sCT,  
161 molecular weight 3.4 kDa, freely soluble in water, isoelectric point of 8.86 (Torres-Lugo and  
162 Peppas, 1999) and net charge at pH 7.4 of approximately 3+) was obtained from PolyPeptide  
163 Laboratories (Denmark). CellTiter 96<sup>®</sup> Non-Radioactive Cell Proliferation Assay was obtained  
164 from Promega Corporation (USA). Other cell culture reagents were provided by Sigma Aldrich  
165 (Ireland). All other reagents, chemicals and solvents were of analytical grade.

166 The molecular weight of CHON was determined using a gel permeation chromatography system  
167 previously described (Umerska et al., 2012). Briefly, CHON was dissolved in a mobile phase  
168 composed of 0.2M NaCl and 0.01M NaH<sub>2</sub>PO<sub>4</sub> brought to pH 7.4 with NaOH solution. Pullulan



169 standards (PL Polymer Laboratoires, Germany) were used to construct the calibration curve.  
170 Standards and samples were prepared as 1 mg/ml solutions in the mobile phase. 100 µl of the  
171 standard or sample was injected into the Plaquagel-OH mixed 8µm 300 × 7.5 mm column  
172 (Polymer Laboratories Ltd., UK) using a flow rate of 1 ml/min. A Waters 410 refractive index  
173 detector was employed. Data collection and integration were accomplished using CLASS-VP  
174 software (version 6.10) with GPC for Class VP (version 1.02) (Shimadzu, Japan). The weight  
175 average molecular weight (Mw) of CHON was 58.6±0.23 kDa.

## 176 **2.2 Preparation of CHON/PROT carriers and CHON/PROT/sCT NPs**

177 The CHON solutions with concentrations of 1, 2, 3 or 5 mg/ml as well as the PROT solutions with  
178 concentrations of 0.4-12 mg/ml were prepared in deionised water. NP carriers (NPs without the  
179 cargo) were formed by adding 4 ml of an aqueous PROT solution to 10 ml of a CHON solution at  
180 room temperature under magnetic stirring. The stirring was maintained for 10 minutes to allow  
181 stabilisation of the system. A dispersion of particles was instantaneously obtained upon mixing of  
182 the polymer solutions. As a range of CHON/PROT ratios were used, the NP dispersions that were  
183 formed had different compositions of CHON (equivalent to 0.71 mg/ml, 1.43 mg/ml 2.14 mg/ml or  
184 3.57 mg/ml in the final formulation) and PROT (equivalent to 0.11-3.43 mg/ml in the final  
185 formulation).

186 NPs containing sCT were formed following the above procedure. An appropriate quantity of the  
187 peptide, resulting in the final sCT concentration in the NP dispersion of 0.5 and 1.0 mg/ml, was  
188 dissolved in the CHON solution prior to mixing with the PROT solution.

## 189 **2.3 NPs characterisation and stability**

### 190 **2.3.1 Transmittance measurements**

191 The transmittance of the NP dispersions was measured using an UV-1700 PharmaSpec UV-  
192 Visible spectrophotometer (Shimadzu) at an operating wavelength of 500 nm in optically  
193 homogenous quartz cuvettes (Hellma, Germany) with a light path of 10 mm (Umerska et al.,  
194 2012).

### 195 **2.3.2. Dynamic viscosity and quantification of associated in NPs CHON**

196 Dynamic viscosity measurements of CHON and PROT solutions as well as NP dispersions were  
197 carried out with an SV-10 Vibro Viscometer (A&D Company Limited) at  $25\pm 0.2$  °C. The instrument  
198 was calibrated using deionised water before measurements and the analysis was done at least  
199 in triplicate for each batch of the liquid sample. The amount of free/unassociated with NPs CHON  
200 was determined from viscosity measurements of continuous phases of NP dispersions. The  
201 viscosity value of pure CHON solution, for a given polymer concentration, was taken as containing  
202 100% free CHON, while the viscosity of water was taken as containing 0% free CHON, since the  
203 viscosity of the continuous phase of a colloidal dispersion compared to that of water can be  
204 correlated with the quantity of free polymer present in the liquid (Umerska et al., 2012). The  
205 amount bound/associated in NPs CHON (expressed as %) was calculated as a difference  
206 between the starting quantity of CHON used in formulation (using the initial CHON concentration)  
207 and the quantity of free/unassociated CHON.

### 208 **2.3.3 Particle size and zeta potential analysis**

209 The intensity-averaged mean particle size (hydrodynamic particle diameter), polydispersity index  
210 (PDI) and zeta potential (ZP) values of the NPs were determined as previously described by  
211 Umerska et al., (2014b). Samples, in their native dispersions, were placed into folded capillary  
212 cells without dilution. Each analysis was carried out at 25 °C with the equilibration time set to 5  
213 minutes. The readings were repeated at least three times for each batch and the average values  
214 of at least three batches are presented. The results obtained were corrected for the sample  
215 viscosity measured at described above.

## 216 **2.4 Salmon calcitonin (sCT) loading studies**

### 217 **2.4.1 Separation of non-associated sCT**

218 Non-associated sCT was separated from the NPs using a combined ultrafiltration-centrifugation  
219 technique (Amicon® Ultra-15, MWCO of 30 kDa, Millipore, USA). A total of 5 ml of sample was  
220 placed in the sample reservoir (donor phase) of the centrifugal filter device and centrifuged for 1

221 hour at 3,000 rpm (4,537 g). After centrifugation, the volume of the solution in the filtrate vial  
222 (acceptor phase) was measured, and the filtrate was assayed for sCT content via high  
223 performance liquid chromatography (HPLC), as described below. This quantity of sCT was  
224 referred to as the non-associated sCT.

225 The NP suspension from the sample reservoir was standardized to 5 ml with deionised water. A  
226 total of 0.75 ml of the NP suspension from the sample reservoir was mixed with 0.75 ml of 0.1  
227 mM NaOH (this NaOH concentration was optimised and did not cause sCT degradation) to break  
228 up the NPs and release sCT, and the mixture was centrifuged for 30 minutes at 13,000 rpm  
229 (16,060 g). The supernatant was assayed for sCT content via HPLC, and this portion of the  
230 peptide was referred to as the extracted sCT. The remainder of the dispersion from the sample  
231 reservoir was analysed for particle size, zeta potential and transmittance. The particle size, zeta  
232 potential, transmittance, pH and viscosity of NPs before separation of the non-associated sCT  
233 were also measured.

234 The association efficiency (AE) and drug loading (DL) were calculated using the following  
235 equations (Umerska et al., 2014b):

$$236 \quad AE = \left[ \frac{A-B}{A} \right] * 100\% \quad (\text{Eqn. 1})$$

237 where A is the total amount (mass) of the sCT, and B is the mass of the non-associated sCT

$$238 \quad DL = \left[ \frac{A-B}{C} \right] * 100\% \quad (\text{Eqn. 2})$$

239 where C is the total weight of all components of the NPs (the associated sCT and the mass of  
240 CHON and PROT used for the preparation of NPs).

#### 241 **2.4.2 Colloidal behaviour of CHON/PROT/sCT NPs in different media**

242 Aliquots of 250  $\mu$ l of sCT-loaded CHON/PROT NPs (CHON conc. 2.1 mg/ml, sCT conc. 1 mg/ml,  
243 CHON/PROT mass mixing ratio (MMR)=6.3) were added to 2.25 ml of the dispersant. The  
244 following dispersants were used:

- 245 • phosphate-buffered saline (PBS) pH=7.4 (137 mM NaCl, 2.7 mM KCl, 1.4 mM NaH<sub>2</sub>PO<sub>4</sub>,

- 246 1.3 mM Na<sub>2</sub>HPO<sub>4</sub> adjusted to pH 7.4 with NaOH solution),
- 247 ● PBS pH=5 (137 mM NaCl, 2.7 mM KCl, 1.4 mM NaH<sub>2</sub>PO<sub>4</sub>, 1.3 mM Na<sub>2</sub>HPO<sub>4</sub> adjusted to
- 248 pH 5 with HCl solution),
- 249 ● diluted PBS pH=7.4 (1:10 dilution with deionised water of pH 7.4 PBS),
- 250 ● acetate buffer pH=5 (0.0375M CH<sub>3</sub>COOH and 0.0643M of CH<sub>3</sub>COONa pH=5),
- 251 ● acetate buffer pH=7.4 (0.0375M CH<sub>3</sub>COOH and 0.0643M of CH<sub>3</sub>COONa adjusted to pH
- 252 7.4 with NaOH solution),
- 253 ● diluted acetate buffer pH=5 (1:10 dilution with deionised water of pH 5 acetate buffer),
- 254 ● deionised water
- 255 ● HCl 0.07M pH=1.2
- 256 ● Eagle's Minimal Essential Medium (MEM), pH=7.4
- 257 ● aqueous NaCl solution with the following concentrations: 0.09%, 0.23%, 0.45%, 0.9% and
- 258 1.8% w/v

259 Samples were incubated at 37 °C at 100 rpm in a reciprocal shaking water bath (model 25,  
260 Precision Scientific, India). Size and zeta potential measurements (Section 2.3.2) were performed  
261 after 1 hour of incubation.

### 262 **2.4.3 Release studies**

263 Aliquots of 250 µl of sCT-loaded CHON/PROT NPs (CHON conc. 2.1 mg/ml, sCT conc. 1 mg/ml,  
264 CHON/PROT mass mixing ratio=6.3) were added to 2.25 ml of dispersant. The following  
265 dispersants were used: PBS (pH 5 and 7.4), diluted PBS (pH 7.4), acetate buffer (pH 5 and 7.4),  
266 diluted acetate buffer (pH 5), deionised water and 0.07M HCl (pH 1.2). The samples were  
267 incubated at 37 °C at 100 rpm in a reciprocal shaking water bath (model 25, Precision Scientific,  
268 India). After 1, 2, 4, 6 and 24 hours, 2.5 ml aliquots were withdrawn, and the released sCT was  
269 separated using the combined ultrafiltration-centrifugation technique as described above. The  
270 samples were centrifuged at 4,500 rpm (6,805 g) for 15 minutes. After centrifugation, the volume

271 of the solution from the filtrate vial (acceptor phase) was measured, and the filtrate was assayed  
272 for sCT content by HPLC (released sCT; the HPLC method is described below). The NP  
273 dispersion from the sample reservoir was made up to 2.5 ml with the dispersant and returned to  
274 the water bath to continue the release studies.

