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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 h. The media and composition of the nanocarriers were found to affect the release of sCT.

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., 2012). 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)

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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 0.2 M NaCl and 0.01 M 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).

2.3. Preparation of CHON/CHIT, CHON/CHIT/sCT and CHON/sCT nanoparticles

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

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

2.4. Nanoparticle characterization

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

The intensity-averaged mean particle size (hydrodynamic particle diameter) and polydispersity index were determined by dynamic light scattering (DLS) using 173° 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, and Tajber (2014).