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Development and characterisation of chondroitin sulfate- and hyaluronic acid-incorporated sorbitan ester nanoparticles as gene delivery systems

I. Fernandez-Piñeiro[a], A. Pensado[a,1], I. Badiola[b], A. Sanchez[a,c,*]

[a] Department of Pharmacy and Pharmaceutical Technology, Faculty of Pharmacy, University of Santiago de Compostela (USC), Campus Vida, 15782 Santiago de Compostela, Spain

[b] Department of Cell Biology and Histology, Faculty of Medicine and Odontology, University of Basque Country, B° Sarriena, s/n, 48940 Leioa, Spain

[c] Genetics and Biology of the Development of Kidney Diseases Unit, Sanitary Research Institute (IDIS) of the University Hospital Complex of Santiago de Compostela (CHUS), Travesía da Choupana, s/n, 15706 Santiago de Compostela, Spain

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ABSTRACT

Glycosaminoglycans (GAGs) are natural polymers that are broadly used in gene delivery systems to increase stability as well as decrease toxicity and nonspecific interactions, thereby increasing transfection efficiency. In this work, we propose sorbitan ester-based lipid nanoparticles (SENS) functionalised with the GAGs chondroitin sulfate (CS) and hyaluronic acid (HA) as gene delivery systems. For this purpose, we describe the design and evaluation of these nanosystems loaded with plasmid DNA, including an evaluation of their physicochemical characteristics, stability properties, ability to protect and efficiently transfect cells with Enhanced Green Fluorescent Protein plasmid (pEGFP) *in vitro,* and biocompatibility both *in vitro* and *in vivo*. We confirm that molecules with high biological value and targeting potential, such as HA and CS, can be successfully incorporated into our recently developed sorbitan ester-based nanoparticles (SENS) and that this incorporation leads to effective stabilisation of both nanosystems as well as protects plasmid DNA. We demonstrated that the aforementioned incorporation of HA and CS enables long-term stability of the nanosystems in both liquid and lyophilised states, which is a remarkable property that can aid in their transfer to industry. The ability of these functionalised nanosystems to transfect the A549 cell line without compromising cell viability was also shown, as well as their innocuous safety profile *in vivo*. Thus, we provide valuable evidence of the suitable properties and potential of these hybrid nanoparticles as gene delivery systems.

1. Introduction

Gene therapy consists of the delivery of nucleic acids to specific cells to replace or silence malfunctioning genes with the aim of curing or favourably altering pathological progression [1]. Deregulation of gene expression is associated with the origin of multiple diseases. Thus, recent advances in identification of the molecular basis of numerous inherited genetic disorders have made them susceptible to gene therapy treatment. Although gene therapy can be applied to treatment and prevention of multiple pathologies, such as infectious diseases, Parkinson's disease or cystic fibrosis, most efforts and clinical trials have been focused on cancer therapy [2]. Cancer comprises a group of diseases with multifactorial origin. Despite considerable advances in the field of cancer therapy, chemical-based therapeutics, radiotherapy and surgery have been insufficient in addressing the diverse cancer aetiologies, leading to a high rate of treatment failure. Gene-based therapies could solve this problem by targeting specific pathways that are altered in cancer while avoiding most of the side effects associated with conventional therapies [3,4].

However, gene delivery to cancer cells remains a major challenge. Nucleic acid delivery must overcome numerous barriers and obstacles before its therapeutic effect can be exerted. Nucleic acids are negatively charged macromolecules with a hydrophilic nature, characteristics that explain their poor ability to penetrate biological barriers and their susceptibility to degradation by enzymes in the body. These properties make necessary the development and inclusion of nucleic acids into gene delivery systems. Non-viral delivery systems have been extensively studied due to their safety, low cost and scalability in comparison with viral vectors [4–6].