275 The data from the release studies were fitted to the first-order equation:

$$276 \quad W = W_{\infty}(1 - e^{-kt}) \quad (\text{Eqn. 3})$$

277 where W is the amount of the peptide released at time t (based on cumulative release),  $W_{\infty}$  is the  
278 amount of the peptide released at infinity and k is the release rate constant (Corrigan et al., 2006).

#### 279 **2.4.4 Quantification of sCT**

280 Analysis of sCT content was performed using an HPLC system as described previously (Umerska  
281 et al., 2014b). Briefly, standard solutions of sCT (1.5–50 µg/ml) were prepared in deionised water,  
282 and 50 µl of the standard or sample was injected into the Jones Chromatography Genesis 4µ C18  
283 150x4.6 mm column. A flow rate of 1 ml/min was employed using a mobile phase composed of  
284 0.116% w/v NaCl, 0.032% v/v trifluoroacetic acid and 34% v/v acetonitrile. The UV detection was  
285 carried out at 215 nm. The sCT peak had a retention time of ~5 min. Data collection and  
286 integration were accomplished using CLASS-VP software (version 6.10, Shimadzu, Japan).

#### 287 **2.5 Solid state characterisation**

288 Solutions of CHON (5 mg/ml) and PROT (12 mg/ml) as well as a range of CHON/PROT NP  
289 dispersions (without sCT) were prepared as described in Section 2.2. The following CHON/PROT  
290 MMRs and CHON concentrations were tested: complex 1 (COM1, MMR=3.1, final CHON  
291 conc.=0.7 mg/ml), complex 2 (COM2, MMR=3.1, final CHON conc.=1.4 mg/ml), complex 3  
292 (COM3, MMR=12.5, final CHON conc.=1.4 mg/ml), complex 4 (COM4, MMR=3.1, final CHON  
293 conc.=2.1 mg/ml), complex 5 (COM5, MMR=5, final CHON conc.=3.6 mg/ml) and complex 6  
294 (COM6, MMR=0.2, final CHON conc.=0.16 mg/ml). In addition, samples of CHON/PROT/sCT  
295 NPs were collected after 6h of release studies (release studies are described in Section 2.4.3).  
296 All liquid samples were freeze dried following the procedure presented by Umerska et al. (2012)

297 and analysed by powder X-ray diffraction (PXRD), differential scanning calorimetry (DSC) and  
298 attenuated total reflectance Fourier transform infrared spectroscopy (ATR-FTIR) (Paluch et al.,  
299 2010, 2013). Briefly, a Miniflex II Desktop X-ray diffractometer Rigaku with a Haskris cooling unit  
300 was employed. The tube output voltage used was 30 kV and tube output current was 15 mA. A  
301 Cu-tube with Ni-filter suppressing  $K\beta$  radiation was used. Measurements were taken from 5 to 40  
302 on the 2 theta scale at a step size of  $0.05^\circ$  per second. DSC experiments were performed using  
303 a Mettler Toledo DSC 821e with a refrigerated cooling system LabPlant RP-100. Nitrogen was  
304 used as the purge gas. Aluminium sample holders were sealed with a lid and pierced to provide  
305 three vent holes. Sample weights were approximately 3 mg and measurements were carried out  
306 at a heating rate of  $10^\circ\text{C}/\text{min}$ . The unit was calibrated with indium and zinc standards. ATR-FTIR  
307 spectra were recorded on a PerkinElmer Spectrum 1 IR Spectrometer and evaluated using  
308 Spectrum v5.0.1 software. The spectral range was  $650\text{--}4000\text{ cm}^{-1}$  and a resolution of  $4\text{ cm}^{-1}$ . Six  
309 scans were collected, averaged and normalised to obtain good quality spectra.

## 310 **2.6 Cell culture and *in vitro* cytotoxicity studies**

311 Intestinal epithelial cells (Caco-2) were obtained from the European Collection of Cell Cultures.  
312 Cells were cultured as a monolayer in Eagle's Minimal Essential Medium (MEM) supplemented  
313 with 20% fetal bovine serum (FBS), penicillin (0.006 mg/ml), streptomycin (0.01 mg/ml),  
314 gentamicin (0.005 mg/ml), sodium bicarbonate (2.2 g/l) and sodium pyruvate (0.11 g/l) in a 5%  
315  $\text{CO}_2$  and  $37^\circ\text{C}$  humidified atmosphere ( $\text{CO}_2$  incubator series 8000DH, ThermoScientific, USA).  
316 Cells were supplied with fresh medium every second day and split after detachment with EDTA-  
317 trypsin twice a week. The passage number range was maintained between 20 and 30.

### 318 **2.6.1 MTS assay**

319 The Caco-2 cells were seeded into flat-bottom 96-well plates in  $100\ \mu\text{l}$  of MEM containing 20% of  
320 FBS at a density of 25,000 cells per well and incubated at  $37^\circ\text{C}$  for 24 hours. The medium was  
321 replaced with  $100\ \mu\text{l}$  of the sample dispersed or dissolved in serum-free media. After 72 hours of  
322 incubation, the supernatant was removed from the wells and replaced with serum-free media. An

323 amount of 20  $\mu$ l of MTS reagent prepared according to the manufacturer protocol was added into  
324 each well; in the case of the positive control (0% viability), the media was replaced with a 10%  
325 SDS solution in serum-free media 30 min before the addition of MTS reagent. After 4 hours, the  
326 UV absorbance of the formazan product was measured spectrophotometrically (FLUOstar  
327 Optima microplate reader, BMG Labtech) at 492 nm. The positive control was treated as a blank,  
328 and its absorbance was subtracted from each reading. The cell viability was expressed as the  
329 ratio of the absorbance reading of the cells treated with different samples and that of the negative  
330 control (cells treated with serum-free MEM), which was assumed to have 100% of cell viability.  
331 Experiments were repeated 3 times.

## 332 **2.7 Statistical analysis**

333 The statistical significance of the differences between samples from *in vitro* release studies was  
334 determined using one-way analysis of variance (ANOVA) followed by the post-hoc Tukey's test  
335 using Minitab software. Differences were considered significant at  $p < 0.05$ . As regards cell culture  
336 studies, statistical differences between groups were determined by Student's t-test. A two-tailed  
337 p-value  $< 0.05$  was considered as statistically significant.

## 338 **3. Results**

### 339 **3.1 CHON/PROT NPs: formation and formulation variables**

340 Depending on CHON/PROT mass mixing ratio (MMR) and the concentration of both  
341 polyelectrolytes, different types of CHON/PROT dispersions, i.e. solution (transmittance above  
342 95%), opalescent (transmittance 85-95%), turbid (transmittance 0.1-85%) dispersion or a phase  
343 separation were observed. Fig. 1 shows the properties of macroscopically homogeneous (i.e.  
344 either having the appearance of a solution or being opalescent or turbid) CHON/PROT  
345 polyelectrolyte complexes dispersed in the form of colloidal particles.

346 For each concentration of CHON tested the systems containing more PROT (lower CHON/PROT  
347 MMRs) were generally more turbid (Fig. 1A). All dispersions that are included in Fig.1 were  
348 visually homogenous. The phase separation (observed as particle flocculation) occurred at

349 CHON/PROT MMRs of 1.0, 1.1, 1.0 and 1.1 for CHON concentrations of 0.7, 1.4, 2.1 and 3.6  
350 mg/ml, respectively.

351 Fig. 1B shows the mean hydrodynamic diameter of CHON/PROT NPs. CHON/PROT NPs had the  
352 size ranging from  $78\pm 3$  or  $78\pm 12$  nm (CHON of 1.4 mg/ml, CHON/PROT MMR=3.0 and CHON of  
353 0.7 mg/ml, CHON/PROT MMR=3.2, respectively) to  $296\pm 16$  nm (CHON of 3.6 mg/ml,  
354 CHON/PROT MMR=1.2). The particle size increased with an increasing amount of PROT in the  
355 formulation (decreasing CHON/PROT MMR). The concentration of CHON was also found to  
356 affect the particle size of CHON/PROT NPs, especially when it increased from 1.4 mg/ml to 2.1  
357 mg/ml.

358 Fig. 1C presents PDI of CHON/PROT NPs. The PDI values varied from  $0.11\pm 0.00$  (CHON of 1.4  
359 mg/ml and CHON/PROT MMR=1.4) to  $0.43\pm 0.02$  (CHON of 3.6 mg/ml and CHON/PROT  
360 MMR=10.6). The CHON/PROT MMR had a considerable influence on the size distribution of NPs.  
361 The higher the amount of PROT in the dispersion (i.e. the lower CHON/PROT MMR), the more  
362 homogenous the size distribution of the particles was. Also, the PDI values generally increased  
363 with the increasing concentration of CHON and decreased with an increase in the particle size.

364 All physically stable (i.e. non-sedimenting and non-aggregating) dispersions were characterised  
365 by a negative surface charge between  $-27.6\pm 5.99$  mV (CHON of 2.1 mg/ml, CHON/PROT MMR=  
366 1.2) and  $-63.1\pm 8.59$  mV (CHON of 3.6 mg/ml, CHON/PROT MMR=10.6) (Fig. 1D). The surface  
367 charge of CHON/PROT NPs decreased as the CHON/PROT MMR decreased. This decrease  
368 was marginal at high MMRs (approximately 2-20) and more significant at lower MMRs  
369 (approximately 1.2-2).

370 The efforts to obtain physically stable (for at least 24h) positively charged CHON/PROT  
371 dispersions of NPs were unsuccessful. When CHON solution (0.16 mg/ml) was mixed with PROT  
372 solution (0.7 mg/ml) at CHON/PROT MMR of 0.2, a very homogeneously dispersed (PDI of  $0.014\pm$   
373  $0.010$ ) small ( $211\pm 6$  nm) NPs bearing positive surface charge of  $11.6\pm 0.78$  mV were obtained  
374 (Fig. 1b, c and d). Aggregation of the positively charged NPs forming larger NPs and later



375 microparticles was observed after a few hours of storing the dispersion at room temperature (Fig.  
376 2).

