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Spermine modified starch-based carrier for gene delivery: Structure-transfection activity relationships

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ABSTRACT

This study aims at developing starch based gene carriers with low cytotoxicity and high transfection efficiency. Starch molecules with molecular weight of about 50 kDa were cationically modified by spermine to obtain spermine modified starch (SMS) based gene carriers. Plasmid pAcGFP1-C1 (pDNA) was chosen as the model gene material and formed self-assembly nanocomplexes with SMS. The cytotoxicity and transfection efficiency of SMS/pDNA complexes were tested in HepG2 cell lines. Results showed that SMS/pDNA complexes formed by SMS-DS3 with the highest primary amine content (1.17 μmol mg^{-1}) and pDNA at the weight ratio of 25 displayed the highest transfection efficiency (~40%) with low cytotoxicity. Dynamic Light Scattering (DLS) and Small Angel X-ray Scattering (SAXS) investigation under simulated acidified endosomal environments (pH 5.0–7.4) revealed that SMS-DS3/pDNA complexes (~180 nm) with moderately compact structures within acidic environments (i.e. no significant particle size changes, slightly shrunken shapes) showed superior transfection efficiency.

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1. Introduction

Gene therapy is regarded as a promising therapeutic strategy to treat many serious incurable diseases (e.g. cancer and genetic disorders) where therapeutic genes are delivered into the target cells to produce desired proteins or inhibit harmful gene expression (Ibraheem, Elaissari, & Fessi, 2014; Jo et al., 2007). In order to achieve successful gene therapy, gene delivery systems should be employed to protect therapeutic genes from premature degradation during systemic blood circulation and to efficiently transfer the therapeutic genes to target cells. Recent progresses in polymer sciences, nanotechnology and nucleic acid chemistry have accelerated the developments of non-viral gene delivery methods. Among all the polymeric gene delivery vectors, polysaccharide-based polymers such as chitosan, dextran, pullulan, cyclodextrin, hyaluronic acid, etc. have considerable advantages over synthesized polymers (e.g. polyethyleneimine (PEI) and poly (L-lysine)) mainly due to their natural characteristics such as biocompatibility, biodegradability, and low immunogenicity and cytotoxicity (Aranda et al., 2013; Choi, Jang, Kim, & Ahn, 2014; Khan et al., 2012; Lu et al., 2009; Ochrimenko et al., 2014; Priya, Rekha, & Sharma, 2014).

Starch is one of the most abundantly obtainable natural polysaccharides, and has been widely used as pharmaceutical excipients. Our group has demonstrated that starch derivatives could be used as potential drug delivery carriers for a wide range of bioactive components (Bie, Chen, Li, & Li, 2016; Li et al., 2012; Pu et al., 2011; Situ, Li, Liu, & Chen, 2015). Starch molecule is composed of repeated α-glucose units and can be found in two main structural forms, i.e. linear amylose and branched amylopectin. Amylose contains anhydrous glucose units that are linked mainly by α-(1-4)-D-glycoside bonds, while amylopectin is branched with α (1 → 6) linkages. A predominant model defines that the amylopectin and amylose chains (0.1–1 nm) can aggregate into clusters (20–50 nm) which contain alternating crystalline and amorphous lamellae with a thickness of approximate 9–11 nm (Le Corre, Bras, & Dufresne, 2010; Pérez & Bertoft, 2010). It is expected that the unique cluster structure of starch would offer plenty binding sites for effective DNA condensation. Moreover, starch is known to be enzymatic biodegradability, so that some enzymes native to cells may facilitate cargo release once starch based gene delivery vectors have entered the cells. Even though less of a focus has been placed to date on starch as gene delivery vectors, some promising results obtained from a few recent studies have set the scene for using starch as a cost-effective and readily modifiable nanoscale gene

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delivery system. Cationically modifying starch molecules with positively charged amino groups such as quaternary ammonium groups (Amar-Lewis et al., 2014; Engelberth, Hempel, & Bergkvist, 2015), oligoamine residues (Zhou et al., 2012), and polymeric amine (Yamada, Loretz, & Lehr, 2014) is a typical way to engineer starch based gene delivery vectors. Although the structural properties of starch and starch/gene complexes, including the molecular weight of starch, the degree of substitution (DS) of positively charged amino groups, and the sizes and surface charges of the complexes were reported to have great influence on the transfection efficiency (Sieradzki, Traitel, Goldbart, Geresh, & Kost, 2014), less emphasis has been put on investigating the relationships between the internal nano-structure changes of the cationic starch/gene complexes and the transfection activity.

Studies have shown that cationic polymer/DNA polyplexes with a diameter of <200 nm are generally internalized by cells via the clathrin-dependent endocytosis pathway, especially in cell lines (e.g. HepG2 cells) which lack endogenous caveolins responsible for a caveolae-mediated pathway (Rejman, Bragonzi, & Conese, 2005; Von Gersdorff et al., 2006). The clathrin-dependent endocytosis (CME) pathway is initiated by the formation of clathrin-coated vesicles and subsequent formation of early (pH 6.0–6.5) and late endosomes (pH 5.0–5.5), which ultimately fuse with lysosomes (pH 4.5–5.5) (Mindell, 2012; Sorkin & von Zastrow, 2002), and is applicable to polyplex-based gene delivery applications only when the polyplexes could be released from endosomes into the cell's cytoplasm at a relatively early stage (Won, Sharma, & Konieczny, 2009). Moreover, in order to avoid DNA degradation in endo-lysosomal compartments before endosomal escape through endosomal lysis (Haensler & Szoka, 1993), the cationic starch should bind to DNA compactly and form stable polyplexes inside the acidified endosomal compartments. Therefore, the compactness of the structure of cationic starch/DNA complexes in acidic environments could be an important factor determining the transfection efficiency.

