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Systemic heparin delivery by the pulmonary route using chitosan and glycol chitosan nanoparticles

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ABSTRACT

The aim of this study was to evaluate the performance of chitosan (CS) and glycol chitosan (GCS) nanoparticles containing the surfactant Lipoid S100 for the systemic delivery of low molecular weight heparin (LMWH) upon pulmonary administration. These nanoparticles were prepared in acidic and neutral conditions using the ionotropic gelation technique. The size and zeta potential of the NPs were affected by the pH and also the type of polysaccharide (CS or GCS). The size (between 156 and 385 nm) was smaller and the zeta potential (from +11 mV to +30 mV) higher for CS nanoparticles prepared in acidic conditions. The encapsulation efficiency of LMWH varied between 100% and 43% for the nanoparticles obtained in acidic and neutral conditions, respectively. X-ray photoelectron spectroscopy studies indicated that the surfactant Lipoid S100 was localized on the nanoparticle's surface irrespective of the formulation conditions. *In vivo* studies showed that systems prepared in acidic conditions did not increase coagulation times when administered to mice by the pulmonary route. In contrast, Lipoid S100-LMWH GCS NPs prepared in neutral conditions showed a pharmacological efficacy. Overall, these results illustrate some promising features of CS-based nanocarriers for pulmonary delivery of LMWH.

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1. Introduction

Low molecular weight heparin (LMWH) is a linear anionic polysaccharide used as an anticoagulant for the prevention and treatment of deep vein thrombosis, pulmonary embolism and other thromboembolic disorders (Schulman, 2000; Yang et al., 2004). Unfortunately, LMWH exhibits poor oral bioavailability and, consequently, has to be administered *via* parenteral route. Due to poor patient compliance and side effects associated with injections, alternative routes for non-invasive LMWH administration have been actively investigated (Motlekar and Youan, 2006). Among those, the pulmonary route has attracted notable interest as a potential strategy to deliver therapeutically useful amounts of the anticoagulant (Qi et al., 2004). The large alveolar surface area available for drug absorption, the low thickness of the epithelial barrier, its extensive vascularization and relatively low proteolytic activity make pulmonary delivery of drugs of particular interest even for chronic therapy (Hussain et al., 2003; Labiris and Dolovich, 2003; Craparo et al., 2011; Licciardi et al., 2012). Moreover, it has also been observed that LMWH itself causes, upon pulmonary administration, a transient opening of the tight junctions in the lung epithelium, leading to a rapid onset of action and a C_{max} comparable to subcutaneous administration (Qi et al., 2004). Nevertheless, beyond these positive aspects, it is believed that, without the use of penetration enhancers and adequate delivery vehicles, the amount of LMWH overcoming the pulmonary barriers and reaching the systemic circulation might be insufficient for an adequate pharmacological response.

Among the different pulmonary drug delivery vehicles, chitosan (CS)-based nanoparticles are particularly attractive. Indeed, besides promising properties including low toxicity, biocompatibility and high loading of hydrophilic molecules (Garcia-Fuentes and Alonso, 2012; Ieva et al., 2009), CS shows excellent mucoadhesive characteristics and it is capable of opening the tight junctions of epithelial cells, thereby improving the uptake of hydrophilic drugs (Mao et al., 2010). A CS derivative conjugated with ethylene glycol branches, *i.e.*, glycol chitosan (GCS), which is water soluble at neutral and acidic pH values, has also been described (Siew et al., 2012). In addition to its adequate biocompatibility, GCS has been reported to

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retain the mucoadhesive properties inherent to CS (Trapani et al., 2009; Makhlof et al., 2010).

There are some previous reports on the potential of CS nanoparticles for the local and systemic delivery of macromolecules following pulmonary administration. For example, nanoparticles made of CS and hyaluronic acid (HA) have been used for local heparin delivery to the lungs (Oyarzun-Ampuero et al., 2009). The results of this work have shown that CS–HA nanoparticles are able to enhance the inherent ability of heparin to block the degranulation of mast cells (Oyarzun-Ampuero et al., 2009). Similarly, NPs made of CS or GCS have been recently described for pulmonary delivery of DNA (Bivas-Benita et al., 2004) and therapeutic peptides such as insulin (Al-Qadi et al., 2012) or calcitonin (Makhlof et al., 2010).

Taking this into account, the aim of the present work was to develop CS and GCS nanoparticles containing LMWH and to evaluate their performance following pulmonary administration. In addition, we found it critical to incorporate the non ionic surfactant Lipoid S100 to the nanoparticle's structure. Our hypothesis was that the co-encapsulation of LMWH and the penetration enhancer Lipoid S100 in CS and GCS nanoparticles would favour for the pulmonary absorption of macromolecules (Hussain et al., 2003). Moreover, Lipoid S100, being a mixture of natural phospholipids, was thought to improve the biocompatibility of the nanosystems in contact with the alveolar surface. Ultimately, we hypothesized that, by combining the mucoadhesive characteristics of the polymers with the nanoscale dimensions and the absorption enhancing properties of the surfactant and polymers, we could significantly enhance the pulmonary absorption of LMWH. In addition, nanoencapsulation was also conceived as a way to protect the anticoagulant drug from possible enzymatic degradation.

