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Hyaluronic acid-coated chitosan nanoparticles: Molecular weight-dependent effects on morphology and hyaluronic acid presentation

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ABSTRACT

Chitosan nanoparticles are popular carriers for the delivery of macromolecular payloads, *e.g.* nucleic acids. In this study, nanoparticles were prepared *via* complexation with triphosphate (TPP) anions and were successively coated with hyaluronic acid (HA). Key variables of the preparative process (*e.g.* chitosan and HA molecular weight) were optimised in view of the maximisation of loading with DNA, of the Zeta potential and of the dimensional stability, and the resulting particles showed excellent storage stability.

We have focused on the influence of chitosan molecular weight on nanoparticle properties. Larger molecular weight increased their porosity (= decreased cross-link density), and this caused also larger dimensional changes in response to variations in osmotic pressure or upon drying. The dependency of nanoparticle porosity on chitosan molecular weight had a profound effect on the adsorption of HA on the nanoparticles; HA was apparently able to penetrate deeply into the more porous high molecular weight (684 kDa) chitosan nanoparticles, while it formed a corona around those composed of more densely cross-linked low molecular weight (25 kDa) chitosan. Atomic Force Microscopy (AFM) allowed not only to highlight the presence of this corona, but also to estimate its apparent thickness to about 20–30 nm (in a dry state). The different morphology has a significant effect on the way HA is presented to biomolecules, and this has specific relevance in relation to interactions with HA receptors (*e.g.* CD44) that influence kinetics and mechanism of nanoparticle uptake.

Finally, it is worth to mention that chitosan molecular weight did not appear to greatly affect the efficiency of nanoparticle loading with DNA, but significantly influenced its chitosanase-triggered release, with high molecular chitosan nanoparticles seemingly more prone to degradation by this enzyme.

1. Introduction

Chitosan, a linear random copolymer of β-1,4-D-glucose-2-amine and N-acetyl-D-glucose-2-amine, is a versatile biomaterial obtained through the deacetylation of chitin, which is abundantly present in crustacean shells [1], in insect exoskeletons [2] and also in fungal cell walls [3]. Chitosan is generally regarded as a biocompatible [4] and degradable material [5] and is employed in a wide range of applications from tissue engineering [5] to food science [6], specifically including micro- and nano-carrier-mediated delivery of payloads, generally of macromolecular nature [7,8]. In the latter area, the ionotropic gelation with triphosphate (TPP) is a popular method to obtain chitosan nanoparticles [8], due to the ease of performance and the rather benign character both of the polyanion and of the process (absence of chemical reaction, use of only mildly acidic water solutions, *etc.*). Chitosan–TPP nanoparticles typically exhibit a cationic surface, which determines a quick and unselective cellular uptake, above all in phagocytic cells [9]. However, chemical modification of chitosan or decoration with polyanions [10,11] allows for the preferential uptake of the nanoparticles in specific cell types. For example, mannosylated and galactosylated chitosans were used to target dendritic cells and hepatocytes respectively [12,13].

The chitosan/TPP weight ratio is possibly the most important variable to control particle size, morphology and surface charge [14,15]. However, also chitosan concentration and molecular weight (MW),

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ionic strength and pH of the medium play a significant role [16–20] and have been shown to affect the long-term stability of the particles [21–23]. In particular, it is worth pointing out that differences in the degree of deacetylation and/or molecular weight confer to chitosan considerably different physico-chemical properties, which affect its complexation ability [24], the toxicity of its nanoparticles (reportedly lower with decreasing degree of deacetylation and molecular weight) [25], their dimensions (lower with decreasing molecular weight) [26], and their encapsulation efficiency (lower with decreasing molecular weight and with increasing degree of deacetylation), and ultimately also the efficiency in payload delivery *e.g.* of nucleic acids (higher with lower molecular weight) [27] or of proteins (again higher with lower molecular weight) [28].

Our group has previously optimised the conditions for the preparation of nanoparticles through chitosan/TPP electrostatic complexation in diluted solution; *inter alia* we have also showed the importance of variables such as order of addition and mixing of the solutions with identical or different pH [29]. Chitosan/TPP nanoparticles can be coated with hyaluronic acid (HA) to render their surface anionic and in principle biofunctional. The advantages of HA decoration are: A) resistance to protein adsorption [30,31], which is accompanied by prolonged circulation times [32] and therefore confers a relative "stealth" character; we have previously confirmed these effects, showing that macrophages uptake HA-coated chitosan nanoparticles at 2–3 orders of magnitude lower rate than their parent uncoated nanoparticles [9]. B) The possibility to target cells overexpressing HA receptors, such as CD44 [33], which is a possible strategy to target activated inflammatory cells [34,35] or certain types of tumours [36,37]. Indeed we have recently demonstrated that HA-coated nanoparticles are uptaken in a CD44-mediated manner in macrophages [38].

In the present study, we have investigated the effect of chitosan molecular weight on the morphology/structure of chitosan–TPP nanoparticles; further, we have studied how the resulting differences may influence the process of HA adsorption, which can significantly affect its presentation to receptors and thus also nanoparticle cell uptake [38]. We have also assessed whether the coating process is influenced by the presence of a nucleic acid payload, salmon sperm DNA (2000 bp $\approx$ 1.2–1.3 $*$ 10^3 kDa); this is a popular model [39,40] since it offers an intermediate size between plasmid DNA and oligos/siRNA and its ready availability allows studies of maximum loading capacity.

