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Research Paper

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

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ABSTRACT

A new type of self-assembled polyelectrolyte complex nanocarrier composed of chondroitin (CHON) and protamine (PROT) was designed and the ability of the carriers to bind salmon calcitonin (sCT) was examined. The response of sCT-loaded CHON/PROT NPs to a change in the properties of the liquid medium, e.g. its pH, composition or ionic strength was studied and *in vitro* peptide release was assessed. The biocompatibility of the NPs was evaluated in Caco-2 cells.

CHON/PROT NPs were successfully obtained with properties that were dependent on the concentration of the polyelectrolytes and their mixing ratio. X-ray diffraction determined the amorphous nature of the negatively charged NPs, while those with the positive surface potential were semi-crystalline. sCT was efficiently associated with the nanocarriers (98–100%) and a notably high drug loading (13–38%) was achieved. The particles had negative zeta potential values and were homogenously dispersed with sizes between 60 and 250 nm. CHON/PROT NPs released less than 10% of the total loaded peptide in the first hour of the *in vitro* release studies. The enthalpy of the decomposition exotherm correlated with the amount of sCT remaining in NPs after the release experiments. The composition of medium and its ionic strength was found to have a considerable influence on the release of sCT from CHON/PROT NPs. Complexation to CHON markedly reduced the toxic effects exerted by PROT and the NPs were compatible and well tolerated by Caco-2 cells.

1. Introduction

Considerable efforts have been dedicated towards incorporation of bioactive ingredients into nanoparticles (NPs) composed of biodegradable polymers [24]. There are a considerable number of polymers and techniques that are used to produce NPs, which allows a broad differentiation of their internal and external structures as well as composition and biological properties. The choice

Abbreviations: AB, acetate buffer; AE, association efficiency; ANOVA, one-way analysis of variance; Arg, arginine; ATR-FTIR, attenuated total reflectance Fourier transform infrared spectroscopy; CHON, chondroitin; COM1, complex 1, composition: chondroitin/protamine mass mixing ratio = 3.1, final chondroitin concentration = 0.7 mg/ml; COM2, complex 2, composition: chondroitin/protamine mass mixing ratio = 3.1, final chondroitin concentration = 1.4 mg/ml; COM3, complex 3, composition: chondroitin/protamine mass mixing ratio = 12.5, final chondroitin concentration = 1.4 mg/ml; COM4, complex 4, composition: chondroitin/protamine mass mixing ratio = 3.1, final chondroitin concentration = 2.1 mg/ml; COM5, complex 5, composition: chondroitin/protamine mass mixing ratio = 5, final chondroitin concentration = 3.6 mg/ml; COM6, complex 6, composition: chondroitin/protamine mass mixing ratio = 0.2, final chondroitin concentration = 0.16 mg/ml positively charged nanoparticles; dH, enthalpy of process; DL, drug loading; DSC, differential scanning calorimetry; FBS, foetal bovine serum; HA, hyaluronic acid; HPLC, high performance liquid chromatography; kDa, kilodalton; kV, kilovolt; mA, milliampere; MEM, Eagle's Minimal Essential Medium; MMR, mass mixing ratio; MPS, mean particle size; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium; MWCO, molecular weight cut-off; NP, nanoparticle; PBS, phosphate-buffered saline; PDI, polydispersity index; PROT, protamine; PXRD, powder X-ray diffraction; sCT, salmon calcitonin; Tg, glass transition; TR, transmittance; ZP, zeta potential.

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of the nanoparticle manufacturing method is influenced by the solubility of the active compound to be associated/complexed with the NPs as well as the solubility, chemical structure, characteristic chemical groups, molecular weight and crystallinity/amorphicity of the polymer [18]. The most commonly used polymers are polyesters (e.g. poly(lactic acid) and poly(lactic-co-glycolic acid)), either alone or in combination with other polymers [18]. However, the limitation of biodegradable water-insoluble polymers is that they are mostly hydrophobic, whereas nucleic acids, many peptides and proteins, which are recognised to have a great potential in therapeutics, are hydrophilic. This leads to difficulties for the drug to be efficiently encapsulated [48]. Hence, the preparation of NPs with the employment of more hydrophilic and naturally occurring polymers has been explored. Amongst polymeric NPs, those composed of polyelectrolytes (polyelectrolyte complex NPs or nanoplexes) attract particular attention e.g. because of their water soluble character [25]. Amongst cationic polymers used in the formation of nanoplexes, undoubtedly chitosan is the most extensively investigated [4]. Recently, other polycations have also been employed in the formation of nanoplexes, e.g. polyarginine [37] and protamine [52]. Protamine is a naturally occurring and strongly charged cationic protein already used in formulations containing insulin [2]. Protamine (PROT) is rich in arginine and displays a membrane translocation activity [43]. PROT offers a long history of use and established biological effects and safety in humans [43]. It has been demonstrated to form polyelectrolyte complexes with oligonucleotides [27,23] and glycosaminoglycans: hyaluronic acid (HA) [52] and heparin [33].