2. Materials and methods

2.1. Materials

The following chemicals were used as received. Chitosan hydrochloride salt (Protasan, UP CL 113, Mw 110 kDa, deacetylation degree 86%, viscosity = 13 mPa/s according to manufacturer data sheet) was purchased from Pronova Biopolymer (Norway). Lipoid S100 was kindly donated by Lipoid KG (Germany). LMWH (average MW 18 kDa, 177 UI/mg), glycol chitosan (MW 400 kDa according to supplier instructions), mucine from porcine stomach (type II, bound sialic acid, ~1%), glycerol, and pentasodium tripolyphospate (TPP) were purchased from Sigma–Aldrich (Milan, Italy). Ultrapure water was used throughout the study. All other standard chemicals were of reagent grade.

2.2. Analytical determination of low molecular weight heparin

The heparin amounts were directly measured using two colorimetric assays (*i.e.*, Stachrom® Heparin, Diagnostica Stago, Italy (Oyarzun-Ampuero et al., 2009) and Azure A colorimetric method (Ramadan et al., 2011). These assays were performed according to the manufacturer's instructions and linearity was checked in the 140 μg/mL to 1.0×10^{-1} μg/mL concentration range. For Azure A colorimetric assay, the limit of quantification (LOQ) of LMWH was 0.9×10^{-1} μg/mL.

2.3. Preparation of nanoparticles

LMWH loaded CS or GCS-based NPs were prepared according to the ionic gelation technique, as previously reported (Ieva et al., 2009; Mao et al., 2010; Calvo et al., 1997).

2.4. Unloaded CS (and GCS) NPs

CS NPs were spontaneously formed by adding 1 mL of TPP (0.1%, w/v, in NaCl aqueous solution (87 mM)) to 3 mL of CS solution (0.20% (w/v) in NaCl aqueous solution (87 mM) under magnetic stirring (VWR, VMS C-4, Italy). Such concentration of NaCl was previously found to allow the formation of small size CS-based NPs (Goycoolea et al., 2009). The final CS/TPP mass ratio was 6/1. GCS NPs were obtained by mixing 3 mL of TPP aqueous solution (0.07%, w/v) to 3 mL of GCS (0.2%, w/v) dissolved in acetic acid (0.1%, v/v). The final GCS/TPP ratio was 2.9/1 (Gan et al., 2005). For Lipoid S100-containing CS (and GCS) NPs, the surfactant was dispersed to give a final concentration of 0.1% (w/v) in 87 mM NaCl aqueous solution. Lipoid S100 CS NPs were prepared by mixing 0.5 mL of TPP (0.2% (w/v) in 87 mM NaCl aqueous solution) with an aliquot of 0.2 mL of the dispersion of Lipoid S100. The resulting mixture was used to crosslink 3 mL of CS solution (0.20% (w/v) in 87 mM NaCl aqueous solution) under stirring, and thus NPs were formed.

For GCS based NPs (*i.e.*, Lipoid S100 GCS pH 4.3), 3 mL of TPP aqueous solution (0.07%, w/v) were mixed with an aliquot of 0.1 mL of the dispersion of Lipoid S100. The resulting mixture was used to cross-link 3 mL of GCS solution (0.20%, w/v) previously dissolved in acetic acid (0.1%, v/v).

2.5. LMWH loaded NPs

Lipoid S100-LMWH CS NPs were formulated starting from the anionic bearing species consisting of 0.2 mL of Lipoid S100, 0.2 mL of LMWH solution and 0.5 mL of TPP (0.2%, w/v, in 87 mM NaCl aqueous solution). Such mixture was used to cross-link 3 mL of CS solution (0.20%, w/v, in 87 mM NaCl aqueous solution).

Lipoid S100-LMWH GCS NPs were formulated starting from the anionic bearing species consisting of 0.4 mL of Lipoid S100, 0.2 mL of LMWH solution and 1.8 mL of TPP (0.07%, w/v). The resulting mixture was used to cross-link 3 mL of GCS solution (0.20%).

It should be noted that all the nanosuspensions prepared as above displayed acidic pH values (*i.e.*, 3.8 and 4.3 for Lipoid S100-LMWH CS and Lipoid S100-LMWH GCS, respectively). We have also prepared Lipoid S100-LMWH GCS NPs upon neutral conditions. The corresponding Lipoid S100-LMWH CS NPs were not formulated due to the poor water solubility of CS at pH > 6.5. Lipoid S100-LMWH GCS NPs were formed as follows. Briefly, 1.5 mL of TPP aqueous solution (0.07%, w/v) were mixed with an aliquot of 0.52 mL of the dispersion of Lipoid S100 and an aliquot of 0.52 mL of LMWH aqueous solution. The resulting mixture was used to cross-link 8.0 mL of GCS solution (0.20%, w/v) previously dissolved in Tris (10 mM, pH 7).

NPs were isolated by ultracentrifugation (16,000 × g, 45 min, Eppendorf 5415D, Germany) using a glycerol bed in order to facilitate their resuspension in ultrapure water by manual shaking.

2.6. Physicochemical characterization of nanoparticles

For all tested NPs, the mean particle size and distribution were determined in double distilled water by photon correlation spectroscopy (PCS) using a Zetasizer NanoZS (ZEN 3600, Malvern, Herrenberg, Germany). Measurements were performed in triplicate after dilution 1:1 (v/v) in double distilled water and at 25 °C. The autocorrelation functions were analyzed using the DTS v. 5.1 software provided by Malvern. For the determination of zeta-potential values, a laser Doppler velocimetry was adopted by using Zetasizer NanoZS after dilution with 1 mM KCl (pH 7.0) (Montenegro et al., 2012; De Giglio et al., 2012).

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