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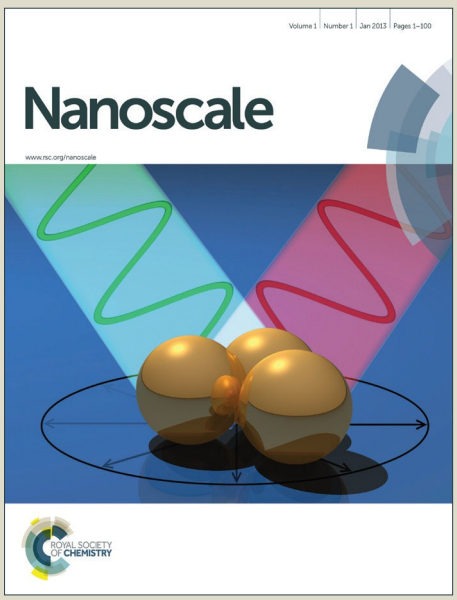

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Cell penetrating peptide-based polyplexes shelled with polysaccharide to improve stability and gene transfection†

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Cell-penetrating peptides (CPP) have been widely developed as a strategy to enhance the cell penetrating ability and the transfection. In this work, octa-arginine modified dextran gene vector with pH-sensitivity was developed via host-guest interaction. α-cyclodextrin was modified with octa-arginine (CDR), which had excellent cell penetrating ability. Dextran was select as backbone and modified with azobenzene as guest units by acid-labile imine bonds (Az-I-Dex). The supramolecular polymer CDR/Az-I-Dex with high C/A molar ratio (molar ratio of CD on CDR to Az on Az-I-Dex) was unfavorable for DNA condensation. Dextran shell of CDR/Az-I-Dex/DNA polyplexes improved the stability in physiological condition. However, once treated with acetate buffer (pH 5.4) for 3 h, large aggregates formed rapidly due to the cleavage of dextran shell. As expected, the vector had cell viability of 80% even when the CDR concentration increased to 100 μg mL-1. Moreover, due to the effective cellular uptake efficiency, CDR/Az-I-Dex/DNA polyplexes had 6~300 times higher transfection efficiency than CDR/DNA polyplexes. It was even higher than high molecular weight PLL-based polyplexes by HEK293T cells. Importantly, chloroquine as endosome escape agent could not improve the transfection of CDR/Az-I-Dex/DNA polyplexes, which indicaded that CDR/Az-I-Dex supramolecular polymer had its own ability for endosomal escape. These results suggested that the CPP-based polyplexes shelled with polysaccharide can be a promising non-viral gene delivery carrier.

Introduction

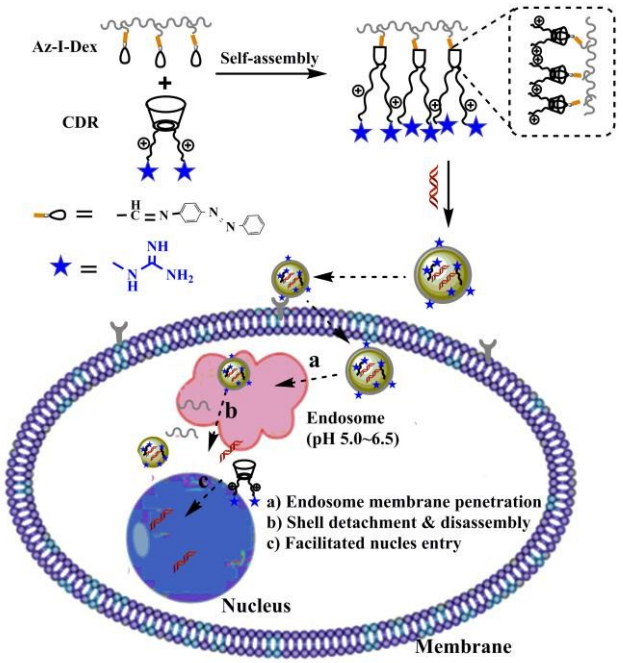
Non-viral vectors have attracted considerable attention due to their lack of immunogenicity, ease of manufacturing, and large nucleic acid loading capacity.[1-3] A great variety of polycations have been developed as non-viral vectors, such as polyethylenimines (PEI), poly-(L-lysine) (PLL), poly(amidoamine) (PAMAM) dendrimers, chitosan, and cationic polysaccharides.[4-10] However, the *in vivo* instability, cytotoxicity, inefficient targeting or intracellular trafficking are still major challenges for their further applications to human gene therapy.[11]

To solve the above problems, poly (ethylene glycol) (PEG),[12-14] polyanions[15] and polysaccharides[16] have been modified onto the surface of the polyplexes by covalent bonds or non-covalent interaction.[17-19] Among the polysaccharides, dextran well-known as its biodegradability and biocompatibility has been widely used in drug delivery systems to enhance the circulation time of drug.[20-22] Subsequently, Remy[23] and Tseng[20] reported that dextran modified with PEI could improve the stability of polyplexes in the presence of serum due to the so called "sugar-induced charge masking", which means the surface charge decrease to nearly neutralization by the water soluble residues such as sugars. However, with the molecular weight or grafting level of dextran increasing, the transfection was decreased due to the hindered DNA release ability.

Cyclodextrins (CDs) have well-known host-guest interactions with a vast array of hydrophobic compounds, which have been developed as a strategy to construct series of carriers for drug and gene delivery.[17, 18, 24-26] Various linear or star cationic polymers composed of CD cores and low molecular weight polycation arms have been developed as non-viral gene delivery vectors.[27-29] Recently, cell-penetrating peptides (CPP), a class of diverse peptides (typically with 5~30 amino acids)[30-32] such as cationic arginines and lysines, have been widely used to conjugate with chitosan, poly(disulfide amine), and dendrimers such as poly(ester-amide)s, and PAMAM to enhance transfection efficiency.[33-38]

By taking advantages of CDs as typical host molecules, we developed a non-viral gene vector based on the self-assembly of octa-arginine conjugated α-CD (CDR) and azobenzene-modified dextran (Az-I-Dex), whose Az units were linked on

dextran through acid-labile imine bonds. Based on host-guest interactions, it would be easy to regulate CD incorporation levels and the positive charge density. Dextran was expected to equip the polyplexes with improved stability and excellent biocompatibility. Once the polyplexes trafficked in endosome, the dextran shell might be detached due to the hydrolysis of imine bonds,[39] which was expected to facilitate efficient DNA release and transfection (Scheme 1). Our previous study found that the properties of vector such as DNA binding ability and transfection efficiency were related to the positive charge and CD grafting-level on polycations.[40] It was easy to adjust the CD grafting-level based on the host-guest interactions. Therefore, the influence of CD incorporation level on particle size was firstly investigated. Chemo-physical properties of polyplexes shelled with polysaccharide including DNA binding capability, particle sizes, and zeta-potentials were characterized. Their potential of pH-sensitivity was tested in acetate buffer (pH~5.4). The ability to mediate the gene expression was further evaluated.



Scheme 1 Schematic illustration of DNA condensation and intracellular trafficking with enhanced uptake by CPP and facilitated DNA release by shell detachment cause by acid-labile linkages.

Materials & Methods

Materials

Dextran 40 (MW=40 kDa), α-cyclodextrin (α-CD), allyl bromide, and sodium periodate were purchased from Aladdin (Shanghai, China). Octa-ariginine modified with cysteine (R8-Cys) was obtained from Apeptide Biotechnology Ltd. (Shanghai, China). 2, 2'-azobis[2-(2-imidazolin-2-yl)propane] dihydro-chloride (VA044), 5,5'-Dithiobis(2-nitrobenzoic acid) (DTNB), and 4', 6-diamidino-2-phenylindole (DAPI) were obtained from J & K Chemical Ltd. 4-aminoazobenzene was obtained from Tokyo Chemical Industry (Shanghai, China) Development Co., Ltd. Polyethyleneimine (PEI, MW=25 kDa) and Poly-L-lysine hydrobromide (PLL, MW= 30~70 kDa) were purchased from Sigma-Aldrich. Deoxyribonucleic acid (DNA, fish sperm, sodium salt) and N-[2-hydroxyethyl] piperazine-N'-[2-ehtanesulfonic acid] (HEPEs, free acid, high pure grade) were obtained from AMRESCO. Cy3-labelled DNA was purchased from Sangon Biotech (Shanghai, China). Plasmid DNA pGL-3 as transfection agent was obtained from Promega (USA). 3-(4, 5-dimethylthiAzl-2-yl)-2, 5-diphenyltetrAzlium bromide (MTT) was purchased from Bio Basic Inc. Loading buffer was obtained from TakaRa Biotechnology Co.Ltd. (Dalian, China). 0.5×TBE buffer was diluted from 4×TBE buffer (0.36 M tris-boric acid, 8 mM EDTA).

Synthesis of CDR and Az-I-Dex

Octa-arginine conjugated α-CD (CDR) was synthesized by thiol-ene coupling reaction according to the previous literature.[41] As shown in Figure 1a, α-CD was firstly functionalized with allyl groups by substitution reaction between bromide and hydroxyl group, which was similar to the analogous β-CD derivative according to the previous report.[42] Then, under a nitrogen atmosphere, 1 equivalent thiol groups of R8-Cys reacted with 1.2 equivalent allyl groups of allyl functionalized CD in the presence of 0.2 equivalent VA044. This reaction was carried out by irradiation (medium-pressure100W Hg lamp with maximum wavelength at 365 nm) in NaAc/HAc buffer solution (pH=4) for different times. The reaction was monitored by the thiol-consumption, which was detected DTNB methods.[43] The resulting product was purified by dialysis using a cellulose membrane (MWCO 2000 Da) for 24 h.

Then, azobenzene linked with dextran by acid-labile imine bond (Az-I-Dex) was performed by reacting 4-aminoazobenzene with aldehyde functionalized dextran (Dex-CHO) at room temperature in ethanol/water (1:4, v/v) for 12h. Dex-CHO was obtained by oxidization with sodium periodate at 4 ℃ according to previous study with some modifications.[44] The final product was purified by dialysis against ethanol/water mixture, then with water using a cellulose membrane (MWCO 2000 Da) for 2 days, and followed by freeze-drying.

The products were characterized by ^{1}H NMR (300 MHz, Varian Spectrometer, USA) and UV-vis Spectrometer (UV-2550，Shimadzu，Japan).

Formulation and Characterization of polyplexes

Preparation of polycations/DNA polyplexes

All the samples were prepared in 20 mM HEPEs buffer solution containing 20 mM NaCl (pH 7.4, short for HEPEs buffere solution). CDR/DNA polyplexes were prepared by vortexing equal volume of CDR solution with DNA solution (100 μg/ml dissolve in HEPEs buffer solution) at various C/P ratios (weight ratio of CDR to DNA). CDR/Az-I-Dex/DNA polyplexes were prepared as follows: Az-I-Dex solution and CDR solution were ultrasonically agitated for 30 min at different C/A ratios (molar

ratio of CD on CDR to Az on Az-I-Dex) ranging from 0.3 ~ 0.9. The progress of the CD/Az inclusion was monitored by UV Spectrometry. The protocol was according to our previous study.[17] Then the above mixture was added to the equal volume of DNA solution by vortexing for 30 s. All the polyplexes were prepared freshly and incubated for 30 min before analysis. The corresponding supramolecular polymer CDR/Az-I-Dex at C/A ratio of 0.4 was named CDR/Az-I-Dex-0.4. For the polyplexes, it was named CDR/Az-I-Dex-0.4/DNA. Others were similar with it.

AGAROSE GEL RETARDATION ASSAY

The DNA condensation capability was examined by gel retardation assay. The polyplexes containing 300 ng pDNA were prepared as above, mixed with loading buffer (5:1 by volume). Then the mixtures were loaded to each well of an agarose gel (1% by weight in 0.5×TBE buffer) and subjected to at 100 V for 50 min. After that, the gel was immersed in ethidium bromide solution (0.5 μg/ml) for 30 min, observed by UV illuminator (Gel Doc, Bio-Rad, USA).

PARTICLE SIZE AND ZETA (Z-) POTENTIAL MEASUREMENTS

The particle sizes and zeta potentials of the polyplexes were measured using a Malvern Zetasizer (Malvern Inst. Ltd., UK) equipped with either a four-side clear cuvette for particle size analysis or DTS 1060C cell for zeta-potential measurement. For particle size analysis, the samples were carried out in 4 serial measurements at 25 ℃ (scattering angle 173 °).

