Design of chondroitin sulfate-based polyelectrolyte nanoplexes: formation of nanocarriers with chitosan and a case study of salmon calcitonin

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Abstract

The aim of this work was to examine the formation and properties of chondroitin sulfate (CHON)-based nanoparticles (NPs), namely CHON/chitosan (CHIT), CHON/CHIT/calcitonin (sCT) and CHON/sCT. Both, positively and negatively charged CHON/CHIT NPs have been successfully obtained with properties that were dependent on the polymer mixing ratio, polymer concentration and molecular weight of CHIT. sCT was successfully loaded into CHON/CHIT NPs with efficiency close to 100% and notably high loading (up to 33%). A new type of NPs composed of CHON and sCT (a binary system) has been successfully developed. CHON/sCT NPs offer the advantage of a very high drug loading up to 73%. The particle size of CHON-based NPs increased in PBS, acetate buffer and in HCl solution compared to that in water, but most of them remained in the nano-range even after 24 hours. The media and composition of the nanocarriers were found to affect the release of sCT.

Keywords

Chondroitin sulfate ; chitosan ; nanoparticles ; polyelectrolyte complexes ; calcitonin ; peptide

1. Introduction

Traditionally, pharmaceutical excipients are treated as “inert” materials and are not expected to have pharmacological activity (Baldrick, 2010), however this view has changed as newly approved excipients cover a range of functions from stabilizing formulations to active roles of enhanced drug uptake and specific drug delivery (Goole et al., 2010). Pharmaceutical polymers bearing a charge, polyelectrolytes, have been extensively studied as components of micro- and nano-sized carriers for the delivery of a range of therapeutic molecules such as peptides and nucleic acids. Among polyelectrolytes, cationic and anionic polysaccharides have received particular attention and some of them also have interesting pharmacological properties. For instance hyaluronic acid (HA) has been shown to act in synergy with salmon calcitonin (sCT) reducing inflammatory biomarkers *in vitro* and inflammatory arthritis *in vivo* (Ryan et al., 2013). Another glycosaminoglycan, chondroitin sulfate (CHON) has been used for the preparation of nanocarriers for drug/gene delivery (Zhao, Liu, Wang & Zhai, 2015). CHON is an unbranched polysaccharide containing two alternating monosaccharides: D-glucuronic acid and N-acetyl-D-galactosamine. It is an abundant glycosaminoglycan found in cartilage, bone and connective mammalian tissue. CHON is a symptomatic slow-acting agent for osteoarthritis, commonly sold together with glucosamine. It has been shown to be absorbed after oral administration in humans as a high molecular weight polysaccharide (Volpi, 2002), therefore it has a potential to be used to increase the absorption of encapsulated molecules. Coating of chitosan (CHIT) NPs with CHON increased the uptake of the encapsulated nucleic acid by COS7 cells (transformed African green monkey kidney fibroblasts) via interaction of CHON with CD44 receptors (Hagiwara, Nakata, Koyama & Sato, 2012).

CHON has weak, carboxylate, and strong, sulfate, moieties attached to the main glycan backbone. Due to its acidic nature CHON is able to produce ionic complexes with cationic molecules (Denuziere, Ferrier & Domard, 1996). Examples of such complexes include CHON complexes with protamine (PROT) (Umerska et al., 2015), lysozyme (van Damme, Moss, Murphy & Preston 1994), trimethylchitosan (Place, Sekyi & Kipper, 2014),

however most of the polyelectrolyte complexes of CHON tested thus far are those with CHIT (Yeh, Cheng, Hu, Huang & Young, 2011; Tsai et al., 2011; Santo, Gomes, Mano & Reis, 2012; Place et al., 2014). CHIT is a linear polysaccharide composed of randomly distributed D-glucosamine (deacetylated unit) and N-acetyl-D-glucosamine (acetylated unit) linked via β-(1→4)-glycosidic bonds. The properties of CHIT can be useful in medicine, as it reduces bleeding (Pusateri et al., 2003) and has antibacterial activity (Benhabiles et al., 2012). Due to its mucoadhesive properties it could be a valuable component of drug delivery systems (Sogias, Williams & Khutoryanskiy, 2008).

Further research on NPs containing CHON and CHIT is required, as to the best of our knowledge no systematic investigation on the formation of both, positively and negatively charged CHON/CHIT NPs, has been published to date. The charge is of a key importance in cellular uptake and cytotoxicity of medical NPs (Fröhlich, 2012). For instance it has been shown that positively charged HA/CHIT NPs exerted toxic effects on Caco-2 cells in contrast to negatively charged NPs (Umerska et al., 2012). Initially the studies on CHON nanocarriers focused on the employment of CHON as an agent to yield positively charged CHIT NPs intended for the encapsulation of molecules like FITC-BSA (Yeh et al., 2011), BSA (Santo et al., 2012), doxorubicin (Hu et al., 2014), Nell-1 protein (Hou, Hu, Park & Lee, 2012). The NPs obtained in those studies were characterized by a positive charge. Recently, CHON/CHIT NPs containing CHON as the main ingredient were produced as aggrecan mimicking NPs (Place et al., 2014). However, none of these studies considered the stoichiometry of CHON/CHIT NPs formation. Hence the aim of this paper was to examine the stoichiometry of molecular interactions between CHON and CHIT within CHON/CHIT NPs. Another objective was to discuss the criteria of carrier selection and to select carriers with optimal properties for the encapsulation of a cationic peptide, sCT. Knowing the principles and approach to loading this peptide, the same criteria could be translated to encapsulating similar cationic therapeutically relevant molecules, such as antimicrobial peptides and growth factors.

Similarly to HA, CHON NPs are interesting as carriers for sCT due to complementary pharmacological action of both molecules (Umerska et al., 2015). As in some instances the presence of CHIT may not be necessary, and there is evidence that CHON forms complexes with sCT (Umerska et al., 2015), the purpose of this paper was to design and characterize CHON/sCT NPs. Cationic molecules, e.g. CHIT, could compete with sCT for binding with CHON molecules. Because the colloidal stability of polyelectrolyte complex NPs depends on their charge, the incorporation of large quantity of sCT could lead to destabilization of the system. Eliminating cationic CHIT from the formulation could offer the advantage of a very high sCT loading. The last objective of this paper was to examine the influence of the composition of the nanocarrier on the stability in different environments and the peptide release.

2. Materials and methods

2.1 Materials

Chondroitin 4-sulfate sodium salt (CHON) was purchased from Sigma (Ireland). Salmon calcitonin (sCT, as acetate salt) was obtained from PolyPeptide Laboratories. Chitosan chlorides were obtained from Chitoceuticals (Germany) (referred to as CL42) and Novamatrix (Norway) (Protasan UP CL113, referred to as CL113). All other reagents, chemicals and solvents were of analytical grade.

2.2 Physicochemical characterization of polymers

The molecular weight of polymers was determined using a gel permeation chromatography system previously described (Umerska et al., 2012). For CHIT samples, the mobile phase was composed of 0.33 M acetic acid and 0.2 M sodium acetate. For a CHON sample, the mobile phase was composed of 0.2M NaCl and 0.01M NaH_2PO_4 brought to pH 7.4 with NaOH solution. Determination of the chloride ions was performed with a Dr Lange LCK 311 test as described earlier (Parojčić et al., 2011). The content of sodium counterion was determined by inductively coupled plasma-mass spectrometry (ICP-MS) (Paluch et al., 2010). NMR experiments on the degree of deacetylation of CHIT was done as described previously (Umerska et al., 2012).

105 **2.3 Preparation of CHON/CHIT, CHON/CHIT/sCT and CHON/sCT nanoparticles**

106 The CHON solutions, CL42 solutions, CL113 solutions and sCT solutions were
107 prepared in deionized water. A predefined aliquot of the sCT solution and/or the CHIT
108 solution was added to a known volume of the CHON solution (one shot addition) at room
109 temperature under stirring; the stirring was maintained to allow for stabilization of the
110 system. A dispersion of particles was instantaneously obtained upon mixing of polymer
111 solutions.

112 Charge mixing ratio (CMR) was calculated by dividing the total number of negatively
113 charged ionizable groups (n^-) by the total number of positively charged ionizable groups (n^+)
114 considering the counterion content, the deacetylation degree of CHIT and pH.

115 **2.4 Nanoparticle characterization**

116 Transmittance and pH of the NP dispersions were measured as described by
117 Umerska et al. (2012). Dynamic viscosity measurements were carried out using an SV-10
118 Vibro Viscometer (A&D Company Limited). The amount of free or NP-associated polymer
119 (CHON for negatively charged NPs; CHIT for positively charged NPs) was determined from
120 viscosity measurements of continuous phases of NP dispersions. The viscosity of pure
121 polymer solution, for a given polymer concentration, was taken as containing 100% free
122 polymer, whereas the viscosity of water was taken as containing 0% free polymer. The
123 percentage of NP-associated polymer was calculated as a difference between the starting
124 quantity of polymer used in formulation (using the initial polymer concentration) and the
125 quantity of free/non-associated polymer (Umerska et al., 2015). The calculations are based
126 on assumptions that the contribution of NPs, any possible soluble complex formed between
127 the polymers and electrolyte ions originating from the polymers to the viscosity of the
128 systems is negligible. This was corroborated by the fact that viscosity of the CHON/CHIT
129 systems with a mass mixing ratio of 1.25 containing stoichiometric quantities of both
130 polymers was 0.89±0.01 mPa·s, which is not significantly different to that of pure water at 25
131 ºC.