In this study, starch molecule with molecular weight of about 50 KDa was cationically modified by spermine, a tetra-amine with two primary and two secondary amino groups, to obtain spermine modified starch (SMS) which are supposed to increase the proton buffering capacity of starch and enhance the endosomal escape of the corresponding polyplexes (Eliyahu et al., 2005). Plasmid pAcGFP1-C1 (pDNA) which is able to express enhanced green fluorescent protein (EGFP) was chosen as the model gene material and formed nanocomplexes with SMS. By mimicking the endo-lysosomal pH variation (7.4 → 5.0), the structural changes of SMS/pDNA complexes, including particle size and internal nanostructure were investigated by DLS and SAXS respectively, which were then correlated to the transfection efficiency of the SMS/pDNA complexes tested in the HepG2 cell line in order to illustrate the relationships between the structural changes of SMS/pDNA complexes and the transfection efficiency.

2. Material and methods

2.1. Materials

Starch with a weight-average molecular weight (M_w) of 4.735×10^4 g mol^{-1}, was acquired from our lab through the degradation of native maize starch (Huanglong Food Industry Co., Ltd., Changchun, China) with thermostable α-amylase, and M_w was measured by gel permeation chromatography (GPC) coupled with multi-angle light scattering (MALS) as previously reported (Zhang, Chen, Zhao, & Li, 2013). 1,1′-Carbonyldiimidazole (CDI) and spermine were purchased from Aladdin Reagent Company (Shanghai, China). Fetal bovine serum (FBS), RPMI-1640 medium, penicillin/streptomycin (10,000 U/ml and 10,000 μg/mL) solution, and trypsin were purchased from Hyclone Co. (Carlsbad, CA, USA). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and 2, 4, 6-trinitrobenzenesulfonic acid (TNBS) were obtained from Sigma-Aldrich (St. Louis, MO, USA). The plamid pAcGFP1-C1 (4.7 kb; Clontech, Palo Alto, CA, USA) encoding enhanced green fluorescent protein (EGFP) was maintained and propagated in DH5αstrain of *Escherichia coli* (*E.coli*), and then purified by using the Endfree plasmid kit (Tiangen, China). The purity and concentration of plasmids were determined by UV spectrophotometry (A260/A280). All other reagents used in this study were of analytical grade and used without further treatment.

2.2. Starch modification

Spermine was introduced to the hydroxyl groups of starch by using CDI as an activator (Jo et al., 2007). Specifically, starch (20 mg mL^{-1} in anhydrous DMSO) was activated by CDI (CDI (mol)/anhydrous glucose units (AGU) of starch (mol)) = 1:12, 1:8 or 1:4 for 2 h at room temperature. Then spermine (CDI (mol)/spermine (mol) = 1/1) was added and the reaction continued at 35 °C for 24 h. When the reaction was completed, the reaction product was dialyzed against pure water (molecular weight cut-off 7000) for 3 days and lyophilized. The resulting samples were named as SMS-DS1, SMS-DS2, and SMS-DS3 respectively according to the DS of SMS. The primary amine (NH_2) content of SMS was measured by the standard protocol of the TNBS method (Snyder & Sobocinski, 1975). Briefly, 20 μL of freshly prepared aqueous solution of TNBS (15 mg mL^{-1}) was separately added into marked tubes containing spermine dissolved in pure water (0–0.5 μmol). Then 150 μL of $NaHCO_3$ (0.8 M, pH = 8.5) was added into the above mixture separately, mixed well and incubated at 37 °C for 2 h. Afterwards, 450 μL of 1 N HCl aqueous solution was added to each tube, vortexed for 1 min, and gently sonicated to remove bubbles. Each sample was further diluted with pure water to obtain a final volume of 10 mL, and absorbance of samples was recorded at 410 nm. SMS (0.5–1 mg) were treated as above, and the NH_2 content was calculated according to the calibration curve.

2.3. Characterization of SMS

The M_w and molecular weight distribution of SMS was determined by gel permeation chromatography (GPC) (Waters China Ltd.) coupled with a multi-angle light scattering (MALS) detector (DAWN HELEOS MALS, Wyatt Technology Corp.) and a refractive index (RI) detector (Optilab rEx, Wyatt Technology Corp.). Shodex OHpak SB-804 HQ column (Waters China Ltd.) was used. Samples were prepared at a concentration of 5 mg mL^{-1} with filtered mobile phase (0.1 M $NaN0_3$ solution with 0.02% w/v NaN_3 which had been filtered through 0.22 μm cellulose nitrate filter). The flow rate of the system was set at 0.5 mL min^{-1}. Data was processed by Astra V software (Wyatt Technology Corp., USA).

FTIR analyses were performed with a Tensor 37 spectrophotometer (Bruker Instrument Co., Germany). Overall, 32 scans/spectrum were acquired in the 4000–400 cm^{-1} range with a resolution of 4 cm^{-1}.

^{1}HNMR analyses were performed in D_2O using a NMR Spectrometer (AVANCE 400, Bruker, Germany). Chemical shifts were referred to the solvent peak, δ = 4.70 ppm. Data was processed by MestReNova software.

The buffer capacity of SMS with different DS was determined by acid-base titration. In brief, SMS was dissolved in 150 mM NaCl with a concentration of 2 mg mL^{-1}. Then the pH of the solution was adjusted to 10.0 using 0.1 M NaOH. Subsequently, this solution was titrated with 0.1 M HCl to pH 3.0 with a pH meter (PB-10, Sartorius, Germany). As reference, the same procedure was applied to native starch.

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