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Nanoparticles based on naturally-occurring biopolymers as versatile delivery platforms for delicate bioactive molecules: An application for ocular gene silencing

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ABSTRACT

Nanoparticles based on naturally-occurring biopolymers, most of them endogenous macromolecules, were designed as a versatile generation of delivery platforms for delicate bioactive molecules. The design of these nanosystems was specifically based on our recent finding about the ability of endogenous polyamine spermine (SPM) to interact with anionic biopolymers (ABs) generating ionically cross-linked nanosystems.

The initial first generation of these delivery platforms, based on glycosaminoglycans and other polysaccharides, showed a very high association capacity for some delicate bioactive proteins such as growth factors, but a limited capacity to associate negatively charged molecules, such as pDNA and siRNA. However, the versatility of these nanosystems in terms of composition allowed us to customise the association of active ingredients and their physicochemical characteristics. Concretely, we prepared and incorporated gelatine cationized with spermine (CGsp) to their composition. The resulting modified formulations were characterised by a nanometric size (150–340 nm) and offer the possibility to modulate their zeta potential (from −35 to 28 mV), providing an efficient association of nucleic acids. The biological evaluation of these optimised nanosystems revealed that they are able to be internalised *in vivo* into corneal and conjunctival tissues and also to provide a significant siRNA gene silencing effect.

1. Introduction

From a historical perspective, a premise of biomaterial-related sciences, when designing new components for medical and pharmaceutical use, was bioinertness as a major requirement; the material should not provoke any reaction from the body (Peppas and Langer, 1994). However, this former position was reconsidered after it became clear that the use of biomacromolecules could improve targeting towards specific tissues/organs and provide an added biological value (Byrne et al., 2008; Farokhzad and Langer, 2009; Phillips et al., 2010; Pirollo and Chang, 2008). As a consequence, biomaterials with specific abilities to interact with biological structures have recently emerged as new potential components of different devices intended for biomedical use (Kamaly et al., 2012). Within this frame, the interest in some naturally-occurring biomaterials as delivery system components has gained increasing attention. However, when designing delivery systems at the nanoscale level, one of the main limitations of these biomaterials is related to the preparation technique, which usually requires organic solvents or harsh conditions that can modify the natural and desired properties of these biomolecules.

With the aforementioned potentials and limitations in mind, we have recently patented new delivery nanosystems based on

Abbreviations: SPM, spermine; ABs, anionic biopolymers; HA, hyaluronic acid; CA, colominic acid; ChS, chondroitin sulfate; DS, dextran sulfate; HS, heparan sulfate; CGsp, modified cationic gelatine with spermine.

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the capacity of the endogenous polyamine spermine to physically cross-link anionic macromolecules (Sanchez et al., 2010). Of course there are numerous nanoparticles based on ionic cross-linking processes. However, two main differences of the nanosystems proposed here can be mentioned. Firstly, such an invention refers to the "back to nature" nanoparticles, based on natural products. Secondly, as the main features of the developed nanosystems, we should mention that preliminary results suggested their potential versatility in terms of modifying their components and including new ones, which opens up the possibility of a tailored-design of stable nanoparticles with a tunable bio-inspired composition.

The objective of the present work has been to verify and to exploit the aforementioned idea and features to develop nanoparticles based on biopolymers as versatile delivery platforms for delicate bioactive molecules in general, and for siRNA in particular, keeping in mind the recent necessity to develop new nanosystems as effective carriers for such molecules (Blow, 2007; de Fougerolles et al., 2007; Whitehead et al., 2009). For this purpose, we decided to select some endogenous anionic biopolymers (ABs) with high biological value as the initial components in the design of our first nanoparticles prototypes. However, we have also considered that, in general, nanocarriers for nucleic acids delivery share a common characteristic: they are based on positively charged polymers or lipids. These key elements play two main roles. First, they provide physical interaction between the positively charged moiety and the negatively charged nucleic acid, protecting them from nuclease degradation. Second, they provide a net positive charge, which in turn enables binding of the nucleic acid complex to anionic cell surface macromolecules (Kang et al., 2012; Li and Szoka, 2007). However, instead of incorporating conventional cationic polymers/lipids in the nanoparticles composition to associate the nucleic acid derivatives, we further continued with the aforementioned philosophy concerning the use of safe materials, and tried to improve the capacity of the described nanosystems to associate genetic material by the introduction of gelatine that was previously aminated by the reaction between the carboxylic acid present in its structure and the endogenous polyamine spermine (Zorzi et al., 2011). To understand the difficulty in developing such an approach, it is necessary to remember that the incorporation of proteins or modified proteins into nanoparticulate carriers often requires the use of organic solvents and/or chemical reagents (Maham et al., 2009). Our challenge has been to incorporate the cationized gelatine into the nanoparticles composition while avoiding all these previously mentioned harmful conditions. Finally, the physicochemical characterisation of the developed formulations was performed and they were biologically evaluated in terms of cellular toxicity and efficiency as siRNA carriers.