### 377 **3.2 Formation and characterisation of sCT-loaded CHON/PROT NPs**

378 The success of loading of sCT into CHON/PROT NPs depended on the concentration of CHON  
379 and PROT solutions as well as their MMR (Table 1).

380 Visually opalescent or turbid dispersions containing NPs characterised by small and  
381 homogeneously dispersed particles were obtained for CHON/PROT MMRs of 2 and greater for all  
382 CHON concentrations tested (i.e. 0.7, 1.4 and 2.1 mg/ml). A further increase in PROT  
383 concentration, and thus a decrease in a CHON/PROT MMR, yielded physically unstable systems,  
384 where phase separation and aggregation were observed. As indicated above, in the case of  
385 CHON/PROT NPs without sCT the colloidal size of the dispersion was maintained even at lower  
386 MMRs of 1.2-1.3. It was impossible to completely dissolve sCT at 1 mg/ml in the 0.7 mg/ml CHON  
387 solution. A clear solution was formed either when 1 mg/ml sCT was dissolved in more  
388 concentrated CHON solutions (i.e. 1.4 and 2.1 mg/ml) or when 0.5 mg/ml sCT was dissolved in  
389 the 0.7 mg/ml CHON solution. Generally, the incorporation of sCT into CHON/PROT NPs resulted  
390 in an increased turbidity of the dispersions (Fig. 1 and Table 1). The turbidity increased with a  
391 decrease in a CHON/PROT MMR and an increase in the concentration of CHON and PROT as  
392 well as the concentration of sCT.

393 The hydrodynamic diameter of the CHON/PROT/sCT NPs particles varied between  $60\pm 6$  and  
394  $245\pm 32$  nm (Table 1). Generally, the incorporation of 0.5 or 1.0 mg/ml of sCT did not impact on  
395 the particle size, but in some cases (i.e. when the CHON/PROT MMR was 2.1 and CHON  
396 concentration was 0.7 or 1.4 mg/ml) loading of sCT increased the particle size compared to  
397 CHON/PROT NPs without sCT (by 50 or 90 nm, respectively). Also, incorporation of 0.5 mg/ml  
398 sCT resulted in a decrease in particle size of CHON/PROT MMR=6.4 NPs (0.7 mg/ml CHON) by  
399 30 nm. Formulations with higher CHON/PROT MMRs (4.2-13) were generally characterised by a  
400 very small particle size below or close to 100 nm, while NPs with CHON/PROT MMRs 2-3 usually

401 had the particle size of 150-250 nm (Table 1). sCT-loaded CHON/PROT NPs were characterised  
402 by moderate values of PDI (0.23-0.45) and a negative surface charge (Table 1).

403 Table 1 shows that CHON/PROT NPs had the sCT association efficiency (AE) values between  
404 98.4 and 99.8%. AEs were slightly higher for formulations containing lower amount of PROT and  
405 higher CHON/PROT MMRs. The differences, although statistically significant, were very small  
406 (less than 1.5%). Peptide loading varied between 13.5 and 37.8%.

### 407 **3.3 Colloidal behaviour of CHON/PROT/sCT NPs in various media**

408 The size of sCT-loaded CHON/PROT MMR=6.3 NPs depended on the composition of the  
409 continuous phase (Table 2). In diluted acetate buffer (diluted AB) or diluted PBS the particle size  
410 was not different compared to that in water, however a decrease in PDI and an increase in surface  
411 charge was observed. The increase in ionic strength of both media (AB with pH=5 and PBS with  
412 pH=7.4) resulted in an increase in particle size by approximately 50 and 120 nm, respectively, in  
413 comparison to the size in water. Even in PBS the hydrodynamic diameter of the particles was not  
414 significantly different from 250 nm. The increase in the ionic strength of the media also increased  
415 the zeta potential and the NPs became more homogeneously dispersed (i.e. PDI values  
416 decreased). The change in pH of the media (AB and PBS pH=5 versus pH=7.4) did not influence  
417 the properties of the NPs significantly (Table 2). The properties of NPs in serum-free medium  
418 (pH=7.4) were comparable to those in PBS. Moreover, the properties did not change within 24  
419 hours of storage. In the acidic medium (0.07M HCl) the particle size increased significantly to 230  
420 nm and, similarly to other media, no further change occurred within 24 hours. A decrease in pH  
421 (HCl solution) led to an increase in the zeta potential to -10 mV.

422 The influence of ionic strength of the properties of NPs was also examined in NaCl aqueous  
423 solutions. The particle size and zeta potential increased, while the PDI decreased, with an  
424 increase in the ionic strength of NaCl solutions.

### 425 **3.4 *In vitro* release of sCT**

426 The amount of sCT released after 24 hours into water or diluted acetate buffer from sCT-loaded

427 CHON/PROT MMR=6.3 NPs was below 5% (Fig. 3). In the other media (acetate buffer, PBS,  
428 diluted PBS and HCl solution) the release was greater, 4-6% of sCT was released after 1 hour  
429 and 10-12% after 2 hours, however no burst release was observed. In HCl solution and PBS  
430  $33\pm 0.1\%$  and  $35\pm 1.8\%$  of the initial amount of sCT was released after 24 hours, respectively. sCT  
431 release in acetate buffer was the same at both values of pH, 5 and 7.4, and a similar behaviour  
432 was observed in PBS (data not shown).

### 433 **3.5 Solid state characterisation**

434 Two amorphous halos, centred at around  $12^\circ$  and  $22^\circ$   $2\theta$ , were visible on each PXRD  
435 diffractograms for CHON and PROT (Fig. 4). The  $22^\circ$   $2\theta$  peak of PROT was more intense than  
436 that at  $12^\circ$   $2\theta$ , however intensity of the two peaks in CHON was comparable. This shows the  
437 amorphous nature of individual polyelectrolytes with the NP carriers (COM1-COM5) also being  
438 disordered. The sample COM6 (MMR=0.2, final CHON conc.=0.16 mg/ml) of the positively  
439 charged NPs showed Bragg peaks at  $11.6$ ,  $20.8$ ,  $29.1$ ,  $29.7$  and  $31.8^\circ$   $2\theta$ . In relation to the sCT-  
440 loaded samples subjected to dissolution studies, only the NPs in water were fully amorphous and  
441 had no single sharp diffraction peaks characteristic of an ordered structure. NPs recovered from  
442 PBS, diluted PBS and HCl solution all contained two crystalline peaks, at  $27.3$  and  $31.8^\circ$   $2\theta$ ,  
443 however the presence of an amorphous halo was apparent. The sample in acetate buffer was  
444 predominantly crystalline, while that in diluted acetate buffer had a faint Bragg peak at  $32.0^\circ$   $2\theta$ .  
445 Results of DSC studies are presented in Fig. 5 and insets show tables with data evaluation. PROT  
446 and COM6 showed a single glass transition ( $T_g$ ) event each, PROT at around  $185^\circ\text{C}$ , while the  
447  $T_g$  of COM6 was 10 degrees lower. CHON and COM1-COM5 showed broad endotherms of  
448 dehydration at  $97^\circ\text{C}$  (CHON) or  $50$ - $59^\circ\text{C}$  (COM1-COM5) followed by an exotherm with an onset  
449 around  $218$ - $232^\circ\text{C}$ . This exotherm had notably different enthalpies, of around  $138\text{ J/g}$  for CHON  
450 and  $39$ - $60\text{ J/g}$  for COM1-COM5. Thermograms of sCT-NPs after dissolution had different shapes  
451 depending on the medium used for the studies. The exotherm was still present in all of them,  
452 however its position and magnitude was dependent on the composition of the dissolution medium.

453 The onset temperatures varied between 210 and 232 °C and the peak enthalpies were between  
454 4 and 30 J/g.

455 ATR-FTIR spectra of CHON, PROT and COM1 (NPs with MMR=3.1, final CHON conc=0.7 mg/ml)  
456 with band assignments and principal peak positions are shown in Fig. 6. The assignment was  
457 done based on studies of Chen et al., (2005); Fajardo et al., (2012); Awotwe-Otoo et al. (2012)  
458 and Bonkovoski et al., (2014). In both polyions one of the most prominent vibrations is that of  
459 amide I. Despite being a sodium salt, the presence of protonated –COOH moiety in CHON was  
460 evident (as a shoulder at app. 1650 cm<sup>-1</sup> of the amide I band and as a group at 1411 cm<sup>-1</sup>).  
461 Symmetric and asymmetric –S=O vibrations were also clear, however they were more intense in  
462 CHON than in PROT. The spectrum of the complex had bands of both components, but position  
463 and intensity of some peaks, such as those of –COOH, amide I and amide II, was different.

### 464 **3.6 Cytotoxicity of CHON and CHON/PROT NPs**

465 CHON even at a concentration of 5 mg/ml did not exert any toxic effects on Caco-2 cells (125±10  
466 % of viable cells). PROT complexed to CHON was significantly less cytotoxic than the same  
467 amount of PROT dissolved in serum-free medium (Fig. 7). The cytotoxicity of PROT has been  
468 described by Umerska et al., (2014a) and its IC<sub>50</sub> was found to be 0.24±0.01 mg/ml. 83±5% of  
469 cells remained alive after 72 hours of exposure to CHON/PROT NPs containing 0.25 mg/ml of  
470 PROT, compared to 61±10% for solution containing an equivalent content of PROT. No cytotoxic  
471 effects were observed after first 1:1 v/v dilution of the NPs with the serum-free medium (98±5%  
472 of viable Caco-2 cells). At this concentration (0.125 mg/ml) of PROT a significant toxic effect was  
473 still observed for PROT dissolved in the medium.