The intensity-averaged mean particle size (hydrodynamic particle diameter) and polydispersity index were determined by dynamic light scattering (DLS) using 173º backscatter detection. The electrophoretic mobility values measured by laser Doppler velocimetry (LDV) were converted to zeta potential by the Smoluchowski equation. Both DLS and LDV measurements were done as described by Umerska et al., (2012). The obtained results were corrected for the sample viscosity measured as described above.

2.5 Salmon calcitonin (sCT) loading studies

2.5.1 Separation of non-associated sCT

Non-associated sCT was separated from the NPs using a combined ultrafiltration-centrifugation technique (Amicon® Ultra-15, MWCO of 30 kDa, Millipore, USA) as described by Umerska et al., (2015). The filtrate containing non-associated sCT was assayed via high performance liquid chromatography (HPLC), as described in Section 2.5.4. The association efficiency (AE) and drug loading (DL) were calculated as described by Umerska, Corrigan & Tajber (2014a).

2.5.2 Colloidal behavior of CHON/CHIT/sCT and CHON/sCT NPs in different media

Aliquots of 250 µl of the NPs were added to 2.25 ml of the dispersant. The following dispersants were used: (1) phosphate-buffered saline (PBS) pH=7.4, (2) PBS pH=7.4 diluted with deionized water (1:10), (3) acetate buffer pH=5, (4) acetate buffer pH=5 diluted with deionized water (1:10), (5) deionized water and (6) HCl 0.07M pH=1.2 (Umerska et al., 2015). Samples were incubated at 37 ºC at 100 rpm. Size and zeta potential measurements (Section 2.4) were performed after 1 hour and 24 hours of incubation.

2.5.3 Release studies

Aliquots of 250 µl of sCT-loaded NPs were added to 2.25 ml of dispersant and incubated at 37 ºC at 100 rpm (see Section 2.5.2). After 1, 2, 4, 6 and 24 hours, 2.5 ml aliquots were withdrawn, and the released sCT was separated using the combined ultrafiltration-centrifugation technique as described by Umerska et al. (2015). The data from the release studies were fitted to the first-order equation:

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

where W is the amount of the peptide released at time t (based on cumulative release), W_{∞} is the amount of the peptide released at infinity and k is the release rate constant.

2.5.4 Quantification of sCT

Analysis of sCT content was performed using an HPLC system as described previously (Umerska et al., 2014a). Briefly, 50 µl of the standard or sample was injected into the Jones Chromatography Genesis 4µ C18 150x4.6 mm column. A flow rate of 1 ml/min was employed using a mobile phase composed of 0.116% w/v NaCl, 0.032% v/v trifluoroacetic acid and 34% v/v acetonitrile. The UV detection was carried out at 215 nm.

2.6 Statistical analysis

The statistical significance of the differences between samples was determined using one-way analysis of variance (ANOVA) followed by the post-hoc Tukey's test using Minitab software. Differences were considered significant at $p<0.05$.

3. Results and discussion

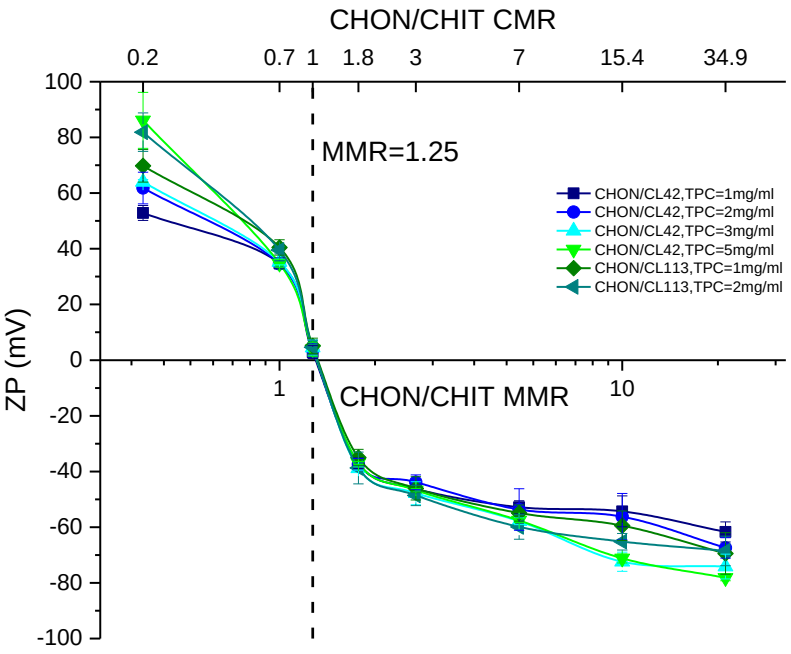
3.1 Formation of CHON/CHIT nanocarriers

The properties of the polymers, particularly the molecular weight, affect the formation of NPs and their characteristics (Umerska et al., 2012; Polexe and Delair, 2013; Wu and Delair, 2015). The relevant properties of the polymers are shown in Table 1.

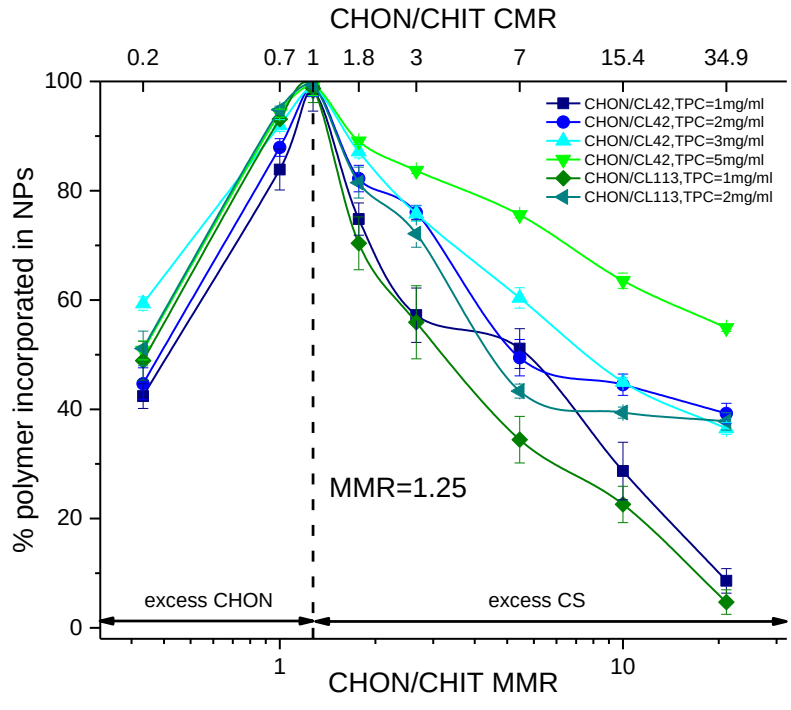
Table 1 Physicochemical characteristics of chondroitin sulfate and chitosan salts used in the studies. DD NMR - degree of deacetylation calculated from nuclear magnetic resonance data, Mn - number average molecular weight, Mw - weight average molecular weight, Mw/Mn – dispersity and N/A – not applicable.

