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Self-assembled ternary complexes stabilized with hyaluronic acid-green tea catechin conjugates for targeted gene delivery

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ABSTRACT

Nanosized polyelectrolyte complexes are attractive delivery vehicles for the transfer of therapeutic genes to diseased cells. Here we report the application of self-assembled ternary complexes constructed with plasmid DNA, branched polyethylenimine and hyaluronic acid-green tea catechin conjugates for targeted gene delivery. These conjugates not only stabilize plasmid DNA/polyethylenimine complexes *via* the strong DNA-binding affinity of green tea catechin, but also facilitate their transport into CD44-overexpressing cells *via* receptor-mediated endocytosis. The hydrodynamic size, surface charge and physical stability of the complexes are characterized. We demonstrate that the stabilized ternary complexes display enhanced resistance to nuclease attack and polyanion-induced dissociation. Moreover, the ternary complexes can efficiently transfect the difficult-to-transfect HCT-116 colon cancer cell line even in serum-supplemented media due to their enhanced stability and CD44-targeting ability. Confocal microscopic analysis demonstrates that the stabilized ternary complexes are able to promote the nuclear transport of plasmid DNA more effectively than binary complexes and hyaluronic acid-coated ternary complexes. The present study suggests that the ternary complexes stabilized with hyaluronic acid-green tea catechin conjugates can be widely utilized for CD44-targeted delivery of nucleic acid-based therapeutics.

1. Introduction

Gene therapy has emerged as a potent therapeutic approach for the treatment of chronic diseases including cancer, monogenic and cardiovascular disease [1]. The success of gene therapy ultimately relies on the development of delivery systems capable of efficiently transferring exogenous genes into target cells. One major approach in gene delivery is to exploit non-viral vectors based on the polyelectrolyte complexes self-assembled through electrostatic interactions between cationic polymers and anionic nucleic acids. A variety of cationic polymers, such as poly(L-lysine), poly(β-amino ester) and poly(amido amine) dendrimers have been utilized to condense plasmid DNA (pDNA) into nanoscale polyelectrolyte complexes favorable for cell internalization [2–4]. Among the cationic polymers, polyethylenimine (PEI) has attracted significant interest because of its excellent transfecting ability with an intrinsic endosomolytic activity. It has been reported that the strong buffering capacity of PEI can facilitate the endosomal escape of pDNA by rupturing the endosome membrane *via* the "proton sponge" effect [5,6]. Despite the desirable features, there are several limitations to the practical applications of pDNA/PEI complexes: 1) strong cytotoxicity due to disruption of the cell membrane with positively charged PEI chains [7,8], 2) agglutination with blood components (*e.g.*, erythrocytes and complements) [9,10], and 3) dissociation of pDNA from the complexes due to competitive binding of polyanions (*e.g.*, heparin and plasma proteins) and subsequent degradation of the liberated DNA by serum nucleases [11,12].

Electrostatic polymer coating has recently been proposed as a promising strategy to overcome the aforementioned limitations of pDNA/PEI complexes. Various anionic polymers such as poly(acrylic acid), poly(γ-glutamic acid) and carboxylated poly(ethylene glycol) derivatives have been electrostatically deposited on a cationic pDNA/PEI complex to alter its surface properties [13–15]. Kurosaki et al. have discovered that the poly(γ-glutamic acid) coating effectively reduces the cytotoxicity and agglutination of pDNA/PEI complexes by shielding their positive surface charges [14]. In addition, carboxylated poly(ethylene glycol) derivatives have been shown to protect pDNA/PEI complexes against non-specific interaction with plasma proteins [15]. Hyaluronic acid (HA), a natural polysaccharide, has also been investigated as a shielding material for pDNA/PEI complexes. HA is a linear non-sulfated glycosaminoglycan with non-immunogenic, biocompatible and biodegradable nature [16]. Since HA serves as a ligand for CD44, HA not only provides a protective coating on pDNA/PEI complexes, but also enables these complexes to target the cancer cells and liver endothelial cells that express high levels of CD44 [17,18]. HA-coated pDNA/PEI complexes have been reported to exhibit superior gene transfection efficiency compared to uncoated complexes in CD44-overexpressing cells *via* HA receptor-mediated uptake [19,20]. Recently, considerable efforts have been focused towards

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developing chemically modified HA derivatives to further improve the stability, serum tolerance and transfecting ability. Ito et al. have described the transcription-enhancing activity of amphoteric spermine-HA conjugates mimicking the high-mobility-group (HMG) proteins essential for initiating transcription [21]. Furthermore, a disulfide-modified HA (HA-SS–COOH) has been explored to selectively trigger the release of pDNA in the intracellular reductive environment to enhance gene delivery efficiency [22].