## 474 **4. Discussion**

### 475 **4.1 CHON/PROT NPs: formation and formulation variables**

476 CHON/PROT NPs were spontaneously formed using mild fabrication conditions, by mixing  
477 aqueous solutions of polyelectrolytes at room temperature. Similar methods were used previously  
478 to obtain other polyelectrolyte complex NPs such as HA/chitosan (Umerska et al., 2012),

479 chondroitin/chitosan (Yeh et al., 2011, Place et al., 2014), HA/PROT (Umerska et al., 2014a) and  
480 HA/polyarginine (Oyarzun-Ampuero et al., 2011). The process of preparation of polyelectrolyte  
481 complex NPs described above does not involve the use of cross-linkers, organic solvents or  
482 surfactants and a homogenisation step is not needed. This is an advantage over the methods  
483 commonly used to fabricate NPs of polyesters, e.g. PLGA, which require the use of organic  
484 solvents, surfactants and sometimes high temperature or homogenisation (des Rieux et al.,  
485 2006).

486 One of the interesting characteristics of polyelectrolytes is their ability to produce stable  
487 interpolymer complexes between oppositely charged species (Dautzenberg, 2000). CHON has  
488 multiple negative charges at physiological pH coming from sulphate and carboxyl groups. Due to  
489 the high arginine content protamines are strongly basic and have isoelectric point around 12,  
490 therefore in all pH conditions tested in this work (pH between 1.2 and 7.4) PROT has a net positive  
491 charge and can be considered as a polycation. Polyion complexation was evident by ATR-FTIR.  
492 First of all, the band at app.  $1650\text{ cm}^{-1}$  of carbonyl moiety of CHON disappeared completely in the  
493 spectrum of complex, either due to complexation or overlap with the amide I group (Fig. 6). Amide  
494 I and II vibrations in CHON and PROT were seen to change positions indicating a change in  
495 intermolecular interactions. The band of  $\text{-S=O}$  stretching vibrations in the complex was less  
496 intense than in pure CHON, although it did not move. Electrostatic interactions between the  
497 sulphate groups of CHON and the amine moieties of chitosan were seen in the range of  $1150\text{--}$   
498  $1300\text{ cm}^{-1}$  by Kaur et al. (2010) and shifts in the sulphate absorptions for carrageenan/PROT  
499 complexes were noted by Dul et.al. (2015). On the other hand, skeletal vibrational modes ( $\text{-C-O-}$   
500  $\text{C-}$  and  $\text{-S-O-C-}$  groups) of CHON in the complex remained in the same positions inferring that  
501 CHON and PROT interact with each other electrostatically. In summary, CHON/PROT NPs are  
502 formed by electrostatic interactions between negatively charged sulphate and carboxylate groups  
503 of CHON and positively charged amino acid residues in arginine-rich PROT molecules.

504 It is commonly accepted that polyelectrolyte complex formation is mainly caused by strong

505 interactions between oppositely charged polyelectrolytes, whereby the gain in entropy due to the  
506 release of the low molecular counterions, initially bound to polyelectrolytes, plays a decisive part  
507 (Dautzenberg and Jaeger, 2002; Schatz et al., 2004). A similar mechanism (i.e. via electrostatic  
508 interactions) has already been confirmed to be responsible for formation of the complex NPs (e.g.  
509 Umerska et al., 2012, 2014a and 2014b).

510 Polyelectrolyte complex dispersions may have the appearance of a solution, an opalescent or  
511 turbid system or a two-phase system consisting of precipitated complex and supernatant liquid.  
512 Usually, there is a narrow window of physicochemical conditions where the complexes are formed  
513 and stay in the form of colloidal dispersion (de Kruif et al., 2004). The process of NP formation is  
514 best conducted in diluted solutions in order to prevent macroscopic gelation, which may lead to  
515 bulk hydrogels (Oh et al., 2009). In this work, the concentration of both polyelectrolytes in the final  
516 dispersion was below 1% w/v (0.1-0.7% w/v). HA nanocomplexes described previously were  
517 formed at comparable conditions (0.1-0.2% w/v) (Umerska et al., 2012, 2014a). It was possible  
518 to obtain CHON-based NPs at higher polyanion concentrations (i.e. between 0.3 and 0.7% w/v)  
519 than the HA-based NPs possibly due to the lower molecular weight of CHON (58.6 kDa) and  
520 consequently lower viscosity of its solutions. It was observed however, that at a higher  
521 concentration (i.e. 2.1 mg/ml or 3.6 mg/ml of CHON) the particles were markedly larger and were  
522 characterised by a broader size distribution compared to lower CHON concentrations (0.7 mg/ml  
523 or 1.4 mg/ml), possibly due to more frequent interparticulate interactions resulting from their  
524 increased numbers.

525 When both polyions are used in equivalent net charge amounts, the particle yield is maximal  
526 (Boddohi et al., 2009). On the other hand, it has been shown that, as a result of charge  
527 neutralisation, aggregation of polyelectrolyte complex NPs occurs and a phase separation takes  
528 place (Boddohi et al., 2009; Umerska et al., 2012). The formation of stable polyelectrolyte complex  
529 NPs requires that the constituent polyelectrolyte solutions are mixed in non-stoichiometric ratios  
530 (Boddohi and Kipper, 2010). The amount of CHON incorporated in NPs is presented in Figure 1S

531 and it is clear that the nanocomplexes are stabilised by the excess of the polyanion.

532 The stability of polyelectrolyte complex NPs depends on the repulsion between similarly charged  
533 particles. Consequently, the net charge of the system must be either sufficiently positive or  
534 negative if the system is to be physically stable. The polyanion/polycation MMRs, which produced  
535 phase separation, were lower for CHON/PROT systems, compared to HA/PROT polyelectrolyte  
536 complexes described previously (Umerska et al., 2014a) (polyanion/polycation MMRs of 1 and  
537 1.4-1.6, respectively). Therefore CHON is capable of neutralising more PROT in the form of  
538 colloidal dispersion compared to HA and/or this reaction is more efficient in CHON/PROT  
539 systems. This may be due to the difference in the density of acidic sites between both molecules.  
540 CHON has a low charge density and contains residues of weak and strong acid groups present  
541 alternately every two monosaccharide, while hyaluronic acid is a weak polyacid with a charge  
542 density much lower than CHON, since only one charge can be present every two residues in HA  
543 molecules (Denuziere et al., 1996).

544 The particle size increased with an increasing amount of PROT in the formulation (decreasing  
545 CHON/PROT MMR) due to neutralisation of CHON by the polycation and attainment of a more  
546 compact structure of NPs. A similar tendency was observed for HA/PROT NPs (Umerska et al.,  
547 2014a). The magnitude of size of CHON/PROT NPs (between 78 and 296 nm) was similar to  
548 CHON/polyethylenimine NPs (between 92 and 207 nm) (Pathak et al., 2009) and generally  
549 smaller than CHON/chitosan NPs (between 178 and 370 nm (Yeh et al., 2011) or between 230  
550 and 260 nm (Place et al., 2014)) or N,N,N- trimethylchitosan (between 380 and 540 nm (Place et  
551 al., 2014)). Yeh et al., (2011) also observed that at a fixed MMR (of CHON and chitosan) the  
552 particle size increased with increasing polymer concentration, which is in agreement with our  
553 observations.

554 Zeta potential magnitude is a surrogate marker for the colloidal stability of NPs. The charge  
555 develops as a function of the excess polymer and is controlled by an ordered polyelectrolyte  
556 complex assembly process (Hartig et al., 2007). The negative zeta potential of these NPs is similar

557 to those obtained for HA/PROT NPs (Umerska et al., 2014a). NPs with positive zeta potential had  
558 very poor colloidal stability and thus were not regarded as suitable nanocarriers.

559 CHON/PROT NPs and HA/PROT NPs (Umerska et al., 2014a) differ from chitosan polyelectrolyte  
560 complexes, e.g. HA/chitosan (Boddohi et al., 2009, Umerska et al., 2012), heparin/chitosan  
561 (Boddohi et al., 2009) and chitosan/dextran (Schatz et al., 2004), because the hydrodynamic  
562 diameter of chitosan-based NPs decreases during titration with an oppositely charged  
563 polyelectrolyte, until a secondary aggregation was obtained, preceding irreversible flocculation.

564 The semi-crystalline nature of the positively charged NPs (COM6, MMR=0.2, final CHON  
565 conc.=0.16 mg/ml) was surprising, considering that the starting polyelectrolyte compounds and  
566 negatively charged NPs were PXRD amorphous (Fig. 4). The peak at  $31.8^\circ 2\theta$  can possibly be  
567 ascribed to sodium chloride, however the rest of Bragg peaks did not match the positions of  
568 diffraction peaks of sodium sulphate, anhydrous or hydrates. Sodium sulphate is expected to be  
569 formed as CHON is used as a sodium salt, while PROT as a sulphate salt. Published reports  
570 indicate that polyelectrolyte complexes could be either amorphous, such as chitosan/CHON  
571 (Denuziere et al., 1996), chitosan/HA (Denuziere et al., 1996), poly[(2-dimethylamino) ethyl  
572 methacrylate]/CHON (Bonkovoski et al., 2014) or crystalline, for instance chitosan/quinoa protein  
573 (Abugoch et al., 2011) and pectin-NH<sub>2</sub>/CHON (Fajardo et al., 2012).

574 The thermogram of CHON (Fig. 5) was consistent with that published previously by Jo et al.  
575 (2012). Bonkovoski et al. (2014) stated that CHON thermally degrades around 230 °C, thus the  
576 exothermic event by DSC is of decomposition. CHON complexation to PROT resulted in a change  
577 in hydration properties of the complexes, as evidenced by the shift of the peak of dehydration  
578 endotherm from app. 97 °C to 50-59 °C for the negatively charged NPs and it was absent for  
579 COM6 (positively charged NPs). Furthermore, COM6 had a visible Tg event, appearing at a lower  
580 temperature than that of pure PROT. Complexation of PROT to CHON resulted in a significant  
581 decrease of enthalpy of the exothermic transition, which can be due to the changing strength of  
582 interactions in the complex. A similar observation was made by Wen et al. (2012) for dermatan



583 sulphate/alginate polyelectrolyte complexes. Nevertheless, no correlation was discerned between  
584 the thermal parameters of the carriers and formulation variables such as MMR or CHON  
585 concentration.

