International Journal of Pharmaceutics 477 (2014) 102–112

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm

Pharmaceutical nanotechnology

Intermolecular interactions between salmon calcitonin, hyaluronate, and chitosan and their impact on the process of formation and properties of peptide-loaded nanoparticles

Anita Umerska, Owen I. Corrigan, Lidia Tajber *

School of Pharmacy and Pharmaceutical Sciences, Trinity College Dublin, Dublin 2, Ireland

ARTICLE INFO

Article history:
Received 14 August 2014
Received in revised form 3 October 2014
Accepted 7 October 2014
Available online 13 October 2014

Keywords:
Hyaluronate
Chitosan
Salmon calcitonin
Nanoparticles
Polyelectrolyte complex
In vitro release

ABSTRACT

The principal aim of this work was to study the formulation of a ternary complex comprising salmon calcitonin (sCT), hyaluronate (HA), and chitosan (CS) in a nanoparticle (NP) format. As interactions between the constituents are possible, their presence and component mass mixing ratio (MMR) and charge mixing ratio (CMR) were investigated to tune the properties of NPs.

Intermolecular interactions between sCT and HA as well as sCT and CS were studied by infrared spectroscopy (FTIR) and dynamic viscosity. The impact of MMR, CMR, and HA molecular weight on the sCT loading capacity in NPs and *in vitro* release properties was determined.

sCT complexes to HA *via* electrostatic interactions and a support for hydrophobic interactions between sCT and HA as well as sCT and CS was found by FTIR. The sCT/HA complex is soluble but, depending on the mass mixing ratio between sCT and HA, NPs and microparticles were also formed indicative of associative phase separation between HA and sCT. The negatively charged HA/CS/sCT NPs were characterized by very high values (above 90%) of peptide association for the systems tested. Also, high sCT loading up to 50% were achieved. The peptide loading capacity and *in vitro* release properties were dependent on the NP composition. The zeta potential of the NPs without sCT was negative and ranging from −136 to −36 mV, but increased to −84 to −19 mV when the peptide was loaded. The particle size was found to be smaller and ranging 150–230 nm for sCT/NPs in comparison to NPs without sCT (170–260 nm). Short-term storage studies in liquid dispersions showed that the colloidal stability of NPs was acceptable and no release of sCT was observed for up to 3 days.

In conclusion, a range of NP systems comprising sCT, HA, and CS was successfully developed and characterized. Such NPs may be considered as a suitable nanoparticulate format for the delivery of sCT.

1. Introduction

Salmon calcitonin (sCT) is commercially available as an injectable and in a nasal spray form (Guggi et al., 2003). It is indicated for the treatment of bone diseases, *e.g.*, osteoporosis, Paget's disease and bone metastasis (Guggi et al., 2003; Makhlof et al., 2010). sCT is also a promising candidate to be used in osteoarthritis (OA) (Manicourt et al., 2005) and in combined therapy with alendronate in patients with rheumatoid arthritis (RA) (Ozoran et al., 2007).

It has been reported that a major issue associated with development of efficient intra-articular (IA) delivery systems is the rapid clearance of many compounds from the joint (Morgen et al., 2013
). Therefore, it is desirable that such IA systems have a longer duration of action to minimize the number of injections due to the discomfort and pain associated with administration as well as possible risk of infection (Gerwin et al., 2006). A range of particulate carriers have been investigated to achieve this goal, including liposomes, microparticles (MPs), and nanoparticles (NPs) (Morgen et al., 2013). The use of MPs and NPs, *e.g.*, albumin- or poly(lactic-*co*-glycolic acid) (PLGA)-based systems has been investigated (Gerwin et al., 2006). Horisawa et al. (2002) described that PLGA NPs should be more suitable for the delivery into the inflamed synovial tissue than MPs due to their ability to penetrate synovium. PLGA-based particles can provide local therapy actions in joint diseases in a different manner depending on the particle size (Horisawa et al., 2002). Other examples of sCT sustained

* Corresponding author. Tel.: +353 1 896 2787; fax: +353 1 896 2810.
E-mail addresses: lidia.tajber@tcd.ie, ltajber@tcd.ie (L. Tajber).

http://dx.doi.org/10.1016/j.ijpharm.2014.10.023
0378-5173/

release systems, but not indicated for IA delivery, includemonolithic depot formulations prepared using lactide:glycolide copolymers (Millest et al., 1993), poly(ethylene glycol)-terephthalate and hydrophobic poly(butylene terephthalate) matrices (van Dijkhuizen-Radersma et al., 2002) and PLGA microspheres incorporated into calcium phosphate cement (Zhong et al., 2012).

Hyaluronate (HA) is administered *via* the IA route in OA to help restore viscoelastic properties of synovial fluid (*e.g.*, Synvisc injections), but HA has also been demonstrated to have a multiplicity of biological actions on cells *in vitro*, *e.g.*, anti-inflammatory and direct anti-nociceptive effects (Gerwin et al., 2006). HA is also an ingredient of synovial fluid and it is known to interact with CD44 receptors of the cells, especially chondrocytes, playing an important role in functions of cartilage (Ishida et al., 1997). One of the attempts to increase the retention time of drugs within the knee cavity and to improve the interactions between cells and particles is the use of HA-functionalised poly(lactic acid)-PLGA particles (Zille et al., 2010).