In this study, we described the development of self-assembled ternary complexes constructed with pDNA, PEI and HA-epigallocatechin gallate (HA-EGCG) conjugates. EGCG is a major constituent of green tea catechins with beneficial properties including antioxidant [23], anti-inflammation [24] and anticancer effects [25]. Importantly, EGCG has been shown to bind strongly to biological macromolecules such as DNA and proteins *via* hydrogen bonding, π–π stacking and hydrophobic interactions [26–28]. Moreover, EGCG is known to inhibit the activity of various enzymes such as nucleases, collagenase and hyaluronidase by blocking their active sites irreversibly [29–31]. We have recently reported that macromolecular EGCG derivatives can form a compact nanocomplex with proteins and effectively protect them from degradation by serum proteases *in vivo* [27]. Inspired by the desirable properties of EGCG, we have designed nanoscale ternary complexes stabilized with HA-EGCG conjugates for CD44-targeted gene delivery. Not only did HA-EGCG conjugate stabilize pDNA/PEI complexes *via* the strong DNA-binding affinity of EGCG, it also assisted their transport into CD44-overexpressing cells *via* HA receptor mediated endocytosis. The stabilized ternary complexes exhibited enhanced protection of pDNA against nuclease attack and polyanion-induced dissociation. We demonstrated that these ternary complexes enable highly efficient transfection of the difficult-to-transfect HCT-116 colon cancer cell line even in serum-supplemented conditions. This was due to their enhanced stability, resistance to nuclease and CD44-targeting ability, which led to more effective nuclear transport of pDNA as compared to binary complexes and HA-coated ternary complexes. The current study suggested that the HA-EGCG-stabilized ternary complexes could be potentially applied for CD44-targeted delivery of nucleotide therapeutics to diseased cells or tissues.

2. Materials and methods

2.1. Materials

HA (M_w = 90 kDa) was kindly donated by JNC Corporation (Tokyo, Japan). Branched PEI (M_w = 25 kDa), heparin sodium salt and 2,2-diethoxyethylamine were purchased from Sigma-Aldrich (St. Louis, MO). EGCG was obtained from DSM Nutritional Products Ltd. (Basel, Switzerland). Green fluorescent protein (GFP)-encoding pDNA (pEGFP-C1, 4.7 kbp) was purchased from Clontech Laboratories (Mountain View, CA). SYBR® Safe DNA gel staining dye, AlamarBlue® cell viability assay reagent, Lipofectamine 2000 transfection reagent and LysoSensor® Green DND-153 (Life Technologies, Carlsbad, CA) were used according to the manufacturers' instructions. DNase I and DNase I reaction buffer were obtained from New England Biolabs (Ipswich, MA). Anti-human CD44 antibody, isotype control antibody and fluorescein isothiocyanate (FITC)-tagged secondary antibody were purchased from Bio-Rad Laboratories (Hercules, CA). Cy5 Label IT® Tracker intracellular nucleic acid localization kit was purchased from Mirus Bio (Madison, WI). Lab-Tek® II chamber slides were obtained from Thermo Fisher Scientific (Waltham, MA). All other chemicals and reagents were of analytical grade.

2.2. Synthesis of HA-EGCG conjugates

HA-EGCG conjugates were synthesized in a two-step process established previously [30,32]. Firstly, EGCG was reacted with 2,2-diethoxyethylamine (DA) to form ethylamine-bridged EGCG dimers. In brief, 145 μL of DA (1 mmol) was added to 1.2 mL of cold MSA:THF (1:5) while stirring. The mixture was then added to EGCG (2.29 g, 5 mmol) dissolved in 3.8 mL of THF containing 1.7 μL of MSA and stirred overnight in the dark at room temperature. The unreacted EGCG was removed by multiple extraction cycles with ethyl acetate until no free EGCG was detected. The concentration of the purified ethylamine-bridged EGCG dimer was determined by absorbance at 274 nm and was found to be 84 mg/ml (yield = 88%).