Polymer	Counter ion	Counter ion content (%, w/w)	DD NMR	Mn (kDa)	Mw (kDa)	Mw/Mn
CHON	Na^+	5.55	N/A	33±0.4	59±0.2	1.76±0.011
CL42	Cl^-	13.6	84.0%	14±0.1	42±0.9	3.96±0.037
CL113	Cl^-	15.5	83.5%	33±7.3	110±6.8	3.38±0.544

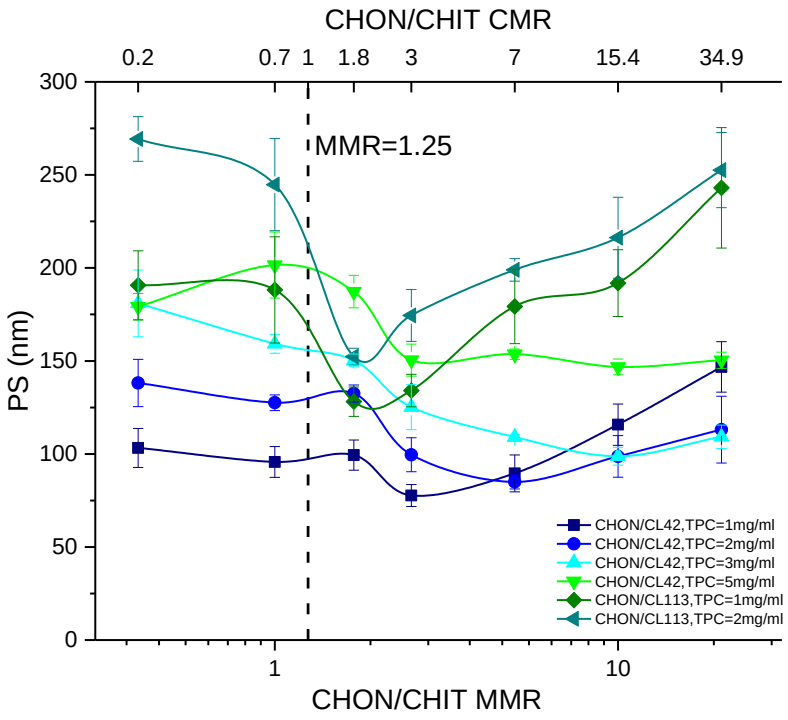
After mixing the CHON and CHIT solutions it was possible to observe solutions, opalescent or turbid macroscopically homogenous systems or even a phase separation depending on the polymer mixing ratio and concentration. Similar phenomena were observed for other polyelectrolyte complexes, e.g. HA/CHIT (Umerska et al., 2012), HA/PROT (Umerska et al., 2014b), carrageenan/PROT (Dul et al., 2015) and CHON/PROT (Umerska et al., 2015). Interestingly, phase separation for the CHON/CHIT pair was observed only when the CHON/CHIT mass mixing ratio (MMR) was 1.25, regardless of the total polymer concentration (TPC) or the molecular weight of CHIT. The MMR of 1.25 corresponds to a theoretical n^-/n^+ charge mixing ratio (CMR) of 1.20-1.24, where n^- and n^+ are the number of moles of negative and positive charges, respectively. However, the fraction of charged groups on the polyelectrolyte molecules that dissociate depends on pH of medium. The pKa of the amino group of CHIT is approximately 6.5, whereas the pKa of sulfate and carboxyl groups of CHON are 2.6 and 4.6, respectively (Fajardo, Lopes, Valente, Rubira & Muniz, 2011). pH of the formulations varied between 4 and 6.5 depending on the mixing ratio and the higher the content of CHIT, the lower the pH of the medium was. In this pH range more than 95% of sulfate groups of CHON should be dissociated, however the dissociation degree varies from 20 to 99% for carboxyl groups of CHON and from 48% to 100% for the amino groups of CHIT. Taking into account the pH and dissociation degree, the actual CMR values that corresponded to MMR=1.25 were between 0.99-1.09, as opposed to 1.20-1.24 (theoretical CMR). Indeed, as shown in Figure 1a, the charge inversion occurred close to the CHON/CHIT MMR of 1.25, therefore aggregation at this MMR may be attributed to charge neutralization.


CHON/CHIT CMR
0.2
0.7
1
1.8
3
7
15.4
34.9
100
80
60
40
20
0
-20
-40
-60
-80
-100
ZP (mV)
MMR=1.25
CHON/CHIT MMR
1
10
CHON/CL42,TPC=1mg/ml
CHON/CL42,TPC=2mg/ml
CHON/CL42,TPC=3mg/ml
CHON/CL42,TPC=5mg/ml
CHON/CL113,TPC=1mg/ml
CHON/CL113,TPC=2mg/ml

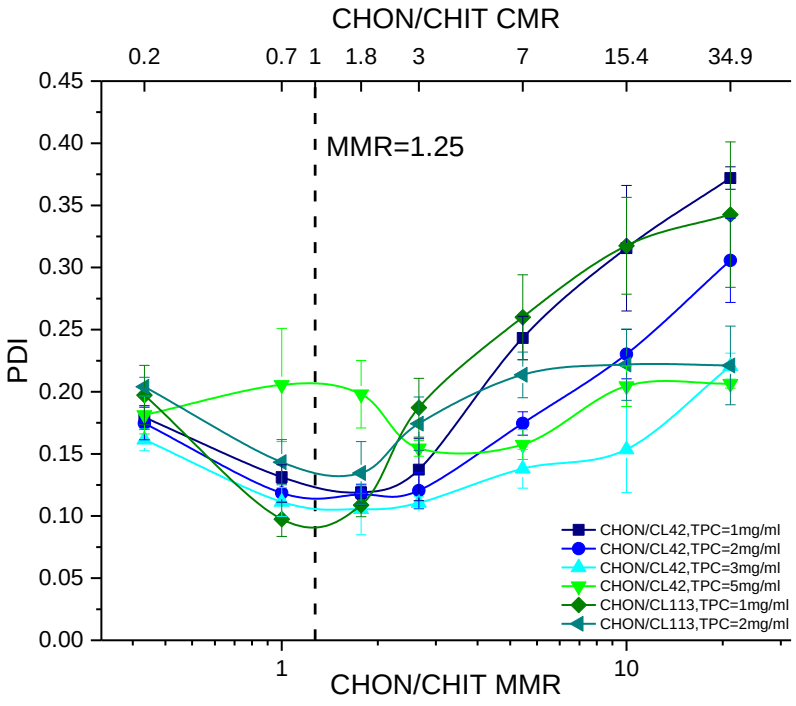
205 a)


CHON/CHIT CMR
0.2
0.7
1
1.8
3
7
15.4
34.9
100
80
60
40
20
0
% polymer incorporated in NPs
MMR=1.25
excess CHON
excess CS
1
10
CHON/CHIT MMR
CHON/CL42,TPC=1mg/ml
CHON/CL42,TPC=2mg/ml
CHON/CL42,TPC=3mg/ml
CHON/CL42,TPC=5mg/ml
CHON/CL113,TPC=1mg/ml
CHON/CL113,TPC=2mg/ml

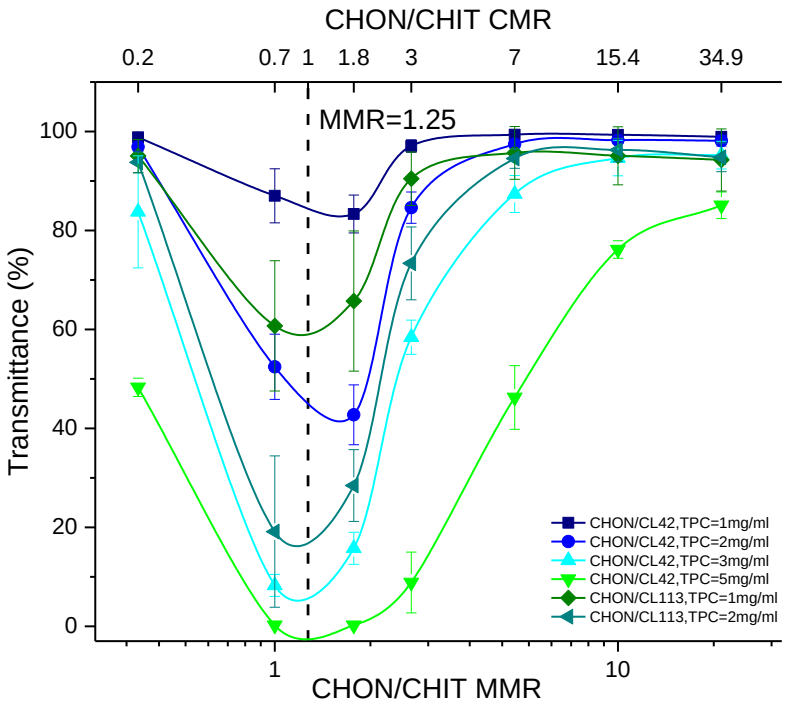
206 b)


CHON/CHIT CMR
0.2
0.7
1
1.8
3
7
15.4
34.9
MMR=1.25
PS (nm)
300
250
200
150
100
50
0
1
10
CHON/CHIT MMR
CHON/CL42,TPC=1mg/ml
CHON/CL42,TPC=2mg/ml
CHON/CL42,TPC=3mg/ml
CHON/CL42,TPC=5mg/ml
CHON/CL113,TPC=1mg/ml
CHON/CL113,TPC=2mg/ml

207 c)


CHON/CHIT CMR
0.2
0.7
1
1.8
3
7
15.4
34.9
MMR=1.25
PDI
0.45
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
1
10
CHON/CHIT MMR
CHON/CL42,TPC=1mg/ml
CHON/CL42,TPC=2mg/ml
CHON/CL42,TPC=3mg/ml
CHON/CL42,TPC=5mg/ml
CHON/CL113,TPC=1mg/ml
CHON/CL113,TPC=2mg/ml

208 d)



e)

Figure 1. Properties of CHON/CHIT NPs: a) zeta potential (ZP), b) percentage of polymer incorporated into the NPs, c) particle size (PS), d) polydispersity index (PDI) and e) transmittance (TR). MMR – mass mixing ratio, CMR – charge mixing ratio, TPC – total polymer concentration.