2. Materials and methods

2.1. Materials

The anionic biopolymers (ABs) used for the development of the nanosystems were hyaluronic acid (HA, 136 kDa – Bioibérica, Spain), colominic acid (CA, 30 kDa – Sigma, Spain), chondroitin-4-sulfate from bovine trachea (ChS, 40 kDa – Calbiochem, CA, USA), dextran sulfate (DS – 40 kDa – TdB Consultancy, Sweden), heparan sulfate (HS) (Sigma, Spain) and gelatine (type A, 137 kDa – Nitta Gelatin, Canada). The polyamine spermine hydrochloride was purchased from Fluka (Spain). The model drugs associated to the nanosystems were albumin (bovine serum albumin), kinetin and bFGF (Sigma, Spain), plasmid that codifies the fluorescent green protein (pEGFP) (Elim Biopharmaceutics, CA, USA), siRNA against GAPDH containing the sequence 5′-UGG UUU ACA UGU UCC AAU ATT-3′ (sense), 3′-UAU UGG AAC AUG UAA ACC ATG-5′(antisense), siRNA against GAPDH labelled with Cy3®, non-specific siRNA against siEGFP containing the sequence 5′-GCA AGC UGA CCC UGA AGU UCTT-3′ (sense), and 5′-GAA CUU CAG GGU CAG CUU GCTT-3′ (antisense), a negative control siRNA containing the sequence 5′-GCA AGC UGA CCC UGA AGU UCTT-3′ (sense) and 3′-GAA CUU CAG GGU CAG CUU GCTT-5′ (antisense) (Ambion, CA, USA). *N*-(3-Dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDC), glycerol and all other chemicals not specified above were of the highest purity available, purchased from Sigma (Spain).

2.2. Preparation and characterisation of the ABs nanoparticles

Polymer solutions of HA (1–1.5 mg/mL), ChS (2–2.5 mg/mL), HS (1.5–2 mg/mL), CA (1–1.2 mg/mL) or DS (1–1.5 mg/mL) were prepared in MilliQ water. The nanoparticles were formed spontaneously by adding an aqueous solution of the cationic spermine (0.1–1.5 mg/mL) over the polyanionic biopolymers solution, under magnetic stirring (volume ratio of 2:1; polymer: spermine). In the case of nanoparticles associating the selected bioactive molecules, these molecules were incorporated depending on their charge either in the anionic polymeric solution (albumin 5 mg/mL, bFGF 100 ng/mL, pDNA 5 mg/mL or siRNA 3 mg/mL) or in the cationic SPM solution (kinetin 1 mg/mL), in order to avoid ionic interactions prior to the formation of nanoparticles. Albumin was incorporated in ChS–SPM and DS–SPM nanoparticles at the theoretical loading of 13% w/w. Kinetin was incorporated in ChS–SPM and HA–SPM nanoparticles at the theoretical loading of 5% w/w. On the other hand, ChS–SPM and DS–SPM nanoparticles were selected to associate the growth factor bFGF at 0.006 and 0.009% w/w, respectively. pDNA and siRNA were incorporated at 5 and 3% w/w, respectively. The yield of production was determined previously for the selected formulations of nanoparticles: ChS–SPM (40:8) 40%, DS–SPM (20:5) 64% and DS–SPM (30:5) 70%, respectively. For kinetin formulations the mass ratio of HA–SPM was 8:1 and ChS:SPM 8:1 (yield of 48 and 52%, respectively).

The mean particle size was determined by photon correlation spectroscopy (PCS). Each analysis was carried out at 25 °C with a detection angle of 173°. The zeta potential was obtained by Laser Doppler Anemometry (LDA). For this purpose, the samples were diluted with a millimolar solution of KCl. Both analyses were performed with a Zetasizer Nano (Malvern, UK).

The association efficacy was calculated indirectly by the difference between the total amount of bioactive molecules incorporated and the amount that was not associated, which remained in the preparation medium. For this purpose, free albumin and bFGF were recovered in supernatant samples collected by centrifugation of the nanoparticles (60 min, 10,000 rcf) (Beckman CR412, Beckman Coulter, Spain). The association efficacy of the albumin was determined using a micro BCA kit (Pierce co, USA) and bFGF was quantified using an ELISA kit (Anogen, Canada). The association efficacy of kinetin was determined by spectrophotometry ($\lambda = 265$ nm) after separation of the free kinetin by ultrafiltration (Amicon Ultra membranes 5000 MWCO, Millipore, Irland) using a centrifuge (11,000 rcf, 30 min) (Beckman CR412, Beckman Coulter, Spain).

The association of siRNA and pDNA to the nanoparticles was determined by an agarose gel electrophoresis assay (see Supplementary Data) ($n = 3$). The % siRNA associated was quantified by Ribogreen® (see Section 2.3.2).

The drug loading content (L.C.) and the association efficiency (A.E.) were calculated using the following equations, respectively: