Development of Supramolecular Pseudo-Block Conjugates Based on Star-Shaped Polycation for DNA Delivery

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Supramolecular assembly provides a new strategy for designing various gene carriers for deoxyribonucleic acid (DNA) delivery. In this work, supramolecular pseudo-block conjugates based on star-shaped polycation are developed as novel nonviral gene vector. First, cyclodextrin (CD) is selected as core and grafted with low molecular weight polyethylenimine (PEI1800) as arms to obtain high molecular weight star-shaped polycation CD-PEI8 (SCP). Then, azobenzene (Az) modified poly(ethylene glycol) is synthesized and named as azobenzene modified poly(ethylene glycol) (Az-PEG). Subsequently, the supramolecular pseudo-block conjugates are prepared via host–guest interaction between CD and Az. The result suggests that SCP/Az-PEG condenses DNA into spherical nanoparticles with sizes of around 150 nm. The in vitro experiment shows that the cell viability of DNA/SCP/Az-PEG polyplexes is significantly improved compared with that of PEI25k/DNA polyplexes. The PEGylation does not decrease the cellular uptake efficiency and transfection efficiency. Furthermore, the cellular trafficking shows that PEGylation does not significantly influence nuclear entry. The star-shaped architecture of polycation plays an important role in gene delivery.

1. Introduction

In recent years, nonviral vectors have been proposed as a potential strategy to protect and deliver therapeutic nucleic acid for gene therapy and gained great interest due to the low cost, easy manipulation, and large loading capacity of nucleic acid.[1,2] Polyethylenimine (PEI) has been used as a typical and efficient gene vector due to the so-called proton sponge effect.[3,4] The transfection efficiency of PEI extremely relies on the molecular weight. Specifically, high molecular weight PEI shows effective transfection efficiency with high cytotoxicity, while low molecular weight PEI has the opposite effect. Therefore, much progress has been made in assembling low molecular weight cationic segments into high molecular weight polycation.[5–7] Recent reports indicated that the architecture of polycation showed great influence on the transfection.[8,9] For example, Xu and co-workers[10] developed various linear or star-shaped poly(aspartic acid)-based gene vectors. They found star-shaped polycation exhibited much better transfection performances. Star-shaped CDPD polycation consisting of β-cyclodextrin (CD) core and poly(2-(dimethylamino) ethyl methacrylate) (P(DMAEMA)) arms was also reported as gene vector.[11] It was found CDPD exhibited higher gene transfection efficiency than...
P(DMAEMA) homopolymer, which suggested that the unique star-shaped architecture could promote gene expression. Therefore, many biocompatible molecules including cyclodextrin,$^{[12,13]}$ glucose,$^{[14]}$ and polyethylene,$^{[15]}$ were selected as core, and then grafted with cationic segments as arms to prepare star-shaped polycation.

On the other hand, PEI/deoxyribonucleic acid (DNA) polyplexes tend to rapidly aggregate in physiological salt condition.$^{[16,17]}$ PEGylation is a common approach to improve the colloidal stability and prolong the blood circulation time in vivo.$^{[18,19]}$ Host–guest interaction provides a facile strategy to construct PEGylated polyplexes due to easy preparation process.$^{[19–21]}$ Recently, we developed poly(ethylene glycol) (PEG)-detachable polyplexes and multifunctional nanoparticles via the light-regulated host–guest interaction between CD and azobenzene (Az).$^{[19,22]}$ In this work, supramolecular pseudo-block host–guest interaction between CD and azobenzene and multifunctional nanoparticles via the light-regulated PEGylated polyplexes tend to rapidly aggregate in physiological salt condition.$^{[16,17]}$ PEGylation is a common approach to improve the colloidal stability and prolong the blood circulation time in vivo.$^{[18,19]}$ Host–guest interaction provides a facile strategy to construct PEGylated polyplexes due to easy preparation process.$^{[19–21]}$ Recently, we developed poly(ethylene glycol) (PEG)-detachable polyplexes and multifunctional nanoparticles via the light-regulated host–guest interaction between CD and azobenzene (Az).$^{[19,22]}$

In this work, supramolecular pseudo-block host–guest interaction between CD and azobenzene and multifunctional nanoparticles via the light-regulated PEGylated polyplexes were investigated. The cellular trafficking was further investigated by confocal laser scattering microscope (CLSM).