The main factor determining the zeta potential was the mixing ratio of the polymers (Figure 1a). The zeta potential was highly negative for CHON/CHIT MMRs between 1.7 and 20 (CMRs of 1.8-35) and at CHON/CHIT MMRs of 1 and 0.4 (CMRs of 0.7 and 0.2, respectively) the charge was highly positive. The molecular weight of CHIT appeared to affect zeta potential of positively charged NPs; CL113-based NPs were characterized by significantly higher zeta potential values than CL42-NPs at the same CHIT and NP concentration. The absolute value of zeta potential also increased corresponding to an increase in TPC, particularly in the case of positively charged NPs. The stability of polyelectrolyte complex NPs depends on the repulsion between similarly charged NPs and the net charge must be sufficient if the system is to be physically stable (Umerska et al., 2015).

The amount of polymers incorporated within the NPs is presented in Figure 1b. The polymer in excess was either CHIT or CHON for positively and negatively charged NPs,

respectively. It can be observed, that nearly 100% of the polyelectrolytes were incorporated within the CHON/CHIT MMR=1.25 complexes, therefore this ratio can be considered as stoichiometric. The amount of polymers incorporated within the NPs increased when the MMR was approaching 1.25. The amount of incorporated polymer was also dependent on the TPC- the higher the TPC was, the more polymer was contained within the NPs. The formation of stable polyelectrolyte complex NPs requires that the polyelectrolytes are mixed in non-stoichiometric ratios (Boddohi & Kipper, 2010). Although the dispersions of particles with the MMRs close to 1.25 (i.e. CHON/CHIT MMRs of 1.7 or 1) contain very small amount of free polymer, and for that reason they seem to be more convenient from the quality control point of view, they should be avoided as potential candidates for a drug delivery system, because a small change in pH of the system or addition of a polycationic/polyanionic molecule (for example an active ingredient- a peptide or a nucleic acid) could cause charge neutralization, aggregation and destabilization of the system.

Apart from aggregated samples with the CHON/CHIT MMR of 1.25, all tested dispersions were characterized by particles with a small size that varied between 80 and 270 nm (Figure 1c). The particle size was influenced by the molecular weight of CHIT, CL113-NPs were larger than CL42-NPs. The particle size was also affected by the TPC. Generally, the higher TPC, the larger was the hydrodynamic diameter of the NPs, however some deviations from this trend were observed. The particle size was also influenced by the CHON/CHIT MMR and the effect exerted by the MMR depended on TPC and molecular weight of CHIT. Generally CHON/CHIT NPs had a homogenous size distribution with PDI values below 0.25 for most of the samples (Figure 1d). Only samples with low TPCs (1 mg/ml and in the case of CL42 NPs- also 2 mg/ml) and high CHON/CHIT MMRs (10 for TPC 1 mg/ml and 20 for TPC 1 and 2 mg/ml) were characterized by PDI values higher than 0.3. Interestingly, these dispersions contain high percentage of CHON which did not react with CHIT, so the increased PDI values may be due to the contribution of free CHON molecules.

Samples with CHON/CHIT MMR=1.25 were not suitable for transmittance measurements. The transmittance of the remaining dispersions is shown in Figure 1e. It can

256 be observed that the turbidity of the samples increased corresponding to an increasing
257 polymer concentration and an increasing molecular weight of CHIT. Another important factor
258 influencing the turbidity of the dispersions was the mixing ratio of the polymers- the turbidity
259 increased when the mass mixing ratio was approaching to 1.25. The transmittance depends
260 on the concentration and the particle size of the complexes. Decreasing value of
261 transmittance corresponding to the CHON/CHIT MMR approaching 1.25 and to an
262 increasing TPC may be an indicator of the formation of larger number of the NPs, particularly
263 for lower TPCs.

264 **3.3 CHON/CHIT/sCT nanoparticles**

265 CHON/CL42 NPs with MMRs of 2.5, 5 and 10 mg/ml at TPCs 1, 2 and 3 mg/ml were
266 selected as potential carriers for sCT (Table 2). Because TPC of 5 mg/ml resulted in the
267 most viscous nano-suspensions with the largest particles, they were not considered as
268 carriers for sCT.

269

Table 2 Composition, properties (TR - transmittance, PS - particle size, PDI - polydispersity index and ZP - zeta potential), total n^-/n^+ CMR (charge mixing ratio), percentage of CHON incorporated into the NPs, association efficiency (AE) and drug loading (DL) of sCT-loaded CHON/CL42 NPs. *p<0.05, **p<0.01, ***p<0.001 versus NPs without sCT.

TPC [mg/ml]	CHON/CL42 mass ratio	Initial conc. sCT (mg/ml)	Total n^-/n^+ CMR	CHON incorporated into NPs (%)	AE (%)	DL (%)	TR (%)	PS (nm)	PDI	ZP (mV)
3	2.5	0.5	2.08	80±1**	99.8±0.03	14.26±0.004	19±2***	180±6*	0.214±0.027**	-43.1±1.3
3	5	0.5	3.71	65±1*	99.9±0.02	14.27±0.003	57±3***	220±6**	0.268±0.019**	-59.4±1.4
3	10	0.5	6.12	52±1***	99.8±0.01	14.26±0.001	71±4**	248±11*	0.309±0.038**	-70.0±0.8
3	2.5	1.0	1.84	83±1***	99.5±0.27	24.91±0.068	15±4***	177±13**	0.200±0.016**	-38.5±0.6*
3	5	1.0	3.05	68±1**	99.8±0.04	24.96±0.010	54±2***	211±9*	0.293±0.064*	-52.0±0.9**
3	10	1.0	4.55	55±1***	99.7±0.17	24.94±0.043	72±2**	249±15*	0.356±0.082*	-63.0±1.8*
2	2.5	0.5	1.95	82±1**	99.4±0.23	19.90±0.046	64±4**	140±17*	0.150±0.037	-37.2±2.2*
2	5	0.5	3.34	59±1**	99.7±0.07	19.95±0.014	89±4*	150±34**	0.217±0.060	-52.0±3.4
2	10	0.5	5.21	49±2*	99.8±0.04	19.97±0.008	94±3	162±34*	0.251±0.068	-58.2±8.0
2	2.5	1.0	1.67	83±2**	98.9±0.61	33.09±0.204	34±7***	142±3**	0.182±0.012**	-32.5±1.1**
2	5	1.0	2.61	64±2**	99.6±0.16	33.24±0.053	81±2***	155±2***	0.234±0.017**	-46.0±2.5
2	10	1.0	3.65	49±2*	99.7±0.15	33.27±0.050	90±2**	173±1***	0.306±0.066	-55.1±2.7
1	2.5	0.5	1.67	79±7*	99.3±0.29	33.18±0.097	81±5**	101±13	0.149±0.028	-31.4±2.3**
1	5	0.5	2.61	56±4	99.5±0.08	33.22±0.027	96±1**	95±26	0.215±0.033	-43.8±2.0**
1	10	0.5	3.65	36±2	99.7±0.08	33.27±0.027	98±1	100±22	0.279±0.075	-50.7±4.0

sCT was very efficiently associated with CHON/CL42 NPs, with the yield close to 100% (Table 2). Moreover, high peptide loading (14-33%) was also achieved. Similar drug loading was accomplished for the previously described HA- and CHON-based nanocarriers (Umerska et al., 2014a; Umerska et al., 2014b; Umerska et al., 2015). The incorporation of an active ingredient can change the properties of the nanocarrier. This is true particularly for the polyelectrolyte complex NPs, which are characterized by a high drug loading. Despite loading of cationic sCT into CHON/CL42 NPs, all tested formulations had strongly negative zeta potential ranging between -31 and -72 mV; in some cases a significant decrease in the absolute value of zeta potential was observed compared with CHON/CHIT NPs with the same TPC and CHON/CHIT MMR (Table 2). The n^-/n^+ CMR calculations confirmed that there is a stoichiometric excess of negative charges in relation to positive charges. The amount of CHON incorporated within CHON/CHIT/sCT NPs was significantly higher than that of CHON/CHIT NPs. Due to its net positive charge the presence of sCT allowed binding of a larger number of CHON molecules within the NPs, decreasing the amount of free polymer. This phenomenon could prevent the charge neutralization and destabilization of the system. For this reason, formulations containing a larger excess of an anionic polymer (CHON in this case) should be preferred for the encapsulation of cationic molecules. This statement can also be true for the opposite situation: in the case of encapsulation of anionic molecules into positively charged NPs the formulation containing higher excess of cationic polymer would be better candidates.

