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Multiarm cationic star polymers by atom transfer radical polymerization from β-cyclodextrin cores: Influence of arm number and length on gene delivery

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ABSTRACT

Controlled β-cyclodextrin (β-CD) core-based cationic star polymers have attracted considerable attention as non-viral gene carriers. Atom transfer radical polymerization (ATRP) could be readily used to produce the star-shaped polymers. The precise control of the number of initiation sites on the multifunctional core was of crucial importance to the investigation of the structure–property relationship of the functional star gene carriers. Herein, the controlled multiarm star polymers consisting of a β-CD core and various arm lengths of poly(2-(dimethylamino)ethyl methacrylate) (PDMAEMA) were prepared via ATRP from the chloroacetylated β-CD with well-designed initiation sites. Generally, these star polycations can condense plasmid DNA into 100–150 nm nanoparticles with positive zeta potentials of 30–40 mV at N/P ratios (star polymer to DNA ratios) of 17 or higher. The effects of arm numbers and lengths on gene delivery were investigated in detail. With a fixed length of the PDMAEMA arm, the fewer the number of arms, the lower the toxicity. The star polycations with suitable arm numbers possess the best transfection ability. On the other hand, with the fixed molecular weights, the shorter the arms, the lower the toxicity. The polymers with 21 arms possess the lowest transfection efficiency.

1. Introduction

Viral vectors, nonviral vectors, physical methods and the naked plasmid DNA (pDNA) method without vectors are usually used for gene transfer [1]. In comparison with viral vectors and cationic lipids, cationic polymers as the major type of non-viral gene delivery vectors show low host immunogenicity and can be produced on a large scale. Natural polysaccharides are very suitable candidates for the design of novel biomaterials, because they are renewable, non-toxic, biodegradable and biocompatible [2]. Various polycations based on different polysaccharides including dextran [2,3], chitosan [4] and cyclodextrin (CD)-based cationic carriers [5–8], have been reported to deliver pDNA. CDs are a series of cyclic oligosaccharides composed of six, seven or eight D(+)-glucose units linked by α-1,4-linkages and named α-, β- or γ-CD, respectively [9]. Due to their excellent biocompatibility and non-immunogenicity, CDs were quite often used to produce the synthetic polycations [10].

Star-shaped cationic polymers have recently attracted considerable attention as non-viral gene carriers, due to their dense molecular architecture with moderate flexibility [11,12]. Atom transfer radical polymerization (ATRP) is a recently developed "controlled" radical polymerization method, which has been readily used to prepare different controlled star-shaped polymers [13,14]. Such well-designed structures are of particular importance to establishing structure–property relationships [15]. CDs have the unique steric structure of a truncated cone [16]. The substitutable hydroxyl groups on the outside surface of β-CD can be converted into ATRP initiation sites to produce star-shaped cationic polymers [11,17,18]. The precise control of the number of initiation sites on the multifunctional core was mandatory for the preparation of uniform structures with controlled arm lengths. However, due to the different reaction activities of hydroxyl groups at the different positions of CDs, it is very difficult to precisely control the number and position of substituted initiator groups while substitution degrees were below 21 [19].

Recently, based on the different reaction activities of hydroxyl groups of CDs, we reported an efficient "protection–deprotection" method to prepare the chloroacetylated β-CD (Cl-β-CD) with well-designed ATRP initiation sites [19,20], which would provide the possibility for investigating the "arm number–property" relationship of CD-based star polymers. In this work, a library of controlled

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multiarm star gene vectors (s-CDPDs) consisting of the β-CD core and 4, 7, 14 or 21 arms of cationic poly(2-(dimethylamino)ethyl methacrylate) (PDMAEMA) were prepared via ATRP from Cl-β-CD. The effects of arm numbers and lengths on gene delivery were studied systematically.

2. Materials and methods

2.1. Materials

2-(Dimethylamino)ethyl methacrylate (DMAEMA, >98%), copper(I) chloride (CuCl, 99%), *N*,*N*,*N*′,*N*″,*N*″-pentamethyldiethylenetriamine (PMDETA, 98%), branched polyethylenimine (PEI, $M_w \sim$ 25000 Da), 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide (MTT), penicillin and streptomycin were obtained from Sigma-Aldrich Chemical Co., St Louis, MO. DMEAMA was used after removal of the inhibitors in a ready-to-use disposable inhibitor-removal column (Sigma-Aldrich). CuCl was purified twice by glacial acetic acid, then washed promptly with ethanol and dried under vacuum. Ultrapure water obtained from a Millipore system with a resistivity of 18.2 MΩ cm was used in all the experiments. All other chemicals used in the experiments were purchased as analytical reagents without further purification. HepG2 and COS7 cell lines were purchased from the American Type Culture Collection (ATCC, Rockville, MD).

2.2. Syntheses of CD-based star PDMAEMA

In this work, the target arm number of the star-like polymers (s-CDPDs) from the CD core is 4, 7, 14 or 21. The starting chloroacetylated β-cyclodextrins (21-,14-, 7- and 4Cl-β-CD) with well-designed 21, 14, 7 or 4 ATRP initiation sites were synthesized based on the efficient "protection–deprotection" method reported by us [19]. The star-shaped PDMAEMA polymer was synthesized via ATRP according to our previous work [20]. The typical process for the DMAEMA polymerization using the representative 7Cl-β-CD initiator was described as follows (Scheme 1a). 7Cl-β-CD (64.2 mg, 0.04 mmol), DMAEMA (3.158 ml, 18.5 mmol) and PMDETA (200 μl, 0.84 mmol) were dispersed in 1.2 ml of a methanol/water (7/1, v/v) mixture. After three freeze–pump–thaw cycles, the reaction mixture was further degassed by bubbling nitrogen for 30 min. Then 84 mg (0.84 mmol) CuCl was added under the protection of nitrogen. The reaction was kept at 50 °C for 8 h. The reaction was terminated by exposing to air, and diluted with 50 ml of *tetrahydrofuran (*THF). After being concentrated in a rotary evaporator, the solution was dialyzed (MWCO 1000) against water for 2 days and then lyophilized to yield the star s-CDPD. For other s-CPPDs, the detailed reaction conditions were summarized in Table 1.