586 The poor physical stability of positively charged NPs was evident from kinetic plots (Fig. 2). As  
587 previously presented, this issue with stability was also seen for HA/PROT NPs (Umerska et al.,  
588 2014a) and attributed to a large difference in the molecular weight of both components (~35-fold).  
589 Boddohi et al. (2009) stated that if the polyion in excess (PROT) has a much lower molecular  
590 weight than the other polymer (HA in the above example), poor colloidal stability of the NPs is  
591 observed. As the difference in the molecular weight of PROT and CHON is approximately 10-fold,  
592 the same factor might be responsible for the physical instability of the positivity charged NPs.  
593 Moreover, PXRD and DSC analyses of these NPs (COM6) indicated a semi-crystalline structure  
594 of the polyelectrolyte complex formed, in contrast to the amorphous nature of the negatively  
595 charged NPs. Therefore the factors compromising further the colloidal stability is the lowering of  
596 surface energy and a decrease in surface area triggered by repulsion of water from the crystalline  
597 segments (Caldwell et al., 2010).

#### 598 **4.2 Formation and characterisation of sCT-loaded CHON/PROT NPs**

599 The charge of polyelectrolytes is the most important factor for their self-assembly in NPs. The  
600 protein and polymers must bear opposite charge in order to form polyelectrolyte complexes  
601 (Cegnar and Kerč, 2010). The results show that using high concentrations of PROT and low  
602 CHON/PROT MMRs is not advised if very small NPs below 100 nm are to be obtained.

603 The high association efficiency and drug loading are in agreement with the results obtained for  
604 sCT-loaded polyelectrolyte complex NPs described previously (Umerska et al., 2014a and  
605 Umerska et al., 2014b). Polyelectrolyte complexes have the ability to undergo reactions of  
606 polyelectrolyte substitution and exchange (Kabanov and Kabanov, 1995). Indeed, it has been  
607 suggested that in ternary HA/PROT/sCT complexes the competition occurs between positively  
608 charged PROT and sCT molecules for binding with negatively charged carboxylic groups of HA

609 (Umerska et al., 2014b) and the interaction between PROT and HA has been suggested to be  
610 stronger than between sCT and HA. A decrease in AE values from 98 to 85% was observed when  
611 the HA/PROT MMR decreased from 6 to 2 consistent with substitution of small fraction of sCT by  
612 PROT. In similar conditions in CHON/PROT NPs the decrease in AE values, although statistically  
613 significant ( $p=0.0274$ ), was much smaller ( $99.7\pm0.1$  and  $98.7\pm0.5\%$  for CHON/PROT MMRs of 6  
614 and 2, respectively). Moreover, binding of sCT by CHON/PROT NPs was significantly higher than  
615 by HA/PROT NPs. Also, the incorporation of sCT into HA/PROT NPs had a more pronounced  
616 impact on their properties (especially the zeta potential) compared to CHON/PROT NPs. This  
617 behaviour may be attributed to the higher charge density of CHON compared to HA.

#### 618 **4.3 The influence of the dispersant on the properties of CHON/PROT/sCT NPs**

619 The properties of polyelectrolyte complex NPs depend on many parameters, also on the  
620 properties of the dispersant such as its ionic strength and pH (Boddohi et al., 2009). It can be  
621 expected that the properties of polyelectrolyte complexes can change depending on the medium,  
622 as polyelectrolyte complexes are self-assembling objects thermodynamically stable under certain  
623 conditions of medium composition, pH and ionic strength (Kabanov and Kabanov, 1995).

624 The values of zeta potential in all slightly acidic or neutral media were negative and close to -30  
625 mV, therefore indicating a good colloidal stability of the NPs (Table 2). Even at acidic pH of 1.2,  
626 which could be encountered e.g. in stomach, the particles maintained the negative charge. The  
627 decrease of zeta potential from -47 mV in water to -10 mV in HCl solution suggests that the  
628 Coulomb interactions between positively charged sCT and negatively charged CHON become  
629 weaker, but are still present. Indeed, the *in vitro* release studies showed that more than 65% of  
630 sCT was associated with the particles after 24 hours of incubation at acidic pH.

631 It is known that two different effects govern the behaviour of polyelectrolyte complexes after a  
632 subsequent change of the ionic strength of the medium, namely secondary aggregation or  
633 dissolution, depending on the nature of the components of the system (Dautzenberg, 2000). In  
634 CHON/PROT NPs a significant increase in particle size was observed, especially in NaCl solution

635 and in PBS (where NaCl is the main component). This is similar to the results obtained by  
636 Oyarzun-Ampuero et al., (2009), who observed an increase in the particle size of heparin-loaded  
637 chitosan/HA NPs after contact of the NPs with PBS or Hank's Balanced Salt Solution (HBSS)  
638 compared to the particle size in water. However, partial dissolution was observed in PBS for  
639 HA/PROT NPs described previously (Umerska et al., 2014a).

640 The interactions between oppositely charged macromolecules depend on the strength of their  
641 acidic or basic groups, their charge density and the degree of neutralisation (Denuziere et al.,  
642 1996). It is known that weak complexes are formed between polyelectrolytes containing weak  
643 acidic and basic groups, while oppositely charged polymers containing anions and cations of  
644 strong acids and bases generally form very strong polyelectrolyte complexes (Denuziere et al.,  
645 1996). CHON, in contrast to HA, also contains strong acid groups (sulphates) and therefore forms  
646 stronger complexes than HA. It is likely that molecules of the dispersant can penetrate the network  
647 of weak nanoplexes easier. This phenomenon appears to dominate in diluted HA/PROT NPs  
648 leading to their dissolution (Umerska et al., 2014a). In stronger and more compact CHON/PROT  
649 complexes the dissociation of the complex as a result of screening of the charges by salt is lower  
650 and the secondary aggregation predominates, which can be observed as the increase in the  
651 particle size or even in the separation of the phases (Table 2).

652 Interestingly, chloride anions (present both in NaCl solution and in PBS) appeared to exert a  
653 stronger effect on the properties (especially the particle size) of the CHON/PROT/sCT NPs than  
654 acetate. CHON/PROT/sCT NPs dispersed in acetate buffer with the ionic strength of 0.1M were  
655 characterised by significantly smaller particle size compared to NaCl solution with the ionic  
656 strength of 0.075M (150 nm and 210 nm, respectively). It may be due to the fact that in the  
657 Hofmeister series acetate anion is more kosmotropic compared to more chaotropic chloride anion.  
658 The kosmotropes, which are able to induce order by creating hydrogen bonding throughout water,  
659 are hydrated ions and exert stabilising and salting-out effects on proteins and macromolecules.  
660 On the other hand, chaotrops are able to disrupt water structure, destabilise folded proteins and

661 give rise to salting-in behaviour (Zhang and Cremer, 2006). Alternatively, the decrease in the  
662 hydrodynamic radius of NPs induced by the acetate ions can be through a salting-out effect as  
663 the CHON chains on the exterior of NPs will find themselves in a poorer solvent, while the increase  
664 in the size caused by NaCl can be explained by a partial dissociation and/or dissolution of the  
665 polyelectrolyte complexes.

#### 666 **4.4 *In vitro* release of sCT**

667 The kinetics of peptide or protein release from ionic hydrogels depends on various structural  
668 parameters of the protein and polyelectrolyte as well as on the environmental conditions inside  
669 the hydrogel (Cooper et al., 2005). The release data (Fig. 3) was fitted to the first order equation,  
670 as no burst phase was observed and confirmed when the data was fitted to the Gallagher-  
671 Corrigan model (Gallagher and Corrigan, 2000), and the parameter estimates obtained and  
672 related statistics are summarised in Table 3.

673 The release rate constant ( $k$ ) was greatest for the NPs in the acetate buffer but the greatest  
674 amount of sCT released at infinity ( $W_{\infty}$ ) was estimated with PBS. The values of release rate  
675 constant ( $k$ ) of sCT from CHON/PROT NPs were markedly lower than those of HA-based NPs  
676 (Umerska et al., 2014a) and the  $k$  parameter was approximately a 4-fold lower for CHON/PROT  
677 NPs in PBS. Therefore the peptide/NP complex is stronger in CHON-based NPs compared to  
678 HA-based NPs, which is also supported by lower amounts of sCT released from CHON/PROT  
679 NPs. This is in agreement with outcomes of studies of Kamiya and Klibanov (2003) on lysozyme  
680 release from its water-insoluble complexes.

681 A good linear correlation between the ionic strength of release medium, but not pH, and sCT  
682 released after 24 h ( $R^2=0.83$ ,  $p=0.007$ ) and the particle size of NPs in the media ( $R^2=0.89$ ,  
683  $p=0.003$ ) was found. However, the release rate ( $k$ ) was not found to be reliant on the ionic strength  
684 of medium. Chen et al., (2007), who examined the release of bovine serum albumin (BSA) and  
685 R6G (Rhodamine 6G) from chitosan/dextran sulphate NPs in PBS with different ionic strength  
686 also observed that the greatest release of both proteins occurred in the release media of a high

687 ionic strength. Sustained release of BSA and doxorubicin from CHON/chitosan polyelectrolyte  
688 NPs was reported by Yeh et al (2011) and Tsai et al. (2011), respectively, with evidence that BSA  
689 release properties were pH sensitive. Release of sCT from CHON/PROT MMR=4.2 NPs was  
690 comparable to those from CHON/PROT MMR=6.3 (data not shown), it may be concluded that  
691 release of the loaded bioactive from CHON-based NPs is influenced mainly by the peptide  
692 interactions with the polyanion (CHON) and not with the polycation.