All tested CHON/CHIT/sCT NPs had particle diameters varying between 95 and 250 nm; in some cases a significant increase was observed compared with CHON/CHIT NPs (Table 2). Inclusion of sCT produced an increase in turbidity of the dispersions, which could be attributed to both, an increase in particle size and in the number of the particles formed. The reduction in negative charge of the NPs and an increase in the turbidity of the system have been observed for different sCT-loaded polyelectrolyte complex NPs, namely CHON/PROT, HA/PROT and HA/CHIT. However the influence of sCT on the particle size varied depending on the type of the carrier. In contrast to CHON/CHIT/sCT, in previously

described HA/CHIT NPs either no change or a decrease in particle size was observed for lower and higher sCT concentration, respectively (Umerska et al. 2014a). Only formulation with the lowest MMR tested (2.5) containing high amount of sCT (0.35 mg/ml) showed an increase in particle size. On the other hand, an increase in size was observed for HA/PROT (Umerska et al., 2014b) and in some of the CHON/PROT NPs (Umerska et al., 2015). The increase in particle size in some CHON/CHIT/sCT NPs after incorporation of sCT may be attributed to neutralization of CHON by the polycations (CHIT and sCT) and attainment of more compact structure of the NPs. Indeed, a significant increase in the quantity of CHON incorporated within the NPs was observed in sCT-containing NPs compared with sCT-free NPs.

3.4 CHON/sCT NPs

CHON/sCT complexes obtained by mixing polymer and peptide solutions at different concentrations and different ratios were examined. Similarly to CHON/CHIT system, the mixing of the polysaccharide and peptide solutions yielded different types of systems: solution, an opalescent or turbid dispersion or two-phase systems containing supernatant liquid and aggregates. As shown in Table 3, sCT was bound very efficiently to CHON forming the NPs. Nearly 100% of sCT was complexed to CHON for CHON/sCT MMRs of 0.7 and 1.4. Comparable AE was obtained for CHON/sCT MMR of 0.35 at 1 mg/ml of sCT. Therefore, in diluted dispersions such as that containing 0.35 mg/ml CHON, the mass of sCT bound by the polysaccharide was 3 times higher than the mass of CHON. It is probably due to much higher charge density (the number of moles of charges per gram of the compound) in CHON molecules compared with that of sCT molecules.

Table 3 Composition, properties (TR - transmittance, PS - particle size, PDI - polydispersity index, ZP - zeta potential), total n^-/n^+ CMR (charge mixing ratio), percentage of CHON incorporated into the NPs, association efficiency (AE), and drug loading (DL) of binary CHON/sCT complexes. MMR – mass mixing ratio.

CHON conc. (mg/ml)	sCT conc. (mg/ml)	CHON/sCT MMR	Total n^-/n^+ CMR	CHON incorporated into NPs (%)	AE (%)	DL (%)	TR (%)	PS (nm)	PDI	ZP (mV)
0.35	1	0.35	1.41	79±5	94.4±3.96	73.0±3.06	12±2	195±18	0.040±0.013	-15.7±0.5
0.35	2	0.175	0.90	98±3	44.1±14.41	71.6±23.4	0±0	aggregates	N/A	-7.3±0.2
0.7	1	0.7	2.42	76±5	99.7±0.23	58.8±0.14	82±12	99±14	0.227±0.021	-36.8±3.7
0.7	2	0.35	1.41	79±8	95.1±2.39	73.1±1.84	0±0	831±145	0.197±0.034	-14.4±0.2
1.4	1	1.4	4.44	10±8	99.9±0.12	41.6±0.05	99±1	163±13	0.508±0.108	-45.1±0.7
1.4	2	0.7	2.42	66±5	99.8±0.16	58.8±0.10	51±10	120±20	0.135±0.016	-38.4±1.8

330 Additionally, CHON molecules only have anionic moieties, whereas sCT, bearing net
331 positive charge at neutral and acidic pH, apart from cationic amino acids also contains
332 anionic amino acids. When the CHON/sCT MMR was further decreased to 0.175, phase
333 separation occurred immediately after mixing the sCT and CHON solutions and 56% of sCT
334 was found in the non-associated fraction. Hence, at CHON/sCT MMR of at least 0.35,
335 CHON was able to efficiently bind sCT, but with a further decrease in CHON/sCT MMR the
336 binding places in CHON molecules became saturated and sCT molecules not involved in the
337 interactions with CHON were separated from the CHON/sCT complex by ultrafiltration-
338 centrifugation. CHON/sCT dispersions had an excellent loading capacity and stable
339 CHON/sCT NPs contained up to 73% (w/w) of sCT.

340 The zeta potential of CHON/sCT complexes was markedly affected by the
341 CHON/sCT MMR (Table 3). All tested dispersions contained negatively charged particles,
342 however in the case of CHON/sCT MMR of 0.175 dispersion, where the aggregation was
343 observed, the value of zeta potential was only -7 mV, indicating neutralization of the anionic
344 groups of CHON by sCT. The negative surface charge can be attributed to the dominant
345 presence of CHON molecules on the particle surface. The quantity of CHON bound within
346 the nanoplexes was dependent on the CHON/sCT MMR; the lower the MMR was, the higher
347 amount of CHON was present in the nanoplexes.

348 The turbidity of the dispersions increased correspondingly to a decrease in
349 CHON/sCT MMR (Table 3). Apart from the dispersion with the CHON/sCT MMR of 0.175,
350 other tested dispersions contained the particles with sizes in the nano range. Similarly to the
351 previously described HA/sCT complexes (Umerska et al., 2014a), the excess of the anionic
352 polysaccharide increased the solubility of its complexes with sCT. Apart from the CHON/sCT
353 MMR of 1.4 dispersion, which was not suitable for DLS analysis as it did not meet quality
354 criteria, all other dispersions were homogenously dispersed with PDI values below 0.25. It is
355 possible that in the CHON/sCT MMR of 1.4 dispersion the excess of CHON was responsible
356 for the increase in the solubility of the CHON/sCT complex and the decrease in the amount
357 of particles. As the amount of sCT was relatively low compared with CHON, the competition

between CHON molecules for binding with cationic groups of sCT may have resulted in the formation of highly disordered NPs with relatively low density.

In summary, after mixing CHON and sCT solutions at CHON/sCT MMRs of 0.35 and 0.7 it was possible to obtain stable nanoparticulate dispersions (Table 3). This phenomenon was not observed for HA/sCT complexes described previously (Umerska et al., 2014a). Although an HA/sCT complex was formed, as demonstrated by transmittance, viscosity measurements and by FTIR, only two types of systems were formed: soluble complexes and unstable NPs (having the appearance of a solution) or two-phase systems. Phase separation was observed at HA/sCT MMRs of 0.44 and 0.33. Interestingly, hyaluronic acid/sCT MMR of 0.33 corresponds to total n^-/n^+ CMR of 0.72, whereas CHON/sCT MMR of 0.35- to total n^-/n^+ CMR of 1.41; the former yielded phase separation, whereas the latter- stable NPs. Difference in the CMRs is due to the presence of two ionizable groups in each disaccharide unit of CHON molecules in contrast to HA, where only one carboxylic group is present in each disaccharide unit. Although the CMR influences the appearance of the system, particularly the phase separation due to charge neutralization which occurs at CMR values close to 1, it does not explain the formation of stable NPs for the CHON/sCT system and not for the HA/sCT system, as in the case of HA/sCT system the formation of stable NPs was not observed for the CMR between 0.33 and 2.83. HA contains only weak acid carboxylic groups in its molecules whereas CHON contains both weak and strong acid carboxylic and sulfate groups, respectively (Denuziere et al., 1996). For this reason he former can be considered as a weak polyelectrolyte, whereas the latter- as a strong polyelectrolyte. Both, the higher charge density of CHON and the higher strength of CHON complexes compared with HA may cause the formation of more compact structures yielding stable CHON/sCT NPs. The smaller molecular weight of CHON (50 kDa) compared with that of HA (257 kDa) may also contribute to the better colloidal stability of the former complexes with sCT.

3.5 Colloidal behavior of CHON/sCT and CHON/CL42/sCT NPs in various media

Apart from the particle characteristics the properties of polyelectrolyte complex NPs also depend on the properties of the dispersant, such as ionic strength and pH (Umerska et al., 2012, Umerska et al., 2015). Three formulations (Table 4) were selected for stability studies: two CHON/CL42/sCT NP formulations with CHON/CL42 MMRs of 5 and 2.5 and sCT of 1 mg/ml and one CHON/sCT formulation (CHON=1.4 mg/ml, sCT=2 mg/ml).

Table 4. Properties of CHON/CL42/sCT and CHON/sCT NPs in different media. The samples were prepared as described in Sections 2.3 and 2.5.2 and measured after 1 hour or 24 hours of incubation at 37º C. TPC – total polymer concentration, MMR – mass mixing ratio, PS - particle size, PDI - polydispersity index, ZP - zeta potential, (D) – diluted. *p<0.05, **p<0.01, ***p<0.001 versus NPs in water, $^{\#}$p<0.05, $^{\#\#}$p<0.01, $^{\#\#\#}$p<0.001 versus NPs stored for 1 hour.