In the second step, the ethylamine-bridged EGCG dimers were conjugated to HA *via* carbodiimide-mediated coupling reaction. In brief, HA (250 mg, 0.62 mmol) was dissolved in 19.8 mL of 0.4 M MES buffer (pH 5.2) containing 2.5 mL of DMF. NHS (89 mg, 0.78 mmol), ethylamine-bridged EGCG dimers (0.205 mmol in 2.7 mL of H_2O) and EDC·HCl (150 mg, 0.78 mmol) were added successively and the pH of the mixture was adjusted to 4.7. The reaction mixture was purged vigorously with N_2 for 10 min and then incubated overnight under N_2. The HA-EGCG conjugates were then purified by three cycles of ethanol precipitation in the presence of NaCl. Subsequently, the precipitates were re-dissolved in 150 mL of H_2O and dialyzed against H_2O in N_2 atmosphere overnight before lyophilization. The final yield was 74.4%. The degree of substitution (*i.e.*, the number of EGCG dimers per 100 disaccharide units in HA) was determined by examining the absorbance of HA-EGCG conjugates at 274 nm using a Hitachi U-2810 spectrometer. The degree of substitution for HA-EGCG conjugates was determined to be approximately 2.5.

2.3. Preparation and characterization of polyelectrolyte complexes

To prepare pDNA/PEI complexes, 1 μg of pDNA was gently mixed with branched PEI in 50 μL of deionized water at various N/P ratios (*i.e.*, the ratios of the number of amino groups in PEI to the number of phosphate groups in pDNA). This mixture was incubated for 15 min at room temperature. The resulting pDNA/PEI complexes were mixed with HA-EGCG conjugates in 50 μL of deionized water at various C/P ratios (*i.e.*, the ratios of the number of carboxylic groups in HA to the number of phosphate groups in pDNA), and then incubated for 30 min to form pDNA/PEI/HA-EGCG ternary complexes. For comparison, pDNA/PEI/HA ternary complexes were prepared by mixing HA with the pDNA/PEI complexes at equivalent C/P ratios and subsequently incubating for 30 min. The hydrodynamic diameters of the polyelectrolyte complexes were measured using a particle size analyzer (Zetasizer Nano ZS, Malvern Instruments, UK). Each sample was diluted with deionized water to produce a final pDNA concentration of 1 μg mL^{-1}. The zeta potential values were measured in 10 mM NaCl solution. All measurements were performed in triplicate.

2.4. Evaluation of pDNA condensation and physical stability of polyelectrolyte complexes

The effect of ternary complex formation on pDNA condensation was assessed by agarose gel electrophoresis. Briefly, 10 μL of the polyelectrolyte complexes formulated with 1 μg of pDNA were applied to a 1.2% (*w*/*v*) agarose gel containing the SYBR® Safe DNA gel staining dye. The gel was subjected to electrophoresis at 100 V for 25 min in TAE buffer solution (40 mM Tris, 20 mM acetic acid and 1 mM EDTA). The gel image was taken under UV illumination by using a VersaDoc 4000 MP instrument (Bio-Rad Laboratories, Singapore). To examine the stability against polyanion-induced dissociation, the polyelectrolyte complexes (1 μg of pDNA) were incubated with heparin sodium salt solution (1 mg mL^{-1}) for 30 min at 37 °C, and then analyzed by agarose gel electrophoresis [33] as described above. To evaluate nuclease resistance, the polyelectrolyte complexes (1 μg of pDNA) were incubated with 16 units of DNase I for 2 h at 37 °C [33]. The mixture was then treated with 0.5 mM EDTA solution for 15 min to inactivate DNase I. The resulting solution was treated with heparin sodium salt solution (2 mg mL^{-1}) for 30 min to liberate pDNA from the complexes prior to agarose gel electrophoresis.