693 As the sCT-loaded sample subjected to dissolution studies in water was fully amorphous and  
694 retained a majority of the loaded sCT, it can be concluded, based on PXRD and DSC data, that  
695 the peptide was molecularly dispersed within the polyelectrolyte complex. NPs recovered from  
696 PBS, diluted PBS and HCl solution had two crystalline peaks characteristic of sodium chloride.  
697 This was expected as PBS contains sodium chloride and in the HCl medium this salt can be  
698 formed from the sodium ion originating from CHON. NPs after dissolution in acetate buffer retain  
699 the buffer salts, showing as anhydrous sodium acetate (Onwudili and Williams, 2010) however,  
700 the sample from diluted acetate buffer was practically fully amorphous (with a very weak peak  
701  $32.0^\circ$   $2\theta$  of either sodium chloride or sodium acetate). Interesting information in relation to the  
702 sCT NPs was obtained from the thermal analysis studies. While the shape of the thermograms  
703 was strongly dependent on the type of medium used, the decomposition exotherm was still  
704 present in the samples (Fig. 5). Upon incorporation of the peptide, the enthalpy further decreased.  
705 Observing that the enthalpy of the exotherm changed when different dissolution media were used,  
706 an attempt was made to correlate this parameter with the amount of sCT remaining in NPs after  
707 6h of dissolution studies. This relationship was found to be statistically significant ( $p < 0.008$ ,  
708  $R^2 = 0.827$ ) and could be described by this linear equation (Eqn. 4):

709

710 *amount of sCT released from NPs* ( $\mu\text{g}$ ) =  $-2.65 * \text{enthalpy of exothermic peak} + 82.7$  (Eqn. 4)

711

712 This implies that thermal analysis can be a very useful tool when assessing the amount of actives

713 released from polyelectrolyte complexes and indeed the thermal decomposition peak might reflect  
714 the strength of intermolecular interactions in such complexes. The amount of sCT not released  
715 from NPs under the conditions of the *in vitro* experiment indeed remained strongly complexed to  
716 CHON.

#### 717 **4.5 Cytotoxicity of CHON and CHON/PROT NPs**

718 The cytotoxicity of CHON and NPs was examined in Caco-2 cells to assess any potential toxic  
719 interactions with cells. The toxicity of the PROT in Caco-2 cells has already been reported by our  
720 group (Umerska et al., 2014a). The positive charge on the cationic polymers is believed to be a  
721 major cause of their cellular toxicity. These polymers were found to destabilise and ultimately  
722 rupture the cell membrane as a result of strong electrostatic interaction (Pathak et al., 2009). The  
723 neutralisation of PROT by the formation of nanocomplex with HA was shown to reduce the toxicity  
724 of PROT (Umerska et al., 2014a). The toxic effects observed were attributed to the free PROT  
725 rather than by the PROT bound to the polyanion, as the presence of low molecular weight ions in  
726 the medium led to the dissociation of a subset of the HA/PROT nanocomplexes, resulting in  
727 release of PROT molecules. CHON/PROT NPs appear to be less toxic than HA/PROT NPs. The  
728 protective effect of polyanion in CHON/PROT NPs was observed in NPs containing 0.25 mg/ml  
729 PROT, while in HA/PROT NPs the protective effect of HA was observed only at lower  
730 concentration. Because CHON forms stronger electrostatic complexes with PROT than HA, the  
731 amount of PROT released from CHON/PROT complexes is expected to be smaller compared to  
732 HA/PROT complexes, and therefore CHON/PROT exerted less significant toxic effects.  
733 Complexation to CHON has also been reported to significantly decrease the cytotoxicity of  
734 polyethylenimine in HepG2 and HeLa cells (Pathak et al., 2009).

#### 735 **5. Summary and conclusions**

736 A new type of a nanocarrier composed of CHON and PROT has been successfully developed  
737 and characterised. The formation and properties of CHON/PROT NPs depended mainly on the  
738 MMR of polyion components, but also on their concentration. By modulation of formulation

739 conditions it was possible to obtain small (100 nm and less) NPs with a narrow size distribution  
740 (PDI below 0.25). sCT was very efficiently (approximately 100%) associated with the NPs and  
741 good peptide loading (14-38%) was achieved. Some of the sCT-loaded CHON/PROT NPs were  
742 as small as 60 nm. CHON/PROT NPs were capable of providing extended release of sCT.  
743 CHON/PROT NPs maintained the negative surface charge and more than 65% of sCT was still  
744 associated with the particles after 24 hours of incubation at acidic pH values (pH=1.2). Ionic  
745 strength and composition of the medium were found to have a significant influence on the release  
746 of sCT from CHON/PROT NPs. PXRD was found to provide an insight into the solid state  
747 properties of the nanoplexes and revealed a semi-crystalline nature of the positively charged  
748 system that was linked to its poor colloidal stability. The effectiveness of thermal techniques in  
749 assessing the amount of actives released from polyelectrolyte complexes was demonstrated. As  
750 complexation to CHON markedly reduced the toxic effects exerted on cells by PROT,  
751 CHON/PROT NPs were found to be compatible and well tolerated by Caco-2 cells showing a  
752 considerable promise of these NPs in clinical applications.

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759

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- 907 Zhang, Y, Cremer, P.S., Interactions between macromolecules and ions: the Hofmeister series.  
908 *Current Opinion in Chemical Biology* 2006; 10: 658-663

- 1 Table 1. Composition and properties association efficiency (AE) and sCT loading of sCT-loaded
- 2 CHON/PROT formulations tested. TR - transmittance, MPS – mean particle size, PDI -
- 3 polydispersity index, ZP -zeta potential.

Initial CHON conc. (mg/ml)	CHON/PROT MMR	CHON incorporated in NPs (%)	Initial sCT conc. (mg/ml)	TR (%)	MPS (nm)	PDI	ZP (mV)	AE (%)	sCT loading (%)
0.7	2.1	89±8	0.5	71±5	144±10	0.42±0.16	-32.6±3.7	98.7±0.5	32.0±0.15
0.7	6.4	73±5	0.5	97±2	60±6	0.42±0.03	-42.5±3.8	99.7±0.1	37.8±0.04
1.4	2.1	92±4	0.5	43±1	195±14	0.35±0.09	-40.0±3.9	98.8±0.3	18.9±0.06
1.4	4.2	83±3	0.5	92±1	87±6	0.35±0.07	-42.6±12.6	99.6±0.1	22.0±0.02
1.4	13.0	72±4	0.5	98±1	76±14	0.45±0.04	-44.9±11.8	99.8±0.1	24.5±0.02
1.4	2.1	95±2	1.0	22±9	168±10	0.27±0.03	-31.3±1.8	98.4±0.3	31.7±0.08
1.4	4.2	85±4	1.0	83±6	87±4	0.23±0.01	-37.7±1.0	99.5±0.1	36.0±0.03
2.1	2.1	95±2	0.5	15±3	224±13	0.30±0.01	-46.6±1.3	98.7±0.3	13.5±0.03
2.1	3.1	90±3	0.5	68±2	154±5	0.29±0.01	-48.6±7.2	99.6±0.1	15.0±0.02
2.1	6.3	83±3	0.5	92±1	95±7	0.30±0.02	-47.0±9.2	99.8±0.1	16.8±0.02
2.1	2.1	95±3	1.0	8±1	245±32	0.45±0.18	-39.6±2.5	98.4±0.1	23.7±0.02
2.1	3.1	91±2	1.0	48±7	161±12	0.28±0.01	-43.2±2.3	99.5±0.1	26.0±0.02
2.1	6.3	83±3	1.0	85±2	100±9	0.24±0.01	-47.3±5.2	99.7±0.1	28.7±0.02

4

5 Table 2. Properties of sCT-loaded CHON/PROT NPs (CHON conc. 2.1 mg/ml, sCT conc. 1 mg/ml,  
6 CHON/PROT MMR=6.3): mean particle size (MPS), polydispersity index (PDI) and zeta potential  
7 (ZP) in different media. The samples were prepared as described in Section 2.4.2 and measured  
8 after 1 hour of incubation at 37 °C. AB - acetate buffer, PBS - phosphate buffer saline, HCl -  
9 hydrochloric acid solution pH=1.2, MEM - Eagle's Minimal Essential Medium.

Medium	MPS (nm)	PDI	ZP (mV)
Water	100±9	0.24±0.01	-47.3±5.2
PBS, pH=7.4	219±17	0.09±0.04	-24.3±4.1
PBS, pH=5	213±11	0.08±0.01	-22.5±1.8
PBS, pH=7.4; 1:10 dil.	116±11	0.16±0.02	-33.7±2.2
AB, pH=5	149±13	0.06±0.03	-29.0±1.0
AB, pH=7.4	142±9	0.10±0.03	-27.8±1.5
AB, pH=5; 1:10 dil.	102±11	0.14±0.05	-32.9±5.1
HCl, pH=1.2	229±26	0.10±0.05	-10.2±1.1
MEM, pH=7.4	222±7	0.06±0.03	-24.4±1.5
1.8% NaCl	295±6	0.08±0.02	-19.8±1.2
0.9% NaCl	242±9	0.08±0.02	-18.4±1.5
0.45% NaCl	210±5	0.10±0.02	-23.1±2.1
0.23% NaCl	172±5	0.17±0.04	-22.8±2.3
0.09% NaCl	111±6	0.18±0.04	-28.9±1.5