The recently described HA/PROT NPs have been shown to successfully encapsulate salmon calcitonin (sCT) with the association efficiency up to 100% and the advantage of high peptide loading (10–40% w/w) [52]. However, the release of sCT was relatively quick as most of the peptide associated with the particles was released within 2–4 h due to the weak electrostatic interactions between the species forming the NPs. Thus the strengthening of intermolecular interactions may decrease the release rate of sCT. Chondroitin sulphate (CHON) can be considered as a suitable candidate to form polyelectrolyte complexes with PROT and also with sCT. CHON has weak (carboxylate) and strong (sulphate) acid residues, in contrast to HA, which only has carboxylate groups. Moreover, the charge density in CHON molecules is higher than in HA [17]. Therefore it is anticipated that the electrostatic interactions between CHON and PROT as well as CHON and a cationic sCT will be stronger compared to HA-based interactions. Due to its acidic nature CHON is able to produce ionic complexes with positively charged molecules. Indeed, similar to HA, CHON has been shown to form polyelectrolyte complexes with chitosan [17,42], trimethylchitosan [42], lysozyme [54] and polyethylenimine [41].

CHON is an abundant glycosaminoglycan found in cartilage, bone and connective mammalian tissue. It exhibits a wide variety of biological functions and is currently used as an anti-inflammatory, chondroprotective and antirheumatic drug. CHON has been shown to be absorbed after oral administration in humans as a high molecular weight polysaccharide [55]. sCT, currently recommended for short term use in Paget's disease, acute bone loss due to sudden immobilisation and hypercalcaemia caused by cancer [20], has also been considered as a promising candidate to be used in osteoarthritis [32] and in combined therapy with alendronate in patients with rheumatoid arthritis [38]. The biological and pharmacological properties of sCT are therefore complementary to those of CHON.

A combination of CHON with its anti-inflammatory and chondroprotective action and PROT, due to its membrane-translocating activity, may be interesting from the therapeutic point of view and such hybrid CHON/PROT NPs may have the potential to form carriers for the oral delivery of peptides, in particular sCT. Patient compliance was identified as one of the major issues of long-term therapies involving parenteral administration of peptides; hence, developing such a delivery system is of significance [31]. The low bioavailability of sCT after oral administration has been attributed to proteolytic enzymatic degradation and low intrinsic intestinal membrane permeability [31]; however, a correlation between enhancement of sCT absorption and mucoadhesion in rats was found by Sakuma et al. [44,45]. The Sakuma's delivery system comprised NPs with hydrophilic, ionic polymeric chains attached to the NP surface and sCT incorporated in the NPs non-covalently. These NPs also protected the peptide against digestive enzymatic degradation *in vitro* and shielding sCT from pepsin and trypsin was also observed for polymeric HA/PROT NPs [52].

Considering the above and no drug delivery system, especially in the nanoparticulate format, comprising CHON and PROT has been reported to date, the aims of the current work were to investigate the conditions of such carrier formation by adopting the previously presented manufacturing process [51,52,53], to evaluate the conditions of NP formation and their properties as well as to explore the ability of CHON/PROT NPs to bind and release sCT. Bearing in mind that CHON/PROT NPs are polyelectrolyte complex NPs, their potential as extended/controlled drug release systems was also studied and evaluation of suitability of solid-state techniques, as methods supporting the peptide release studies, was performed.

2. Materials and methods

2.1. Materials

Chondroitin 4-sulphate sodium salt (CHON) and protamine sulphate (PROT, molecular weight of 5.1 kDa; manufacturer's data) were purchased from Sigma (Ireland). Salmon calcitonin (sCT, molecular weight 3.4 kDa, freely soluble in water, isoelectric point of 8.86 [49] and net charge at pH 7.4 of approximately 3+) was obtained from PolyPeptide Laboratories (Denmark). CellTiter 96® Non-Radioactive Cell Proliferation Assay was obtained from Promega Corporation (USA). Other cell culture reagents were provided by Sigma Aldrich (Ireland). All other reagents, chemicals and solvents were of analytical grade.

The molecular weight of CHON was determined using a gel permeation chromatography system previously described [51]. Briefly, CHON was dissolved in a mobile phase composed of 0.2 M NaCl and 0.01 M NaH_2PO_4 brought to pH 7.4 with NaOH solution. Pullulan standards (PL Polymer Laboratoires, Germany) were used to construct the calibration curve. Standards and samples were prepared as 1 mg/ml solutions in the mobile phase. 100 µl of the standard or sample was injected into the Plaquagel-OH mixed 8 µm 300 × 7.5 mm column (Polymer Laboratories Ltd., UK) using a flow rate of 1 ml/min. A Waters 410 refractive index detector was employed. Data collection and integration were accomplished using CLASS-VP software (version 6.10) with GPC for Class VP (version 1.02) (Shimadzu, Japan). The average molecular weight (Mw) of CHON was 58.6 ± 0.23 kDa.

2.2. Preparation of CHON/PROT carriers and CHON/PROT/sCT NPs

The CHON solutions with concentrations of 1, 2, 3 or 5 mg/ml as well as the PROT solutions with concentrations of 0.4–12 mg/ml were prepared in deionised water. NP carriers (NPs without the cargo) were formed by adding 4 ml of an aqueous PROT solution to 10 ml of a CHON solution at room temperature under magnetic stirring. The stirring was maintained for 10 min to allow stabilisation of the system. A dispersion of particles was instantaneously obtained upon mixing of the polymer solutions. As a

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