2. Experimental Section

2.1. Materials

Branched polyethyleneimine with molecular weight 25k Da (PEI_{25k}), acryloyl chloride, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich. 4-Phenylazobenzoyl chloride (AzCOCl) was purchased from Tokyo Chemical Industry (Shanghai, China) Development Co., Ltd. Methoxypolyethylene glycol amine (mPEG-NH$_2$) with molecular weight 5k Da, α-cyclodextrin, and branched polyethyleneimine with molecular weight 1800 Da (PEI$_{1800}$) were purchased from Amresco. Cy3-DNA and N'-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid] (HEPEs, free acid, high pure grade) were purchased from Sangon Biotech (Shanghai, China). Loading buffer was purchased from TakaRa Biotechnology (Dalian, China) Co. Ltd. 0.5× TBE buffer was diluted from 4× TBE buffer (0.36 m tris-boric acid, 8 × 10$^{-3}$ m EDTA).

2.2. Synthesis and Characterization of SCP and Az-PEG

0.98 g CD was dissolved in 25 mL anhydrous N,N-dimethylformamide (DMF). With stirring, 12 mL acryloyl chloride was added into the above solution by twice with time interval of 4 h under an ice-water bath. The mixed solution was reacted for another 24 h under the ice-water bath. Then the solvent was removed under reduced pressure. The yellow product was precipitated from distilled water and collected by centrifuge. After lyophilized, 0.1 g of the above product was dissolved in 5 mL dimethylsulfoxide (DMSO) and added dropwise into 25 mL DMSO including 3.1 g PEI$_{1800}$ during 3 h. The reaction was performed under nitrogen atmosphere at 70 °C for 3 d. Then the product was dialyzed with a MWCO 2000 membrane for 5 d. The product was lyophilized and determined by $^1$H NMR (300 MHz, Varian Spectrometer, USA).

Az-PEG was synthesized according to the method reported previously.$^{[28]}$ Similarly, 0.3 g mPEG-NH$_2$, 0.06 g MgO, 0.15 g AzCOCl were dissolved in 25 mL anhydrous DMF. The solution was stirred for 24 h under nitrogen atmosphere at room temperature. Then the product was precipitated from ether, washed repeatedly with ether, and purified by dialysis (MWCO 3500) against deionized water for 5 d. The product Az-PEG was lyophilized and determined by $^1$H NMR spectroscopy (300 MHz, Varian Spectrometer, USA) and ultraviolet–visible spectrophotometer (UV-2550, Shimadzu, Japan).

2.3. Formulation of DNA/SCP and DNA/SCP/Az-PEG Polyplexes

All the polyplexes were formed by electrostatic interaction between polycations and DNA in 20 × 10$^{-3}$ m HEPEs buffer solution (pH = 7.4). SCP solution with different concentration was added to the equal volume of DNA solution (100 μg mL$^{-1}$) according to the various N/P ratio, which means the ratio between the
amount of nitrogen in PEI and the amount of phosphate in DNA. Then the solution was vortexed for 30 s and incubated for 30 min to form polyplexes at room temperature.

SCP/Az-PEG supramolecular pseudo-block conjugates were prepared via host–guest interaction. Az-PEG solution and SCP solution were mixed at the Az/CD molar ratio of 4:1. The mixed solution was ultrasonically agitated for 30 min as reported. Then the above solution was added to the equal volume of DNA solution by vortexing for 30 s and incubating for 30 min. DNA/SCP/Az-PEG polyplexes were formed.

2.4. Characterization of Polyplexes

2.4.1. Gel Retardation Assay

Agarose gel electrophoresis was performed to investigate the DNA binding ability of SCP and SCP/Az-PEG. Briefly, polyplexes containing 300 ng plasmid DNA (pDNA) were mixed with loading buffer (5:1 by volume). The electrophoresis was applied on agarose gel (1% w/v) in 0.5× TBE buffer for 50 min at 100 V. DNA bands were visualized and photographed with UV transilluminator and Gel-DOC system (Gel Doc, Bio-Rad, USA) after the gel was incubated in ethidium bromide (0.5 μg mL⁻¹) for 30 min.

2.4.2. Particle Size Measurements

Particle size and zeta potential of polyplexes were measured by dynamic light scattering (DLS) on Malvern Zetasizer (Malvern Instruments Ltd, UK). All experiments were carried out in triplicate.

2.4.3. Transmission Electron Microscopy (TEM)

The morphologies of polyplexes were characterized by transmission electron microscope (JEM-1200EX, NEC, Tokyo, Japan) with an acceleration voltage of 80 kV. A drop of the polyplexes was deposited onto 200-mesh carbon-coated copper grid for 10 min. In order to obtain enough particles on the grid, the above processes were repeated three times.