TRANSMISSION ELECTRON MICROSCOPY (TEM)

TEM images were performed on transmission electron microscope (JEM-1200EX，NEC, Tokyo, Japan) operated at 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.

pH-sensitivity study of the polyplexes

To check the pH-sensitivity, the suparamolecular polymer CDR/Az-I-Dex-0.7 were incubated in acetate buffer (pH~5.4) at 37 ℃ for 3h, then complexed with DNA (C/P 10) and incubated in the presence of 150 mM NaCl for 1 h. The stability of the samples without treatment by acetate buffer was also investigated in the presence of 150 mM NaCl. After the incubation, the size was detected by Malvern Zetasizer.

Cell cytotoxicity assay

HEK293T (Human embryonic kidney cell line) and Cos7 (Cell lines of african green monkey kidney fibroblasts transformed by gene of SV40 virus) cells were cultured in DMEM with 10% FBS and 1% penicillin-streptomycin. Cells were maintained under humidified air containing 5% CO_2 at 37 ℃. Cell cytotoxicity of different polycations was evaluated by MTT assay. Briefly, the cells were seeded into 96-well plates at a density of 8×10^3 cells/well. Following 24 h incubation, various polycations were added into the cells at final concentrations of 0, 10, 20, 40, 80 and 100 μg mL^{-1}. Here, the CDR and CDR/Az-I-Dex were kept at the same weight concentration of CDR. After incubation for 24 h, 20 μL of MTT (5 mg/ml, dissolved in PBS) was added. The cells were incubated for 4 h at 37 ℃. Then the medium was removed, 200 μL of DMSO was added and incubated for additional 15 min at 37 ℃. 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 sextuplicate.

The cellular uptake efficiency of the polyplexes

The cellular uptake efficiency was performed in HEK293T and Cos7 cells. The cells were seeded into 24-well plates at a density of 1×10^5 cells/well and incubated for additional 24 h. Before the addition of polyplexes, the medium was replaced with 0.5 ml of fresh media. Different polyplexes containing 0.5 μg Cy3-DNA were prepared as described above. The nanoparticles were added and incubated for 5 h. Then the cells were washed three times with PBS in order to detach surface-associated nanoparticles, trypsinized and analyzed by flow cytometry. All experiments were performed in triplicate.

***In vitro* transfection efficiency**

HEK293T and Cos7 cells were seeded in 24-well plates at a density of 5×10^4 cells/well. Before transfection, the medium was replaced by fresh DMEM with 10% FBS. Different polyplexes with 2 μg pGl-3 pDNA at C/P ratio of 5, 10, and 20 were added and incubated with or without 100 μM chloroquine for 5 h. PEI/DNA and PLL/DNA polyplexes at N/P ratio of 10 were used as control. Luciferase expression was quantified 48 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.

Intracellular trafficking of the polyplexes

HEK293T cells with initial density of 5×10^4 cells/dish in glass base dishes and maintained overnight in 1.5 mL of medium. Before experiment, fresh culture medium was replaced. Then various polyplexes with 6 μg Cy3-DNA at C/P ratio of 10 were added and incubated for 5 h in 1.5 mL of medium. After that, the media were replaced and the cells were incubated for another 10 h. Then, the cells were fixed with 4% (w/v) paraformaldehyde for 30 min and stained with DAPI (2.5μg/ml) for 20 min. The intracellular trafficking of the polyplexes was analysed by confocal laser scanning microscope using a 63× objective. (CLSM, ZEISS LSM780, Germany).