The most common traditional non-viral systems can be classified as

* Corresponding author at: Department of Pharmacy and Pharmaceutical Technology, Faculty of Pharmacy, University of Santiago de Compostela (USC), Campus Vida, 15782 Santiago de Compostela, Spain.

E-mail address: alejandro.sanchez@usc.es (A. Sanchez).

[1] Present address: Department of Pharmacy and Pharmacology, University of Bath, Bath, United Kingdom.

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lipidic, polymeric or polymer-lipid hybrid systems [3]. Among them, cationic lipids have been broadly used for gene delivery due to their high efficacy in transfecting a variety of cell types with different types of nucleic acids and their reproducibility and ease of use [7]. However, their positive charge is related to high cytotoxicity, nonspecific uptake and short blood circulation time due to interaction with negatively charged serum proteins and eventual phagocytosis by the reticuloendothelial system (RES). Diverse strategies have been applied in an attempt to shield the positive surface charge and avoid these side effects. Pegylation is known to reduce non-specific interactions of nanosystems; however, this strategy might decrease cellular uptake and gene release [8–10]. Alternatively, surface modification of nanocarriers with anionic macromolecules, such as glycosaminoglycans (GAGs), may lengthen their circulation time by reducing complement activation and thus recognition by the RES [11,12]. Therefore, it has been demonstrated that incorporation of GAGs into gene delivery systems increases the stability of the carriers and decreases toxicity and nonspecific interactions, thereby increasing their transfection efficiency *in vivo* [13–18]. GAGs have also been shown to influence intracellular routing of nanosystems by improving endosomal escape and localisation at the nuclear periphery [19]. In our study, the natural anionic polymers chondroitin sulfate (CS) and hyaluronic acid (HA) were employed to stabilise and functionalise the system by taking advantage of their suitable properties for gene delivery. They are the main components of the extracellular matrix and are considered biocompatible and biodegradable polymers, and thus, they are widely used in the biomedical field [15,20–22]. CS and HA are mucopolysaccharides naturally present in the body, and they are composed of glucuronic acid and N-acetylglucosamine repeats connected via a β-1-4 linkage. A sulfate group in at least one of the CS side groups is the primary structural difference between them. HA is present in connective tissue, vitreous humour and synovial joint fluid, while CS has a role in wound healing and chondrogenesis [23].

Despite the large number of lipid-based nanosystems described in the literature, only a few have reached the market [24]. One of the limitations of lipid nanosystems is their low stability in aqueous suspensions, which compromises their transfection efficiency [25]. Thus, more stable systems are necessary to scale production from basic research to the pharmaceutical industry. In addition, advances in characterisation and manufacturing of the vectors are needed for successful therapy.

In this work, we propose sorbitan ester-based lipid nanoparticles (SENS) functionalised with the natural polymers CS or HA as gene delivery systems, in order to fully characterise and compare the effect of each polymer in the nanoparticle properties. For this purpose, we describe the design and evaluation of these nanosystems loaded with plasmid DNA, including their physicochemical characterisation, their stability properties upon storage at different temperatures in both a suspension and as a lyophilised product, their ability to protect the delicate bioactive molecule associated with them, their transfection properties and their biocompatibility both *in vitro* and *in vivo*.

2. Materials and methods

2.1. Materials

Sorbitan monooleate (Span® 80) (SP), oleylamine (OA) (purity ≥ 70%), glucose, sucrose, and sodium dodecyl sulfate (SDS) were purchased from Sigma (Madrid, Spain). Chondroitin sulfate (CS, from bovine trachea, mixture of chondroitin sulfates A, C, D, and E) and hyaluronic acid (HA, pharmaceutical grade, low molecular weight) were provided by Merck (Madrid, Spain) and Bioiberica (Barcelona, Spain), respectively. The pEGFP-C3 plasmid was obtained from Elim Biopharmaceutics (CA, USA). SYBR Safe DNA Gel Stain and SYBR® Gold post-electrophoresis and agarose were provided by Life Technologies (Madrid, Spain). Dulbecco's modified Eagle's medium (DMEM), foetal bovine serum (FBS), penicillin-streptomycin, L-glutamine, DNase I and Lipofectamine 2000 were acquired from Thermo Fisher (Madrid, Spain). XTT cell viability reagent was provided by Roche (Barcelona, Spain). The aspartate aminotransferase AST colorimetric kit was purchased from Randox (Barcelona, Spain).