2.4.4. Particle Size Measurements

Particle size and zeta potential of polyplexes were measured by dynamic light scattering (DLS) on Malvern Zetasizer (Malvern Instruments Ltd, UK). All experiments were carried out in triplicate.

2.5. Cell Culture Experiments

HEK293T cells (human embryonic kidney cells) and SKOV-3 cells (human ovarian carcinoma cells) were required in the cell experiments. HEK293T cells and SKOV-3 cells were cultured in DMEM medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin at 37 °C in a humid atmosphere containing 5% CO₂.

2.5.1. The Cellular Uptake Efficiency

Cells were seeded into 48-well plate at a density of 3 × 10⁴ cells per well and incubated for 24 h. Then the medium was exchanged for 0.25 mL of fresh medium with 10% FBS. Different polyplexes containing 1 μg Cy3-DNA were applied to each well. After incubation for 0.5 h or 4 h, the cells were washed with PBS six times, trypsinized and measured by flow cytometry (BD FACS Calibur, Flow Cytometer, USA). For each sample, the final results were presented as an average value of three replicates.

2.5.2. Cell Viability Assay

Cell cytotoxicity of different polyplexes was evaluated by MTT assay. Briefly, cells were seeded into 96-well plates at a density of 5 × 10⁴ cells per well and incubated for 24 h. Then different polyplexes containing 1 μg DNA were applied to each well. After incubation for another 48 h, the cells were washed with PBS, replaced with 100 μL of fresh medium and 20 μL of MTT solution (5 mg mL⁻¹, dissolved in PBS). The cells were incubated for 4 h at 37 °C. Then the medium was removed, 150 μL DMSO was added and incubated for additional 15 min at 37 °C. The absorbance of 100 μL of the above mixture at 570 nm was measured by microplate reader (550, Bio-Rad, USA). All experiments were performed in five replicates.

2.5.3. In Vitro Transfection Efficiency

pGL-3 was used as reported gene for transfection. Cells were seeded into 48-well plate at a density of 3 × 10⁴ cells per well and incubated for 24 h. Then different polyplexes containing 1 μg pGL-3 were applied to each well. After incubation for 4 h, the medium was replaced with fresh medium. Luciferase expression was quantified 44 h later using a Promega luciferase assay system. Luciferase activity was measured in relative light units (RLU) using luminometer. Results were normalized to total cell protein as determined using a KEYGEN BCA protein assay. All transfection experiments were performed in triplicate.

2.5.4. Confocal Laser Scanning Microscope Observation

Cells with initial density of 5 × 10⁴ cells per dish in glass base dishes maintained overnight in 1.5 mL of medium. Before experiment, fresh culture medium was replaced. Then various polyplexes with 2 μg Cy3-DNA were added and incubated for 4 h, followed by incubation in the absence of polyplexes for another 12 h. Then, the nuclei were stained by DAPI according to the manufacturer’s protocol. The intracellular trafficking of Cy3-DNA was observed using a CLSM (Leica TS SP5, Germany).

3. Results and Discussion

3.1. Synthesis and Characterization of SCP and Az-PEG

CD has many hydroxyl groups outside, which can be modified and grafted with PEI1800 by Michael addition reaction. The hydroxyl groups of CD were reacted with acryloyl chloride, followed by reaction with large excess of PEI1800 to obtain the star-shaped polymer. The synthesis route and ¹H NMR spectrum was shown in Figure 2. The proton peaks at 5.0–5.1 and 2.1–3.0 ppm were, respectively, associated with CD and PEI, which were suggested that star-shaped CD-PEI was successfully synthesized. The graft degree of PEI1800 onto each CD was determined by the integral ratio of these signals. About eight molecules of PEI1800 were grafted on the CD core and the resulted polycation was designated as SCP.
The $^1$H NMR spectrum of Az-PEG indicated that the proton peaks at 3.5 and 7.0–8.0 ppm were, respectively, associated with PEG and azobenzene, which suggested that azobenzene was successfully introduced at the terminal of PEG. UV–vis spectroscopy was also used to confirm the structure of Az-PEG. There was an absorption peak at 325 nm, which was consistent with azobenzene according to the reports in the literature.[19,26]

3.2. Characterization of Polyplexes

It was well documented that $\alpha$-CD could form inclusion with specific guest molecules, such as Az and PEG. Although there are two different guest molecules in our system, it was reported that the strength of the interactions between $\alpha$-CD/Az was much higher than that of PEG/$\alpha$-CD.[27] Meanwhile, the Az/CD molar ratio was kept at 4:1 to confirm sufficient PEG chain on star-shaped PEI, which was prepared according to our previous study.[31]