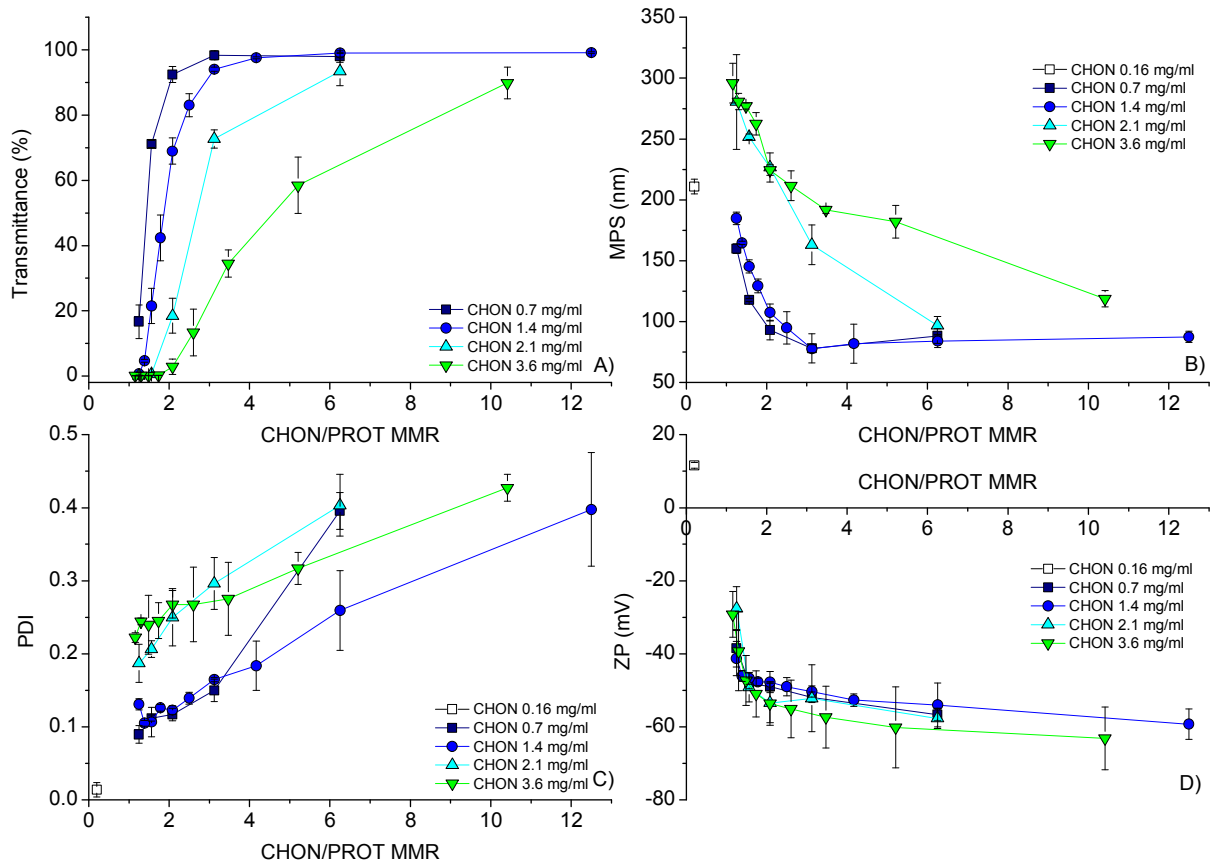
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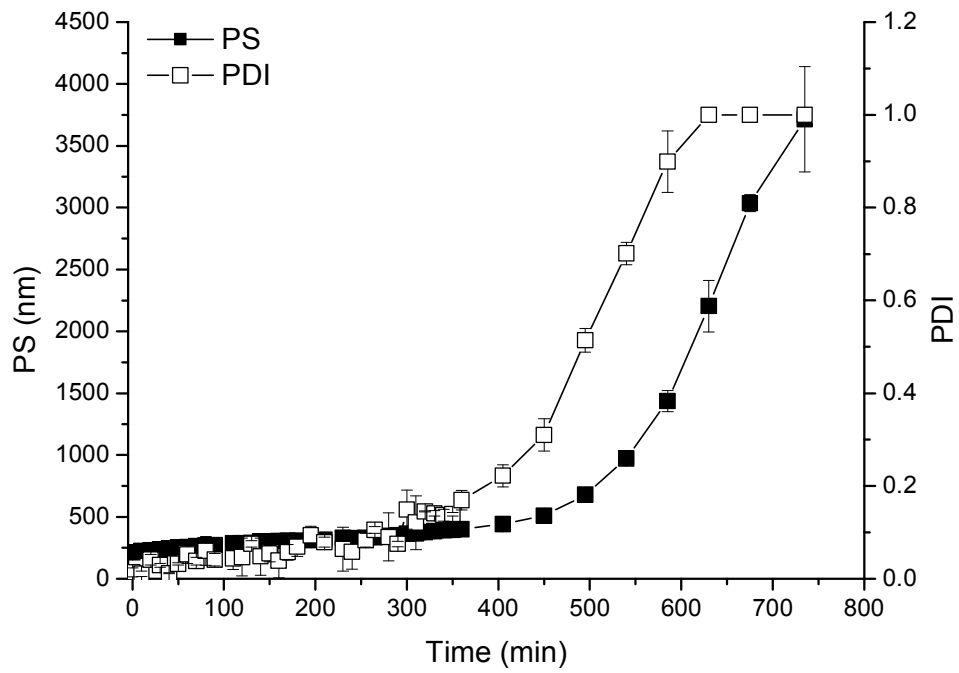
11 Table 3. Model parameter estimates for sCT release data fitted to the first-order model (Eqn. 3,  
 12 Section 2.4.2), where  $W_{\infty}$  is the amount of sCT released at infinity and  $k$  is the release rate  
 13 constant. The composition of the media is shown in Section 2.4.2. AB - acetate buffer, PBS -  
 14 phosphate buffer saline, IS – ionic strength.

Medium	$k$ ( $h^{-1}$ )	$W_{\infty}$ ( $\mu\text{g}/\text{mg}$ of NPs)	Goodness of fit ( $R^2$ )
Water, IS=0.000	0.124±0.054	12.6±4.6	0.9859
HCl, pH=1.2, IS=0.070	0.221±0.011	94.4±0.8	0.9965
AB, pH=5, IS=0.070	0.389±0.012	54.3±11.6	0.9962
AB, pH=5; 1:10 dil., IS=0.007	0.088±0.105	11.6±5.3	0.9972
PBS, pH=7.4, IS=0.140	0.201±0.030	100.2±3.7	0.9961
PBS, pH=7.4; 1:10 dil., IS=0.014	0.250±0.001	72.9±0.8	0.9908

15

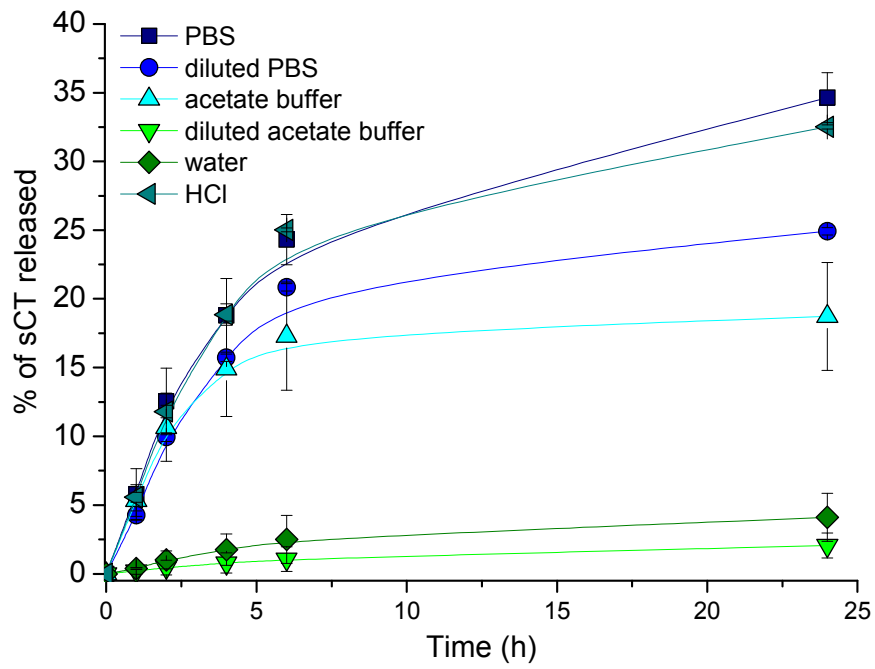


1  
 2 Figure 1. Properties of CHON/PROT NPs: A) transmittance, B) mean particle size (MPS), C)  
 3 polydispersity index (PDI) and D) zeta potential (ZP). Conditions of analyses are presented in  
 4 Sections 2.3.1 and 2.3.3. Lines are for visual guide only.



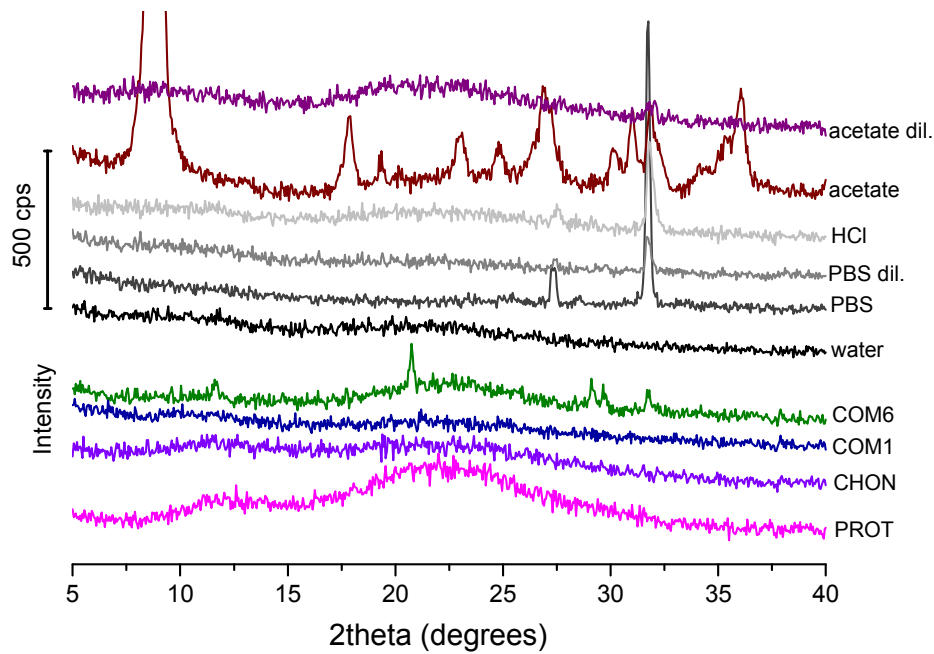
5

6 Figure 2. Kinetics of change in particle size (PS, filled squares) and polydispersity index (PDI,  
 7 empty squares) of positively charged CHON/PROT NPs (PROT of 0.7 mg/ml, CHON of 0.16  
 8 mg/ml).