2. Materials and methods

2.1. Materials

Middle viscosity chitosan (CS, average viscosimetric molecular weight ($\overline{M_v}$) = 684 kDa, deacetylation degree $\approx$ 84% mol, from crab shells; Sigma, UK), Quantipro BCA assay kit, bovine serum albumin (BSA), foetal bovine serum (FBS), sodium nitrite ($NaNO_2$), 1 M hydrochloric acid (HCl), 1 M sodium hydroxide (NaOH) and sodium triphosphate pentabasic (TPP) were obtained from Sigma-Aldrich (Gillingham, UK); 10 mM phosphate buffered saline (PBS) was prepared from appropriate tablets (Oxoid, Basingtoke, UK); hyaluronic acid (HA) with $\overline{M_v}$ = 15, 60, 360, and 1000 kDa was obtained from Medipol SA (Lausanne, Switzerland). Salmon sperm DNA was purchased from Invitrogen (CA, USA). Chitosanase from *Streptomyces griseus* and ovine testis hyaluronidase were obtained respectively from Sigma-Aldrich (Gillingham, UK) and Calbiochem (Gibbston, NJ); PicoGreen® was obtained from Molecular Probes (OR, USA). Glacial acetic acid and sodium acetate were purchased from VWR BDH Chemicals (Poole, UK). The other chemicals were of reagent grade and were used as received.

2.2. Preparative procedures

Chitosan purification, depolymerisation and characterisation are described in Supporting information, Section 1 and Figs. S1 and S2.

2.2.1. Preparation of chitosan/TPP (CS–TPP) nanoparticles

CS–TPP nanoparticles were prepared according to a previously reported procedure [29]. The pH of a 0.069% wt. CS solution in 4.6 mM HCl was adjusted to 5 by the addition of appropriate volumes of NaOH 0.1 M and the solution was sonicated (Branson 200 ultrasonic cleaner, 40 kHz) for 40 min. TPP was prepared as a 0.1% wt. solution in deionised water, correcting the pH to 5 using appropriate volumes of HCl 0.1 M. Both solutions were filtered through a 0.22 μm pore size filter. For a final volume of 3 mL of CS–TPP nanoparticles, 214 μL of TPP solution was pipetted into 2786 μL of CS solution, where the final concentrations of CS and TPP are 0.064 and 0.0071% wt., respectively, resulting in a 9:1 mass ratio of CS:TPP. The complexation was carried under magnetic agitation (750 rpm), for 30 min at 25 °C. The final dispersion was sonicated for 40 min and then left undisturbed for an additional 16 h. Then the nanoparticle dispersion was dialysed against deionised water (MWCO 1000 kDa). For any further experiments, the concentration of nanoparticle dispersions was assessed by sampling a known volume after dialysis and measuring the residual weight after freeze drying.

2.2.2. Preparation of CS–TPP//HA nanoparticles

In a typical experiment, 2 mL of a 0.025% wt. dispersion of CS–TPP nanoparticles in 100 mM acetic buffer at pH = 5 was added under vigorous magnetic stirring (30 min, 1200 rpm) to an equal volume of a 1.5, 1.0, 0.5 or 0.1 mg/mL solution of 1000, 360, 60 or 15 kDa hyaluronic acid in the same buffer. The dispersions were then dialysed against deionised water or PBS (MW cut-off 1000 kDa).

The procedures for the encapsulation of DNA and BSA are described in Supporting information, Section 2, Figs. S3, S4 and S5, and Table S1.

2.3. Characterisation

2.3.1. Nuclear magnetic resonance (^{1}H NMR)

The samples were prepared by dissolving 10 mg of CS in 0.5 mL of 0.5 M DCl in D_2O. ^{1}H NMR spectra were recorded at room temperature on a JEOL EX300 300 MHz NMR spectrometer (Bruker Avance 300, Coventry, UK).

2.3.2. Viscometry

Viscosity measurements were performed in a 0.25 M acetic acid/0.25 M sodium acetate solution using a falling ball automated microviscometer (Anton Parr, Graz, Austria) at 25 °C equipped with a 1.6 mm internal diameter capillary tube at an inclination angle of 30 °C. $\overline{M_v}$ was calculated assuming the parameters of the Mark–Houwink equation for CS to be equal to $K = 1.57 \times 10^{-5}$ L/g and a = 0.79 [41].

2.3.3. Dynamic light scattering (DLS)

Hydrodynamic diameter (Z-average size), size polydispersity (PDI), derived count rate (DCR) and zeta potential measurements were always performed on three independent samples at a temperature of 25 °C using a Zetasizer Nano ZS instrument (Model ZEN3600, Malvern Instruments Ltd., UK) equipped with a solid state HeNe laser (λ = 633 nm) at a scattering angle of 173°.

2.3.4. Atomic force microscopy (AFM)

A drop ($\approx$ 50 μL) of a 50 μg/mL nanoparticle dispersion in deionised water was deposited on a mica surface and left to dry overnight by slow evaporation at room temperature and pressure. A Molecular Force Probe 3D AFM (MFP-169 3D, Asylum Research, Santa Barbara, CA) equipped with a 90 μm scanner and silicon cantilevers (model AC240TS, Olympus; spring constant 2 N/m) was employed in contact mode with a scan rate of 1 Hz.

2.3.5. Analysis of AFM data

2.3.5.1. Substrate. The original images (without flattening or filtering) were analysed with the Igor-pro software (Asylum Research AFM