TPC of 3 mg/ml, CHON/CL42 MMR of 2.5, sCT of 1 mg/ml NPs						
Medium	1h PS (nm)	24h PS (nm)	1h PDI	24h PDI	1h ZP (mV)	24h ZP (mV)
Water	182±13	179±13	0.180±0.029	0.174±0.022	-37.6±1.3	-34.5±6.4
Acetate buffer (D)	190±25	192±23	0.160±0.044	0.143±0.032	-25.1±2.7**	-27.1±4.9
Acetate buffer	228±2**	252±2$^{\#\#\#}$	0.101±0.028*	0.097±0.025	-26.4±0.6***	-26.2±0.7
PBS (D)	175±1	179±1$^{\#\#}$	0.130±0.035	0.159±0.031	-31.4±1.1**	-28.8±0.4$^{\#}$
PBS	aggregation		aggregation		aggregation	
HCl	683±114**	648±147	0.192±0.079	0.149±0.110	4.1±5.6***	5.0±6.3
TPC of 2 mg/ml, CHON/CL42 MMR of 5, sCT of 1 mg/ml NPs						
Medium	1h PS (nm)	24h PS (nm)	1h PDI	24h PDI	1h ZP (mV)	24h ZP (mV)
Water	168±14	175±3	0.250±0.008	0.259±0.004	-48.1±7.2	-47.2±3.4
Acetate buffer (D)	188±5	188±5	0.265±0.008	0.262±0.006	-32.1±1.8*	-32.6±0.6
Acetate buffer	206±9*	224±6$^{\#}$	0.175±0.007***	0.167±0.015	-26.8±0.1**	-28.8±1.8
PBS (D)	162±6	163±8	0.238±0.005	0.240±0.007	-32.0±6.6*	-26.0±9.7
PBS	351±2***	663±13$^{\#\#\#}$	0.155±0.011***	0.256±0.010$^{\#\#\#}$	-25.9±2.7**	-23.8±0.6
HCl	400±113*	435±116	0.144±0.023**	0.149±0.022	-2.5±2.1***	-3.6±0.9
CHON/sCT NPs (CHON of 1.4 mg/ml, sCT of 2.0 mg/ml)						
Medium	1h PS (nm)	24h PS (nm)	1h PDI	24h PDI	1h ZP (mV)	24h ZP (mV)
Water	120±20	126±9	0.139±0.031	0.160±0.029	-39.8±2.0	-38.5±2.8
Acetate buffer (D)	134±7	152±3$^{\#}$	0.096±0.011	0.080±0.031	-27.0±0.1***	-27.2±0.6
Acetate buffer	283±55**	467±42$^{\#\#}$	0.120±0.012	0.230±0.014$^{\#\#\#}$	-25.0±2.0***	-23.3±2.9
PBS (D)	232±39*	276±28	0.328±0.062**	0.455±0.119	-16.2±0.7***	-15.2±6.2
PBS	282±21***	251±34	0.533±0.088**	0.602±0.128	-8.8±1.3***	-5.1±2.5
HCl	761±202**	1120±103$^{\#}$	0.376±0.097*	0.531±0.073$^{\#}$	-12.5±0.1***	-12.6±0.6

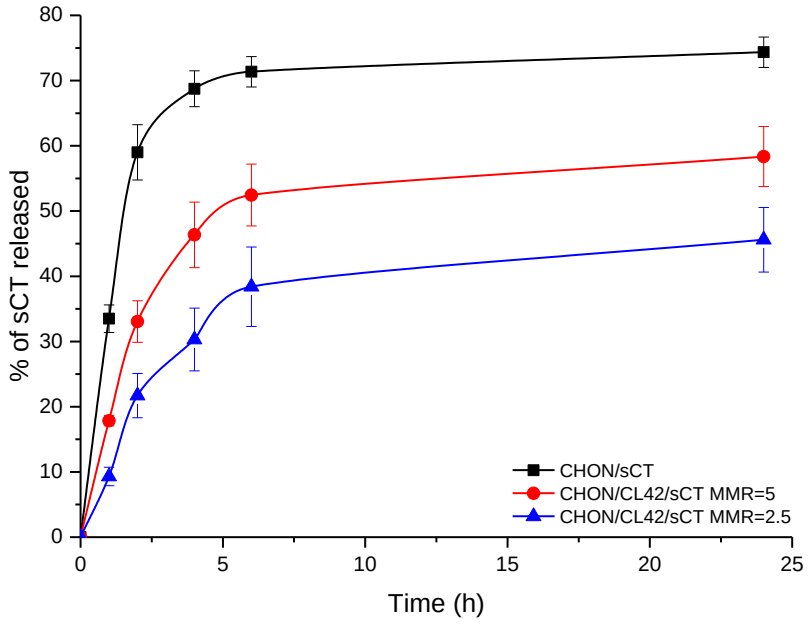
396 It can be observed that the particle size of all tested formulations increased in PBS, acetate
397 buffer and in HCl (Table 4). In the case of CHON/CL42 MMR=2.5 formulation an immediate
398 formation of aggregates was observed in PBS. The increase in particle size can be attributed
399 an increased ionic strength of the medium. Indeed, in diluted acetate buffer or diluted PBS
400 no significant change was observed except CHON/sCT NPs, which significantly increased
401 their size in diluted PBS. A significant decrease in PDI was observed in acetate buffer for
402 both CHON/CL42/sCT formulations; moreover, in the case of formulation with CHON/CL42
403 MMR of 5 a significant decrease was also observed in PBS and HCl. In contrast to
404 CHON/CL42/sCT formulations, CHON/sCT formulations became significantly more
405 polydispersed in diluted PBS, PBS and HCl. The absolute value of zeta potential was
406 significantly reduced in all tested media for all tested formulations. The reduction of surface
407 charge was especially pronounced in HCl for CHON/CL42/sCT formulations; for NPs with a
408 CHON/CL42 MMR=2.5 the inversion from negative to positive zeta potential was observed.
409 This may be attributed to a decrease in ionization of CHON at such low pH values. Similarly
410 to CHON/PROT NPs described previously (Umerska et al., 2015), the chloride anion
411 appeared to exert a stronger effect on the properties of the CHON/CHIT/sCT and
412 CHON/sCT NPs than the acetate.

413 Two different effects govern the behavior of polyelectrolyte complexes after a
414 subsequent change in the ionic strength of the medium, namely secondary aggregation or
415 dissolution, depending on the nature of the components of the system (Dautzenberg, 2000).
416 It has been shown that CHON-based NPs retain sodium chloride from PBS or HCl (in the
417 case of HCl this salt can be formed from the sodium ion originating from CHON) or sodium
418 acetate from the acetate buffer. The salts present in the polymer network can decrease
419 ionization and therefore the charge density of polymer molecules. The secondary
420 aggregation may occur in the case of CHON/CHIT/sCT NPs. The reduction in charge density
421 of polyelectrolytes will induce a decrease in the macromolecules stiffness, as a result of the
422 decreased repulsive electrostatic interactions. Therefore, conformational adaptation required
423 for the charge matching should be easier, thus leading to more compact complexes (Wu &

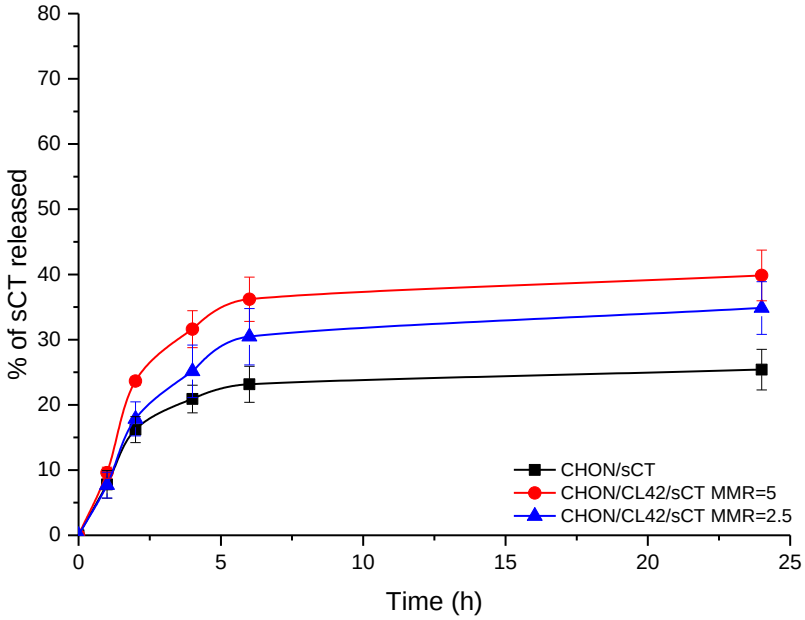
Delair, 2015). A decrease in PDI in CHON/CHIT/sCT NPs could be an indication of the formation of more ordered and compact nanostructures, whereas the increased particle size suggests the incorporation of a larger quantity of polyelectrolyte molecules per nanoparticle. The increase in ionic strength and rearrangement between the polyelectrolyte molecules also leads to release of sCT molecules (see Section 3.6). For CHON/sCT systems, it is likely than in PBS or in HCl dissolution of the complexes occurs. The release of sCT molecules should lead to dissociation of the binary complex, as CHON cannot self-assemble into NPs in an aqueous medium (Zhao et al., 2015). Although an increase in the particle size was observed, high PDI values (0.46-0.6) indicate that these results are not reliable. A change in pH and an increase in ionic strength leads at first to weakening the bonds between CHON and sCT (a fraction of disordered, large particles with low density) and then to dissociation of the complex. Because DLS is more sensitive to larger particles, the obtained result reflects their presence.