2.2. Nanoparticle preparation and characterisation

For construction of the nanoparticles (NPs), a solution of sorbitan monooleate (Span 80, SP) and oleylamine (OA) was prepared in ethanol at a concentration of 6.6 and 0.33 mg/ml, respectively. Then, following our previously used preparation method [26–30], this organic phase was added under magnetic stirring to an aqueous phase containing either chondroitin sulfate (CS) or hyaluronic acid (HA) at a concentration of 0.125 mg/ml in a volume ratio of 1:2. Ethanol was removed under reduced pressure on a rotary evaporator. For encapsulation of Enhanced Green Fluorescent Protein plasmid (pEGFP), the plasmid was incorporated into the aqueous phase. The final formulations obtained were 200 μg/ml pEGFP-loaded NPs.

The mean particle size and the size distribution of the nanoparticles were determined using photon correlation spectroscopy (PCS). Samples were suitably diluted in Milli-Q water. Each analysis was performed at 25 °C with an angle of detection of 173°. The zeta potential of the nanoparticles was determined with laser scattering anemometry (LDA). To that end, the samples were suitably diluted in a millimolar KCl solution. The PCS and LDA analyses were performed with a Zetasizer® 3000HS (Malvern Instruments, Malvern, UK).

The morphology of the nanoparticles was examined with transmission electron microscopy (CM 12 Philips, Eindhoven, The Netherlands) after staining with 2% w/v phosphotungstic acid solution. For this purpose, the samples were placed on copper grids (400 mesh) coated with a Formvar® film.

2.3. Gel electrophoresis

The efficiency of the association of pEGFP with the nanoparticles was determined using agarose gel electrophoresis (Sub-Cell GT 96/192, Bio-Rad Laboratories Ltd., Watford, United Kingdom). Gels with 1% agarose were prepared in TAE (Tris-Acetate-EDTA, 40 mM Tris, 1% acetic acid, 1 mM EDTA, Sigma, Barcelona, Spain). SYBR® Gold Nucleic Acid Gel Stain and SYBR® SAFE DNA Gel Stain were employed as stains, and glycerol was used to aid in loading the gel. A potential difference of 100 mV was applied for 30 min, and free pEGFP was used as the control.

2.4. Lyophilisation and stability studies

Nanoparticles from three different batches were lyophilised in an aqueous solution of cryoprotectant in a 1:1 (v/v) ratio of nanoparticles:cryoprotectant (10% glucose for CS NPs, and 10% sucrose for HA NPs). After sample preparation, vials containing the samples were frozen at −80 °C and further placed on the shelf plates of the freeze-drying equipment (VirTis Genesis 25 ES, S.P. Industries, PA, USA), at a temperature set to −40 °C. The primary drying step (sublimation) was carried out at this temperature under high vacuum (0.3 mBar) for approximately 22 h. Following this step, the temperature gradually rose up to 20 °C (12 h at −30 °C, 5 h at −20 °C and then rising on 10 °C steps of 2 h each). At this point, the secondary drying started maintaining the temperature between 20 and 22 °C for around 3 h under vacuum, to assure the elimination of any residual moisture present in the samples. Afterwards, samples were disposed in sealed eppendorfs to inhibit humidification, and these lyophilised samples, as well as the nanoparticles in suspension, were stored under various temperature conditions (Room Temperature (RT), 4 °C and 37 °C). Besides being the body temperature, 37 °C has been selected taking into account that it is the temperature at which additional *in vitro* experiments were performed, as below