When developing an effective nanoparticulate delivery system, the bioactive loading is a key parameter as often low loading limits the use of such systems, because a substantial amount of the formulation must be administered to achieve the therapeutic effect. This is a disadvantage of *e.g.*, PLGA-based calcitonin NPs. Although the peptide can be very efficiently associated with particles, and for instance Yang et al. (2012) achieved up to 96.7% of loading efficiency, a very low peptide loading, ranging from 0.1 and 0.2% (Yang et al., 2012 and Glowka et al., 2010) to 1.55% (Kawashima et al., 2000) has been achieved. Other common disadvantages of lipid or PLGA-based NPs are the use of surfactants and organic solvents for their preparation. Cetin et al. (2012) produced Eudragit® and Eudragit®-PLGA NPs with a considerably higher sCT loading (upto 9.9%), but surfactants and organic solvents were used in their production, which may adversely affect the peptide stability and activity.

Polyelectrolyte complex NPs offer an attractive alternative, as they do not require organic solvents or surfactants in their manufacturing process. Makhlof et al. (2010) developed calcitonin-loaded NPs by the method of ionic gelation of chitosan (CS)-thioglycolic acid polymer conjugate with tripolyphosphate. Although the process of particle preparation is simple and organic solvent free, the use of a novel polymer raises the need for complicated toxicity studies. Therefore, employment of polymers already approved for the use in drug delivery provides an attractive option.

We have recently, presented studies on an sCT-based nanocomplex able to reduce experimental inflammatory arthritis when delivered intra-articularly in an osteoarthritic murine *in vivo* model (Ryan et al., 2013). The nanocomplex was prepared by polyelectrolyte complex formation between HA, sCT, and CS. The study of Ryan et al. (2013) clearly confirmed therapeutic efficacy and anti-inflammatory effects of sCT and HA by reducing nuclear receptor subfamily 4, group A, member 2 (NR4A2) mRNA expression *in vitro* as well as anti-arthritic effects *in vivo* following IA delivery. Having observed this interesting pharmacological response of sCT and HA, we, therefore, decided to systematically study the formulation process on the properties of the sCT/HA/CS nanocomplex. The factors investigated included the component mass mixing ratio and HA molecular weight, while the characteristics studied comprised physical properties of NPs, their sCT loading and *in vitro* release properties. A possible complex formation between HA and sCT was proposed but not tested by Umerska et al. (2014) and this work presents the evidence and emphasises the implication of intermolecular interactions between the polyelectrolyte complex nanoparticle constituents.

2. Materials and methods

2.1. Materials

Hyaluronic acid sodium salt (HA) from *Streptococcus equi* sp. (sodium content of 3.6% w/w, Umerska et al., 2012) was purchased from Sigma (USA), while chitosan chloride (CS, molecular weight of 110 ± 7 kDa, chloride residue content 16% and degree of deacetylation of ~83%, Umerska et al., 2012) was obtained from Novamatrix (Norway) as Protasan UP CL113. Salmon calcitonin (sCT, as acetate salt) was obtained from PolyPeptide Laboratories (Denmark). All other reagents, chemicals and solvents were of analytical grade.

2.2. Preparation of HA/CS and HA/CS/sCT NPs

HA and CS solutions with concentrations of 0.1 or 0.2% w/v were prepared in deionised water. HA with molecular weights of 176, 257, and 590 kDa, later referred to as HA176, HA257, and HA590, respectively, were obtained by ultrasonication of native HA (2882 ± 24.50 kDa) as previously described (Umerska et al., 2012). Briefly, processing of HA solutions was performed using a 130 W ultrasonic processor (SONICS VC130PB, Sonics, and Materials Inc., USA) equipped with a probe with a diameter of 3 mm. Sonication was carried out at an amplitude of 80 (power of 13 W) on HA solutions contained in a beaker immersed in an ice bath.

NP carriers (NPs without sCT) were formed by adding a predefined aliquot of CS solution (pH 4) to a known volume of HA solution (pH 6), to form NPs with pre-defined HA/CS mass mixing ratios at room temperature under magnetic stirring. The stirring was maintained for 10 min to allow stabilization of the system. A dispersion of particles was instantaneously obtained upon mixing of polymer solutions.

NPs containing sCT were formed following the above procedure. An appropriate quantity of the peptide, resulting in the final sCT concentration in the NP dispersion of 0.1, 0.2, 0.35, 0.5, and 1.0 mg/ml, was dissolved in the HA solution prior to mixing with the CS solution. The ratios reflecting the total number of negatively charged ionisable groups (n^-) to the total number of positively charged ionisable groups (n^+) were calculated considering the counterion content and the degree of deacetylation for chitosan and are presented in Table 2.

2.3. Characterization and stability of NPs

2.3.1. Transmittance measurements

Transmittance of NP dispersions was measured using a UV-1700 PharmaSpec UV–vis spectrophotometer (Shimadzu, Japan) at a wavelength of 500 nm in quartz cuvettes (Hellma, Germany) with the light path of 10 mm (Umerska et al., 2012).

2.3.2. Particle size and zeta potential analysis

The intensity-averaged mean particle size (hydrodynamic particle diameter) and the polydispersity index of the NPs were determined by dynamic light scattering (DLS) with 173° backscatter detection. The electrophoretic mobility values were measured by Laser Doppler Velocimetry (LDV) and converted to zeta potential using the Smoluchowski equation. DLS and LDV measurements were carried out on a Zetasizer Nano series Nano-ZS ZEN3600 fitted with a 633 nm laser (Malvern Instruments Ltd., UK) as previously described (Umerska et al., 2012). Samples, in their native dispersions, were placed into the folded capillary cells without dilution. Each analysis was carried out at 25 °C with the equilibration time set to 5 min. The readings were repeated at least three times for each batch and the average values of at least three