Results and discussions

Synthesis of CDR and Az-I-Dex

The synthetic route was shown in Figure 1(a). Briefly, α-CD was functionalized with allyl groups similar to the analogous β-CD derivative as described previously,[42] which was confirmed by ^{1}H NMR. Figure S1 suggests that there was about 3 allyl groups conjugated to every α-CD. Subsequently, octa-arginine conjugated α-CD (CDR) was synthesized by thiol-ene coupling

reaction according to the methods reported by Davis.[41] The thiol group (-SH) of octa-ariginine was reacted with allyl groups in the presence of water soluble initiator Vazo44 (2,2'-azobis[2-(2-imidazolin-2-yl)propane] dihydrochloride) at molar ratio of 1:1.2:0.2 (-SH:C=C:VA044). The reaction was carried out by irradiation (λ=365 nm) in degassed NaAc/HAc buffer solution (pH=4), and monitored by thiol-consumption detected by DNTB methods (Figure S2).[43] As shown in Figure 1(b), the typical proton signals attributed to the glucose units of α-CD were well observed. Moreover, the peaks at δ=1.3-1.8, 2.9-3.1, and 4.1-4.2 ppm were assigned to the protons of the -$\underline{CH_2}\underline{CH_2}$-, -$\underline{CH_2}$NHC=NH, and (-C=O) $\underline{CH}$ (NH-) of octa-arginine. Based on the ^{1}H NMR spectrum, the average ratio of octa-arginine to α-CD core was estimated by the proton integration. The result indicated that every α-CD had about 2~3 octa-arginine molecules.

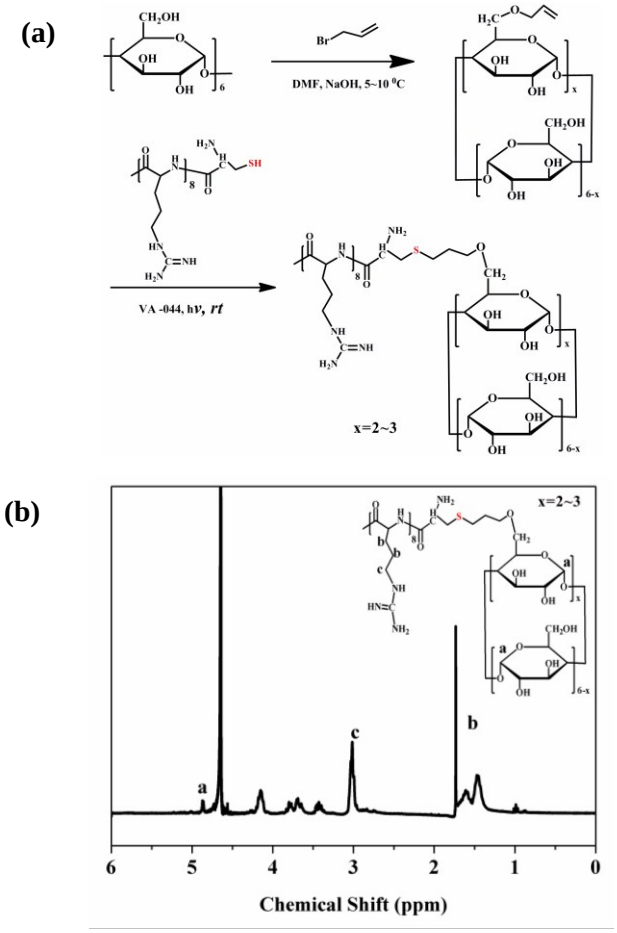


Figure 1 Synthetic route of CDR (a) and ^{1}H NMR spectrum of CDR (b).

We utilized the reaction of amine and aldehyde (imine bond) to conjugate Az with aldehyde-functionalized dextran (Dex-CHO). The imine bond was expected to equip the shell with the pH-sensitivity. Dex-CHO was obtained by oxidization with sodium periodate according to previous report with some modifications.[44] The typical vibration peak of carbonyl group at 1720 cm^{-1} by FT-IR and UV absorbance at 240 nm of Dex-CHO (Figure S3) suggested that Dex-CHO was successfully synthesized. The final product Az-I-Dex was analysed by ^{1}H NMR (Figure S4) and UV-vis spectrophotometer. According to the standard curve of Az obtained by UV-vis spectra, the amount of Az in one dextran polymer was found to be around 45. Due to the short oxidization at low temperature, the decrease of molecular weight of dextran through the oxidation reaction was negligible.[44]

Chemo-physical characterization of CDR/Az-I-Dex/DNA polyplexes

For gene delivery, polycations should condense DNA into small nanoparticles with appropriate size and surface charge to facilitate cellular uptake.[40] Therefore, it is important to carry out characterization of the polyplexes, such as particle size, ζ-potential and DNA binding ability for gene vectors.