3.6 *In vitro* release of sCT

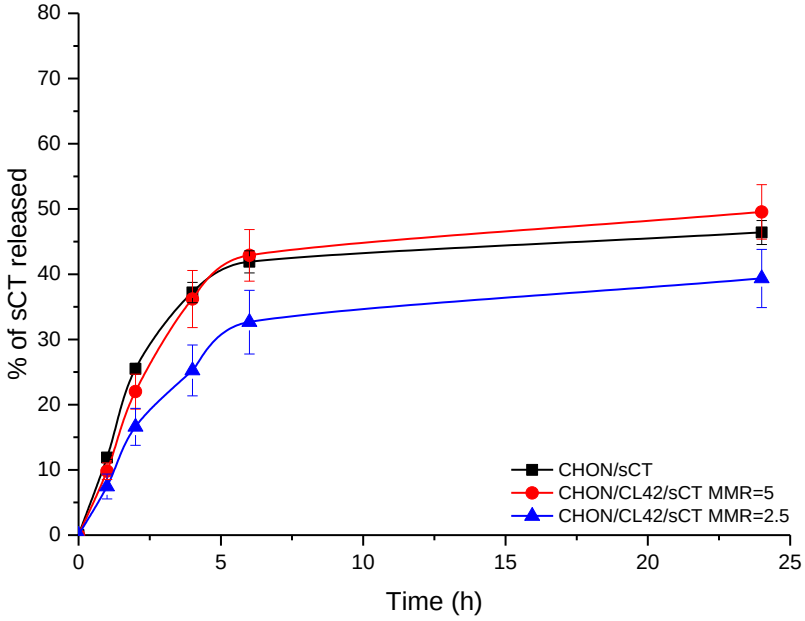
Figure 2 shows the cumulative release of sCT from CHON/CL42/sCT and CHON/sCT NPs.



a)



b)



c)

Figure 2 Cumulative release of sCT from CHON/sCT NPs (CHON of 1.4 mg/ml, sCT of 2.0 mg/ml - black squares), TPC of 2 mg/ml, CHON/CL42 MMR of 5, sCT of 1 mg/ml NPs (red circles) and TPC of 3 mg/ml, CHON/CL42 MMR of 2.5, sCT of 1 mg/ml NPs (blue triangles) into (a) PBS, (b) acetate buffer and (c) HCl solution. MMR – mass mixing ratio.

The release of sCT from its nanocomplex with CHON was influenced by the dispersant and the composition of the nanocarrier. Similarly to the previously described CHON/PROT/sCT NPs (Umerska et al., 2015), almost no sCT was released either to water or to diluted acetate buffer from all tested formulations.

CHON/CHIT/sCT NPs with MMR=2.5 released similar % of sCT into PBS, acetate buffer and HCl after 24 hours (46±5%, 35±4% and 40±4%, respectively, Figure 2). Similar behavior was observed for CHON/CHIT/sCT NPs MMR=5, which released 58±5%, 40±4% and 50±4% after 24 hours into PBS, acetate buffer and HCl, respectively. Release of sCT from CHON/sCT NPs was influenced by the release medium to a larger extent than that of CHON/CHIT/sCT NPs. The amount of sCT released from CHON/sCT complexes after 24 hours into PBS, acetate buffer and HCl was 74±2%, 25±3% and 46±2%, respectively. Interestingly, the size of these NPs in HCl was ~600% of the initial size in water compared to only ~200% in PBS and acetate buffer. Time- and pH-dependent release of CHON from CHON/CHIT complexes was observed by Fajardo et al. (2011) with the greatest release of CHON occurring at pH around neutral (pH=6 and pH=8) and lowest at pH=2. Dissociation and detachment of the principal polymer (CHON) constituting the NPs should lead to breaking up the complex with sCT (and/or CHIT) that occurs in PBS but not in HCl and thus resulting in the greater release of the peptide in PBS. As discussed in Section 3.5, the observed increase in the particle size seen in HCl may be caused by incorporation a quantity of chloride as sodium chloride (the sodium ion originating from CHON used as a sodium salt). However, as the PDI values of these formulations are quite high (0.38-0.60), the particle size measurements are not as reliable as at low PDI values (below 0.25) and should be interpreted with caution.

The most significant differences in sCT release were observed for PBS. The release rate decreased in the following order: CHON/sCT NPs>CHON/CHIT/sCT NPs MMR=5> CHON/CHIT/sCT NPs MMR=2.5, as reflected by decreasing values of the rate constant k (Table 5).

Table 5 Model parameter estimates and related goodness of fit statistics for sCT release from CHON/sCT NPs (CHON of 1.4 mg/ml, sCT of 2.0 mg/ml), TPC of 2 mg/ml, CHON/CL42 MMR of 5, sCT of 1 mg/ml NPs TPC of 3 mg/ml, CHON/CHIT MMR of 2.5, sCT of 1 mg/ml NPs data fitted to the first order model, where W_∞ is the amount of sCT released at infinity and k is the release rate constant.

Medium	k (h^{-1})	W_∞ (μg/mg of NPs)	Goodness of fit (R^2)
CHON/sCT NPs (CHON of 1.4 mg/ml, sCT of 2.0 mg/ml)			
Acetate buffer	0.446±0.021	149±16	0.989
HCl	0.371±0.003	276±11	0.993
PBS	0.689±0.045	434±13	0.992
CHON/CHIT/sCT NPs TPC of 2 mg/ml, CHON/CL42 MMR of 5, sCT of 1 mg/ml			
Acetate buffer	0.387±0.040	134±15	0.984
HCl	0.294±0.003	168±15	0.994
PBS	0.397±0.007	194±16	0.998
CHON/CHIT/sCT NPs TPC of 3 mg/ml, CHON/CHIT MMR of 2.5, sCT of 1 mg/ml			
Acetate buffer	0.320±0.025	88±10	0.991
HCl	0.259±0.022	100±11	0.997
PBS	0.285±0.023	114±13	0.992

This study confirms that release of peptide from ionic hydrogels depend on the composition of the complex and on the environmental conditions. The fact that the quantity of sCT released into acetate buffer was smaller than that released into HCl or PBS may be due to the fact that at pH 5 electrostatic interactions between sCT and CHON are optimal due to maximal ionization of both molecules. At acidic pH ionization of anionic groups of CHON is considerably decreased, whereas at pH 7.4 the degree of ionization of sCT could be smaller- the histidine group (pKa 6.10) should be dissociated at pH=5, but not at pH=7.4. The higher release from CHON/sCT NPs compared with that from CHON/CHIT/sCT NPs could be attributed to the lower density of the former NPs. The dense network of CHON and CHIT chains may retard the diffusion of sCT.

Based on the results of release studies and stability in different buffers it can be concluded that NPs containing large quantities of CHIT are not good candidates for intravenous administration due to the possibility of aggregation. On the other hand, the CHON/CHIT/sCT NPs, particularly those with CHON/CHIT MMR=2.5 are capable of providing a prolonged sCT release and for that reason they could be good candidates for other forms of parenterals, e.g. for intramuscular administration. The advantage of

CHON/CHIT/sCT NPs is the mucoadhesive properties of CHIT and for this reason they could make interesting candidates for oral delivery. Furthermore, the CHON/CHIT/sCT NPs showed better stability in HCl than CHON/sCT NPs. The aggregation process of the CHIT-containing NPs in PBS could be a major disadvantage, however before approaching the intestines the NPs would be diluted and that could prevent possible aggregation. The CHON/sCT NPs would be good candidates as they do not aggregate and the complex dissolves in PBS. Moreover, when administration in a final solid state formulation is necessary, the CHON/sCT NPs with high drug loading are preferred due to dilution issues (excipient addition) associated with forming such dosage forms.

4. Summary and conclusions

Both, positively and negatively charged CHON/CHIT NPs have been successfully developed and characterized. The formation and the physicochemical properties of CHON/CHIT NPs were depended on polymer mixing ratio, polymer concentration and molecular weight of CHIT. NPs containing polymers at the ratio far from stoichiometric would make better carriers for ionized drugs as they are less susceptible to charge neutralization and physical destabilization. sCT was successfully loaded into CHON/CHIT NPs with efficiency close to 100% and notably high sCT loading (up to 33%).

A new type of NPs composed of CHON and sCT (a binary system) has been successfully developed and characterized. Some of these carriers offer the advantage of a very high drug loading up to 73% and high association efficiency (95%).