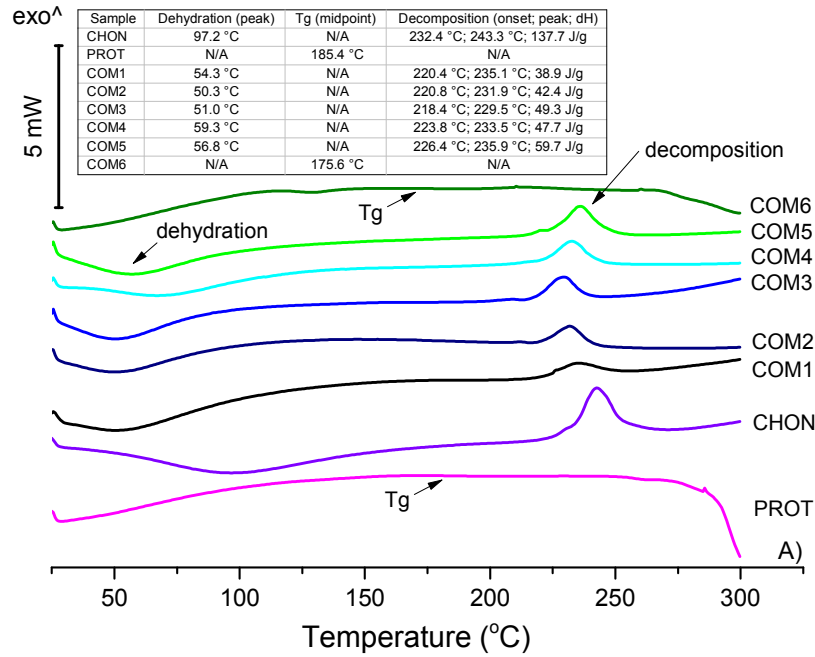
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10 Figure 3. Cumulative release profiles of sCT from sCT-loaded CHON/PROT NPs (CHON conc.  
 11 2.1 mg/ml, sCT conc. 1 mg/ml, CHON/PROT MMR=6.3) into different media. The experiments  
 12 were carried out at 37 °C. The composition of the media is shown in Section 2.4.2.

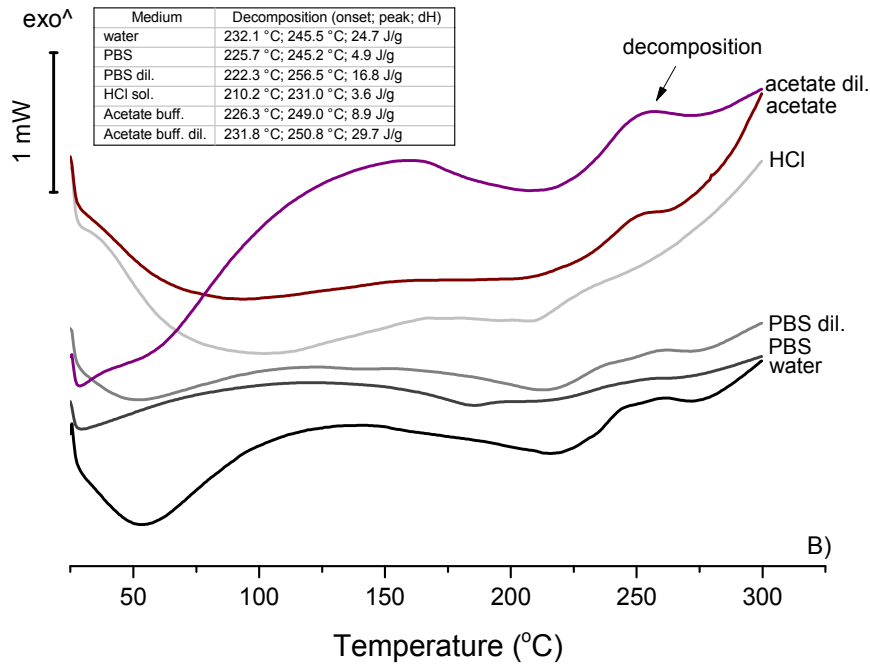


13

14 Figure 4. PXR D patterns of CHON, PROT, CHON/PROT NPs (COM1: MMR=3.1, final CHON  
 15 conc.=0.7 mg/ml; COM6: MMR=0.2, final CHON conc.=0.16 mg/ml) as well as sCT-loaded NPs  
 16 (CHON conc. 2.1 mg/ml, sCT conc. 1 mg/ml, CHON/PROT MMR=6.3) recovered after 6h of  
 17 dissolution studies in various media: deionised water, PBS pH=7.4, diluted (1:10 v/v) PBS, HCl  
 18 sol. pH=2, acetate buffer pH=5 and diluted (1:10 v/v) acetate buffer pH=5.



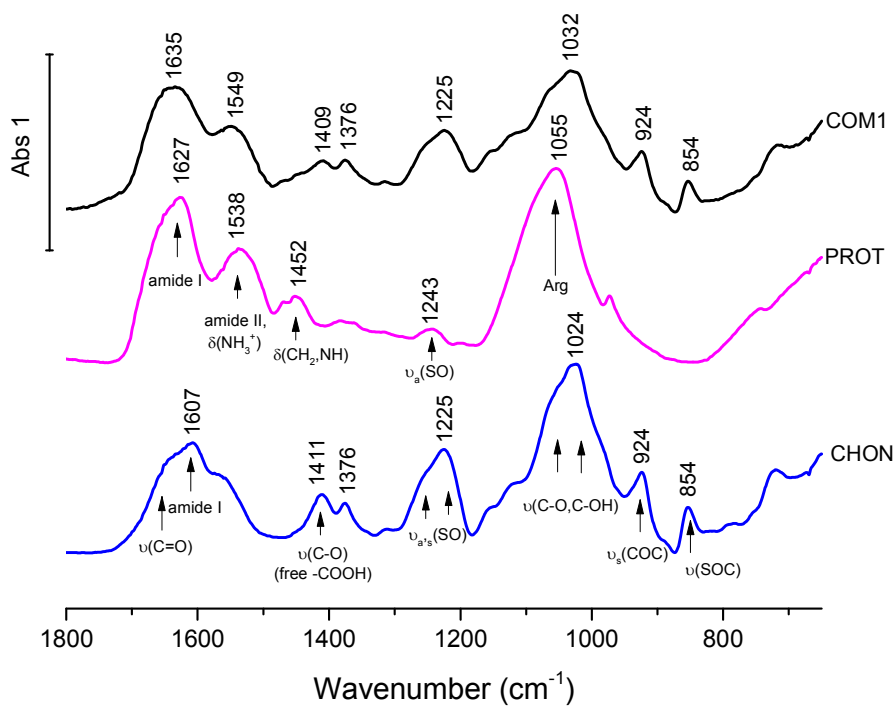
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20

21 Figure 5. DSC thermograms of A): CHON, PROT and CHON/PROT NPs (COM1: MMR=3.1, final  
 22 CHON conc.=0.7 mg/ml; COM2: MMR=3.1, final CHON conc.=1.4 mg/ml; COM3: MMR=12.5,  
 23 final CHON conc.=1.4 mg/ml; COM4: MMR=3.1, final CHON conc.=2.1 mg/ml; COM5: MMR=5,  
 24 final CHON conc.=3.6 mg/ml; COM6: MMR=0.2, final CHON conc.=0.16 mg/ml), B) sCT-loaded  
 25 NPs (CHON conc. 2.1 mg/ml, sCT conc. 1 mg/ml, CHON/PROT MMR=6.3) recovered after 6h of

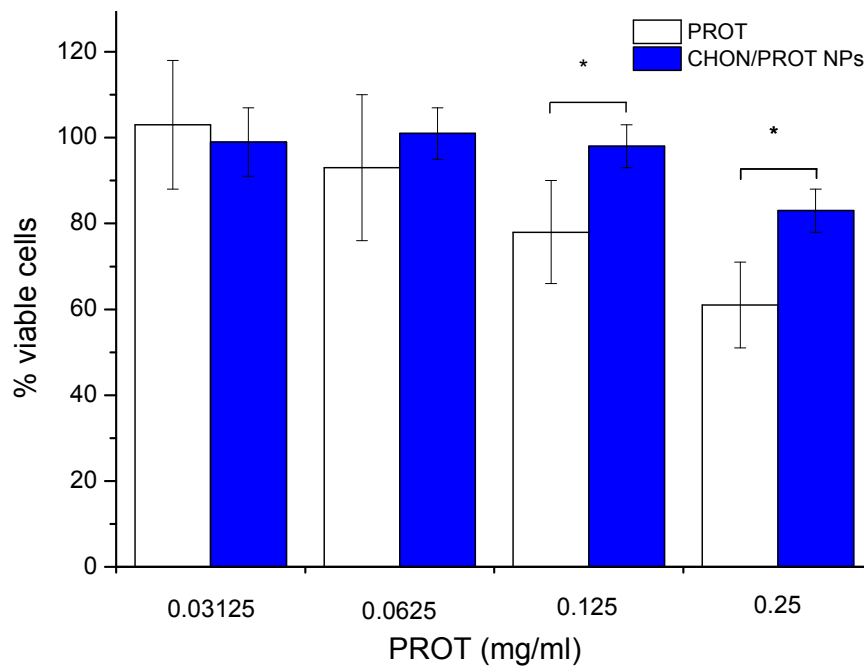
26 dissolution studies in various media: deionised water, PBS pH=7.4, diluted (1:10 v/v) PBS, HCl  
27 sol. pH=2, acetate buffer pH=5 and diluted (1:10 v/v) acetate buffer pH=5. Tables present data  
28 evaluation: Tg - glass transition, dH – enthalpy of process.



29

30 Figure 6. FTIR analysis of CHON, PROT and COM1 (NPs with MMR=3.1, final CHON conc.=0.7  
 31 mg/ml).  $\nu$  – stretching,  $\nu_{s,a}$  – symmetric and asymmetric stretching,  $\nu_s$  – symmetric stretching,  $\nu_a$  –  
 32 asymmetric stretching and  $\delta$  – bending vibrations. Arg – arginine. Numbers above peaks indicate  
 33 the position of principal bands.





34

35 Figure 7. Viability of Caco-2 cells as measured by MTS assay after 72 h of exposure to  
 36 CHON/PROT NPs (CHON/PROT MMR=3.1) and PROT (mean  $\pm$  S.D., n=3). Statistical analysis:

37 \* $p < 0.05$ .