2.3. Polymer characterization

The molecular weights and dispersity of polymers were determined by gel permeation chromatography (GPC) and chemical structure by nuclear magnetic resonance (NMR) spectroscopy. GPC measurements were performed on a *YL9100* GPC system equipped with a UV/vis detector and Waters Ultrahydrogel 250™ (packed with crosslinked hydroxylated polymethacrylate-based gels of 250 Å pore sizes) and Ultrahydrogel Linear™ (packed with crosslinked hydroxylated polymethacrylate-based gels of different pore sizes) 7.8 × 300 mm columns [3]. The Ultrahydrogel 250™ and Ultrahydrogel Linear™ columns allowed the separation of polymers over the molecular weight ranges of 10^3–8×10^4 and 10^3–7×10^6, respectively. A pH 3.5 acetic buffer solution was used as the eluent at a low flow rate of 0.5 ml min^{-1} at 25 °C. Monodispersed polystyrene standards were used to generate the calibration curve. The ^{1}H-NMR spectra were recorded on a 400 MHz Bruker AV-400 NMR spectrometer at room temperature. The ^{1}H-NMR spectral measurements were carried out using an acquisition time of 3.2 s, a pulse repetition time of 2.0 s, and a 30° pulse width. Chemical shifts were referenced to the D_2O solvent peak at 4.70 ppm.

2.4. Characterization of polymer/pDNA complexes

The plasmid (encoding Renilla luciferase) used in this work was pRL-CMV (Promega Co., Cergy Pontoise, France), which was cloned originally from the marine organism Renilla reniformis. The plasmid DNA (pDNA) was amplified in *Escherichia coli* and purified according to the supplier's protocol (Qiagen GmbH, Hilden, Germany). The purity and concentration of the purified DNA were determined by absorption at 260 and 280 nm and by agrose gel electrophoresis. The purified pDNA was resuspended in the tris-EDTA (TE) buffer at pH 7.5, containing 10 mM Tris-Cl (preared from tris base and hydrochloric acid) and 1 mM ethylene diamine tetra acetic acid (EDTA), and kept in aliquots of 0.5 mg ml^{-1} in concentration. All CDPDs and CDPDPE prepared in this work are readily soluble in water at the concentration of 10 mg ml^{-1}. All polymer stock solutions were prepared based on a nitrogen component concentration of 10 mM in distilled water and the pH was adjusted to 7.4. Solutions were filtered through sterile membranes of 0.2 μm in average pore size and stored at 4 °C. Star polymer to DNA ratios are expressed as N/P ratios. All polymer/pDNA complexes were formed by mixing equal volumes of polymer and pDNA solutions to achieve the desired N/P ratios. Each mixture was vortexed and incubated for 30 min at room temperature.

Each cationic polymer was examined for its ability to bind pDNA through agarose gel electrophoresis using procedures similar to those described earlier [3]. The polymer/pDNA complexes at various N/P ratios were investigated. Each mixture was vortexed and incubated for 30 min at room temperature. 3 μl of gel loading buffer was added and the mixture was incubated for 5 min. Then, the mixture was analyzed on 1% agarose gel containing 0.5 μg ml^{-1} ethidium bromide (EtBr). Gel electrophoresis was carried out in the TAE running buffer (containing 40 mM Tris-acetate and 1 mM EDTA) at a voltage of 110 V for 30 min in a sub-cell system (Bio-Rad Lab, Hercules, CA). DNA bands were visualized and photographed by a UV transilluminator and BioDco-It imaging system (UVP Inc., Upland, CA). The particle sizes and zeta potentials of the polymer/pDNA complexes were measured using a Zetasizer Nano ZS (Malvern Instruments, Southborough, MA) with a laser of wavelength of 633 nm at a 173° scattering angle [3].

2.5. Cell viability

The cytotoxicity of the star-like polymers was evaluated via MTT assay in the HepG2 and COS7 cell lines. The cells were cultured in Dulbecco's modified Eagle medium (DMEM), supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 units ml^{-1} of penicillin and 100 μg ml^{-1} of streptomycin at 37 °C, under an atmosphere of 5% CO_2 and 95% relative humidity. The cells were seeded in a 96-well microtiter plate (Nunc Co., Wiesbaden, Germany) at a density of 10^4 cells per well and incubated in 100 μl of DMEM per well for 24 h. The culture media were replaced with fresh culture media containing serial dilutions of polymer complexes, and the cells were incubated for 24 h. Then, 10 μl of sterile-filtered MTT stock solution in PBS (5 mg ml^{-1}) was added to each well, giving a final MTT concentration of 0.5 mg ml^{-1}. After 5 h, the unreacted dye was removed by aspiration. The so-produced formazan crystals were solubilized in dimethyl sulfoxide (DMSO) (100 μl per well). The absorbance