For gene delivery, the ability of polycations to condense DNA into the polyplexes is the primary requirement. Herein, the DNA binding capability of polycations was examined by agarose gel electrophoresis using PEI$_{1800}$ as control. The results are shown in Figure 3. The migration of DNA was retarded when the N/P ratio was above 1.5 for both PEI$_{1800}$ and SCP. But bright DNA bands could be found in the agarose well for PEI$_{1800}$/DNA polyplexes at the N/P ratio of 2.5, indicating that PEI$_{1800}$/DNA polyplexes had looser structure. SCP provided better DNA protection because CD was employed as core to graft PEI$_{1800}$ to form higher molecular weight cationic polymer with unique star-shaped architecture. On the other hand, the introduction of PEG based on host–guest interactions barely affected the DNA condensation ability, which was consistent with our previous study.[19]

Appropriate particle size with positively charged or neutral surface is also necessary for efficient gene transfection. DNA must be condensed into small nanoparticles for cellular uptake. As shown in Figure 4a, the particle size of DNA/PEI$_{1800}$ polyplexes was much larger than that of DNA/SCP polyplexes. At the N/P ratio above 10, DNA/SCP polyplexes could form nanoparticles with size of about 250 nm, while the particle size of PEGylated polyplexes were below 150 nm, which was comparable with DNA/PEI$_{25k}$ polyplexes. As reported in the literature, PEI conjugated with low grafting degree of PEG was able to generate compact and spherical polyplexes with smaller sizes in comparison to PEI$_{25k}$.[28–30] Moreover, the zeta potential of polyplexes was investigated. As shown in Figure 4b, the zeta potential of all polyplexes increased with the increment of N/P ratio. At N/P ratio above 10, the surface charge of DNA/SCP polyplexes was about 20 mV, which was a benefit for endocytosis. Due to the PEG shielding, the zeta potential of DNA/SCP/Az-PEG polyplexes was lower than that of DNA/SCP polyplexes. Subsequently, TEM was used to evaluate the morphology of the polyplexes. As shown in Figure 5, DNA/SCP/Az-PEG polyplexes showed spherical nanoparticles with size of about 100 nm, while DNA/SCP polyplexes were irregular and larger than PEGylated polyplexes. Based on the above results, the significant difference between DNA/SCP polyplexes and DNA/SCP/Az-PEG polyplexes indicated PEGylation were successfully introduced by host–guest interaction between azobenzene and CD.

3.3. The Cellular Uptake Efficiency

Endocytosis is an important step for gene delivery. Herein, polyplexes loading 1 μg Cy3-DNA were prepared and exposed to HEK293T cells or SKOV-3 cells for 0.5 or 4 h, respectively. The cellular uptake efficiency was measured by flow cytometry. The results are shown in Figure 6. The cellular uptake efficiency of DNA/PEI$_{1800}$, DNA/SCP, and DNA/SCP/Az-PEG polyplexes was apparently dependent on N/P ratio in HEK293T cells and SKOV-3 cells for 0.5 h, which might be due to the influence of N/P ratio on the surface charge of the polyplexes. The uptake of DNA/SCP/Az-PEG polyplexes was lower than DNA/SCP polyplexes at N/P ratio of 10 and 30, which may be attributed to shielding PEG layer.[29,31] However, it was interestingly found that PEGylation via host–guest interactions showed little influence on the uptake efficiency especially at N/P ratio of 50 or long-time incubation by both cell lines. We speculated that
the unique star-shaped architecture could be favorable for the cellular uptake. Similar results were also found when PEGylation based on Polyamidoamine(PAMAM) dendrimers.[32]

3.4. The Cytotoxicity of Polyplexes

The cell cytotoxicity of DNA/SCP and DNA/SCP/Az-PEG polyplexes was investigated by MTT assay in HEK293T and SKOV-3 cells. DNA/PEI_{1800} and DNA/PEI_{25k} polyplexes were chosen as controls. The incubation time of polyplexes was 4 h for efficient cell uptake, which might exclude the influence of the uptake efficiency on cytotoxicity. As shown in Figure 7, the cell viability of all polyplexes decreased with increment of N/P ratio. DNA/PEI_{1800} polyplexes had over 70% cell viability even at N/P ratio of 50, indicating lower cell cytotoxicity. The reason may be due to the low molecular weight of PEI_{1800}. On the other hand, for DNA/PEI_{25k} polyplexes, the viability significantly decreased to below 20% at N/P ratio of 30. However, the DNA/SCP and DNA/SCP/Az-PEG polyplexes had higher cell viability of over 80% even at N/P ratio of 50, which was similar to that of DNA/PEI_{1800}
polyplexes. The improved viability was attributed to the excellent biocompatibility of cyclodextrins and PEG.