The particle size of all tested formulations increased in PBS, acetate buffer and in HCl compared to that in water, however most of them remained in the nano-range even after 24 hours. Both, the media and the composition of the nanocarriers were found to affect the release of sCT. In all tested formulations the % of sCT released and the quantity of sCT released per mg of NPs at infinity decreased in the following order: PBS>HCl>acetate buffer. CHON/sCT NPs released the largest % of sCT, whereas CHON/CHIT/sCT MMR=2.5 NPs released the smallest quantity of sCT.

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Conflict of interest

The authors declare that there are no conflicts of interest.

References

Baldrick, P. (2010). The safety of chitosan as a pharmaceutical excipient. *Regulatory Toxicology and Pharmacology*, 56, 290-299.

Benhabiles, M.S., Salah, R., Lounici, H., Drouiche, N., Goosen, M. F. A., & Mameri, N. (2012). Antibacterial activity of chitin, chitosan and its oligomers prepared from shrimp shell waste. *Food Hydrocolloids*, 29, 48-56.

Boddohi, S., & Kipper, M. J., 2010. Engineering nanoassemblies of polysaccharides. *Advanced Materials*, 22, 2998-3016.

Dautzenberg, H. (2000). Light scattering studies on polyelectrolyte complexes. *Macromolecular Symposia*, 162, 1-21

Denuziere, A., Ferrier, D., & Domard, A. (1996). Chitosan- chondroitin sulfate and chitosan-hyaluronate polyelectrolyte complexes. Physico-chemical aspects. *Carbohydrate Polymers*, 29, 317-323.

Dul, M., Paluch, K. J., Kelly, H., Healy, A. M., Sasse, A., & Tajber, L. (2015). Self-assembled carrageenan/protamine polyelectrolyte nanoplexes- Investigation of critical parameters governing their formation and characteristics. *Carbohydrate Polymers*, 123, 339-349.

Fajardo, A. R., Lopes, L. C., Valente, A. J. M., Rubira, A. F., & Muniz, E. C. (2011). Effect of stoichiometry and pH on the structure and properties of Chitosan/Chondroitin sulfate complexes. *Colloid and Polymer Science*, 289, 1739-1748.

553 Fröhlich, E. (2012). The role of surface charge in cellular uptake and cytotoxicity of
554 medical nanoparticles. *International Journal of Nanomedicine*, 7, 5577-5591.
555 Goole, J., Lindley, D. J., Roth, W., Carl, S. M., Amighi, K., Kauffmann, J. M., & Knipp,
556 G. T. (2012). The effects of excipients on transporter mediated absorption. *International*
557 *Journal of Pharmaceutics*, 393, 17-31.
558 Hagiwara, K., Nakata, M., Koyama, Y., & Sato, T. (2012). The effects of coating
559 pDNA/chitosan complexes with chondroitin sulfate on physicochemical characteristics and
560 cell transfection. *Biomaterials*, 33, 7251-7260.
561 Hou, Y., Hu, J., Park, H., & Lee, M. (2012). Chitosan-based nanoparticles as a
562 sustained protein release carrier for tissue engineering applications. *Journal of Biomedical*
563 *Materials Research A*, 100A, 939-947.
564 Hu, C.-S., Tang, S.-L., Chaing, C.-H., Hosseinkhani, H., Hong, P.-D., & Yeh, M.-K.
565 (2014). Characterization and anti-tumor effects of chondroitin sulfate-chitosan nanoparticles
566 delivery system. *Journal of Nanoparticle Research*, 16, 2672
567 Paluch, K. J., Tajber, L., McCabe, T., O'Brien, J. E., Corrigan, O. I., & Healy, A. M.
568 (2010). Preparation and solid state characterisation of chlorothiazide sodium intermolecular
569 self-assembly suprastructure. *European Journal of Pharmaceutical Sciences*, 41, 603-611.
570 Parojčić, J., Stojković, A., Tajber, L., Grbić, S., Paluch, K. J., Djurić, Z., & Corrigan,
571 O. I. (2011). Biopharmaceutical characterization of ciprofloxacin HCl-ferrous sulfate
572 interaction. *Journal of Pharmaceutical Sciences*, 100, 5174-5184.
573 Place, L. W., Sekyi, M., & Kipper, M. J. (2014). Aggrecan-Mimetic,
574 Glycosaminoglycan-Containing Nanoparticles for Growth Factor Stabilization and Delivery.
575 *Biomacromolecules*, 15, 680-689.
576 Polexe, R. C., & Delair, T. (2013). Elaboration of stable and antibody functionalized
577 positively charged colloids by polyelectrolyte complexation between chitosan and hyaluronic
578 acid. *Molecules*, 18, 8563-8578.
579 Pusateri, A. E., McCarthy, S. J., Gregory, K. W., Harris, R. A., Cardenas, L.,
580 McManus, A. T., & Goodwin, C.W. (2003). Effect of a Chitosan-Based Hemostatic Dressing

on Blood Loss and Survival in a Model of Severe Venous Hemorrhage and Hepatic Injury in Swine. *The Journal of Trauma: Injury, Infection, and Critical Care*, 54, 177-182.

Ryan, S. M., McMorrow, J., Umerska, A., Patel, H. B., Kornerup, K. N., Tajber, L., Murphy, E. P., Perretti, M., Corrigan, O. I., & Brayden, D. (2013). An intra-articular salmon-calcitonin based nanocomplex reduces experimental inflammatory arthritis. *Journal of Controlled Release*, 167, 120-129.

Santo, V. E., Gomes, M. E., Mano, J. F., & Reis, R. L. (2012). Chitosan-chondroitin sulfate nanoparticles for controlled delivery of platelet lysates in bone regenerative medicine. *Journal of Tissue Engineering and Regenerative Medicine*, 6, s47-s59.

Sogias, I. A., Williams, A. C., & Khutoryanskiy, V. V. (2008). Why is chitosan mucoadhesive? *Biomacromolecules*, 9, 1837-1842.

Tsai, H.-Y., Chiu, C.-C., Lin, P.-C., Chen, S.-H., Huang, S.-J., & Wang, L.-F. (2011). Antitumor Efficacy of Doxorubicin Released from Crosslinked Nanoparticulate Chondroitin Sulfate/Chitosan Polyelectrolyte Complexes. *Macromolecular Bioscience*, 11, 680-688.

Umerska, A., Paluch, K. J., Inkielewicz-Stepniak, I., Santos-Martinez, M. J., Corrigan, O. I., Medina, C., & Tajber, L. (2012). Exploring the assembly process and properties of novel crosslinker-free hyaluronate-based polyeletrolyte colpmex nanoparticles. *International Journal of Pharmaceutics*, 436, 75-87.

Umerska, A., Corrigan, O. I., & Tajber, L. (2014a). Intermolecular interactions between salmon calcitonin, hyaluronate, and chitosan and their impact on the process of formation and properties of peptide-loaded nanoparticles. *International Journal of Pharmaceutics,* 477, 102-112.

Umerska, A., Paluch, K. J., Santos-Martinez, M.-J., Corrigan, O. I., Medina, C., & Tajber, L. (2014b). Self-assembled hyaluronate/protamine polyelectrolyte nanoplexes: synthesis, stability, biocompatibility and potential use as peptide carriers. *Journal of Biomedical Nanotechnology*, 10, 3658-3673.

Umerska, A., Paluch, K. J., Santos-Martinez, M. J., Medina, C., Corrigan, O. I., & Tajber, L. (2015). Chondroitin-based nanoplexes as peptide delivery systems –Investigations

609 into the self-assembly process, solid-state and extended release characteristics. *European*
610 *Journal of Pharmaceutics and Biopharmaceutics*, 93, 242-253.
611 van Damme, M.-P. I., Moss, J. M., Murphy, W. H., & Preston, B. N. (1994). Binding
612 properties of glycosaminoglycans to lysozyme- effect of salt and molecular weight. *Archives*
613 *of Biochemistry and Biophysics*, 310, 16-24.
614 Volpi, N. (2002). Oral bioavailability of chondroitin sulfate (Condrosulf®) and its
615 constituents in healthy men volunteers. *Osteoarthritis and Cartilage*, 10, 768-777.
616 Wu, D., & Delair, T. (2015). Stabilization of chitosan/hyaluronan colloidal
617 polyelectrolyte complexes in physiological conditions. *Carbohydrate Polymers*, 119, 149-
618 158.
619 Yeh, M.-K., Cheng, K.-M., Hu, C.-S., Huang, Y.-C., & Young, J.-J. (2011). Novel
620 protein-loaded chondroitin sulfate-chitosan nanoparticles: Preparation and characterization.
621 *Acta Biomaterialia*, 7, 3804-3812.
622 Zhao, L., Liu, M., Wang, J., & Zhai, G. (2015). Chondroitin sulfate-based nanocarriers
623 for drug/gene delivery. *Carbohydrate Polymers*, 133, 391-399.