### 3.5. Transfection Experiment In Vitro

In vitro transfection efficiency of different polyplexes was evaluated in HEK293T and SKOV-3 cells using pGL-3 plasmid DNA. PEI<sub>1800</sub> and PEI<sub>25k</sub> were used as control. As shown in Figure 8, the transfection efficiency of polyplexes increased with the increment of the N/P ratio, which resulted from the addition of polycation. The DNA/PEI<sub>1800</sub> polyplexes had the lowest transfection efficiency, which supported the theory that the transfection efficiency for PEI-based polyplexes was significantly dependent on the molecular weight. After grafted with PEI<sub>1800</sub> arms, the star-shaped SCP-based polyplexes had two orders of magnitude higher than DNA/PEI<sub>1800</sub> polyplexes, which was ascribed to the higher molecular weight by the grafting arms. Interestingly, PEGylation via host–guest interactions did not decrease the transfection efficiency, which was related to the star-shaped architecture of polycations. It was also reported that PEGylation based PAMAM dendrimers could increase the transfection efficiency dendrimers. Importantly, DNA/SCP polycplexes and PEGylated polyplexes generated similar transfection efficiency with DNA/PEI<sub>25k</sub> polyplexes at higher N/P ratio, while the cytotoxicity of DNA/SCP polyplexes and PEGylated polyplexes was significantly reduced at high dosage.

### 3.6. Intracellular Distribution

To gain more insight into the relationship between the polyplexes and transfection efficiency, the intracellular distribution of different polyplexes at N/P ratio of 50 was observed by confocal microscopy. The polyplexes containing 2 μg Cy3-DNA were incubated for 4 h with HEK293T cells and followed by incubation in the absence of polyplexes for another 12 h. DAPI was used to stain the nuclei (blue). As shown in Figure 9, for DNA/PEI<sub>1800</sub> polyplexes, red dots from Cy3-DNA was mainly distributed in cytoplasm, while part of Cy3-DNA was observed to enter nuclei for DNA/PEI<sub>25k</sub> polyplexes. Similar phenomenon was observed for both DNA/SCP and DNA/SCP/Az-PEG polyplexes. These results indicated that the high molecular weight of PEI was favorable for nuclei entry, and PEGylation via host–guest interactions did not hinder the nuclei entry of star-shaped polycation. It may be attributed to the unique star-shaped architecture. Therefore, we concluded the unaffected transfection efficiency after PEGylation was due to the favorable nuclei entry caused by the star-shaped architecture of the supramolecular pseudo-block polycation.

### 4. Conclusions

In this work, supramolecular pseudo-block conjugates based on star-shaped polycations were developed as novel nonviral gene vector. Star-shaped polycation SCP and Az-PEG were separately synthesized. Then, the supramolecular pseudo-block conjugates were prepared via host–guest interaction. The result of agarose gel retardation
Figure 6. Cellular uptake efficiency of a,b) HEK293 cells and c,d) SKOV-3 cells for incubation of a,c) 0.5 h or b,d) 4 h. Error bars represent the standard deviation of three measurements (mean ± SD, n = 3, *p < 0.05).

Figure 7. Cytotoxicity of a) HEK293T cells or b) SKOV-3 cells exposed to different polyplexes containing 1 μg DNA after incubation for 48 h. Error bars represent mean ± SD for n = 5.

Figure 8. The transfection efficiency of HEK293T cells or SKOV-3 cells exposed to different polyplexes detected by flow cytometry. Error bars represent the standard deviation of three measurements (mean ± SD, n = 3, *p < 0.05).
assay suggested that PEGylation via host–guest interaction showed little influence on DNA condensation. Moreover, SCP/Az-PEG supramolecular pseudo-block conjugates could condense DNA into spherical nanoparticles with size of around 150 nm. The in vitro experiment by HEK293T and SKOV-3 cells showed that the cell viability of DNA/SCP and DNA/SCP/Az-PEG polyplexes was significantly improved compared with that of DNA/PEI25k polypelexes. It was interestingly found that the PEGylation did not decrease the cellular uptake efficiency and transfection efficiency of the SCP-based polyplexes, which might be due to star-shaped architecture of SCP. Furthermore, the cellular trafficking by CLSM showed that PEGylation based on the star-shaped architecture was favorable for nuclear entry. These results suggested that the structure of polycation played key role in the further application of gene delivery.

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