Recently, we developed CD modified PEI with different CD-grafting levels for DNA delivery.[40] The results suggested that PEI-CD with higher grafting levels had poor DNA condensation ability and could not condense DNA into small nanoparticles at physiological pH, which was contributed to the intermolecular hydrogen bond and rigidity of CD molecules.[40] Herein, to study the influence of CD incorporation level on the particle sizes, various CDR/Az-I-Dex supramolecular polymers were prepared at different molar C/A ratios (CD on CDR to Az on Az-I-Dex). The progress of the CD/Az inclusion was monitored by UV Spectrometry, and the protocol was according to our previous study.[17] Subsequently, the supramolecular polymer CDR/Az-I-Dex with different C/A ratios were complexed with DNA at the same C/P ratio (weight mass ratio of CDR to DNA) of 10. As shown in Figure 2 (a), it was found that the particle size was significantly dependent on the C/A ratios. Specifically, at the first stage of C/A ratios ranging from 0.3 to 0.7, the particle size decreased slowly from around 350 nm to below 200 nm. It was due to the increased positive charge on dextran with the incensement of CDR. However, the particle sizes increased significantly with C/A ratios increasing from 0.7 to 0.9. The reason was probably attributed to the rigidity and hydrogen bonds of CD molecules, which was unfavourable for the electrostatic interaction between DNA and polycations.[40]

Dextran was expected to improve the stability by so called "sugar-induced charge masking" effect, which mean relatively more dextran at the out-layer would be favourable for the stability. Combing the particle size, C/A ratios were fixed at 0.4 and 0.7, designated as CDR/Az-I-Dex-0.4/DNA and CDR/Az-I-Dex-0.7/DNA, respectively. Then the particle sizes of polyplexes at various C/P ratios were investigated. As shown in Figure 2(b), the particle size of all CDR/Az-I-Dex/DNA polyplexes decreased with an increase in C/P ratios. The supramolecular polymer could condense DNA into nanoparticles with diameter of around 300 for C/A=0.4 and 150 nm for C/A=0.7 at C/P ratio of 10, respectively. However, CDR/DNA polyplexes had a diameter over 700 nm even at C/P ratio of 10. ζ-potential was an indicator of surface charges on the polyplexes, and positive surface charge of polyplexes was benefit for the cellular uptake. As given in Figure 2(c), the ζ-

potential of CDR/Az-I-Dex/DNA polyplexes increased with C/P ratios, and the ζ-potentials of CDR/Az-I-Dex-0.4/DNA were all below 10 mV. We also used TEM to evaluate the morphology and particle size of the shelled polyplexes. As shown in Figure 3, all the polyplexes had a regular spherical shape and the size of dextran-shelled polyplexes was smaller than that of CDR/DNA polyplexes.

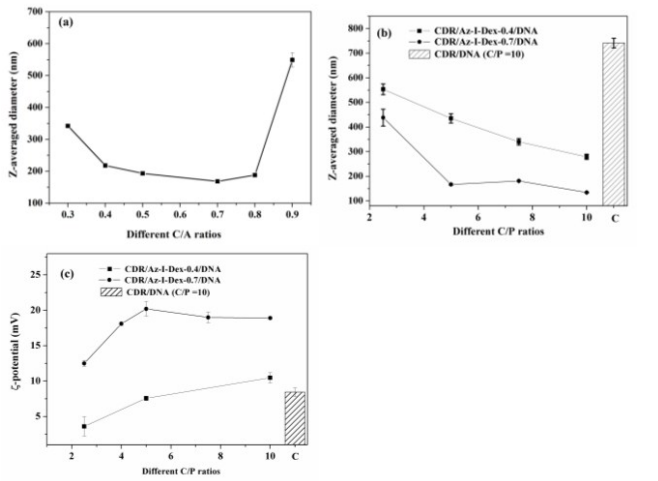


Figure 2 Particle sizes of CDR/Az-I-Dex/DNA polyplexes at C/P ratio of 10 with various C/A ratios (a); Particle sizes (b) and ζ-potentials (c) of CDR/Az-I-Dex/DNA polyplexes with C/A ratios of 0.7 and 0.4 at different C/P ratios.

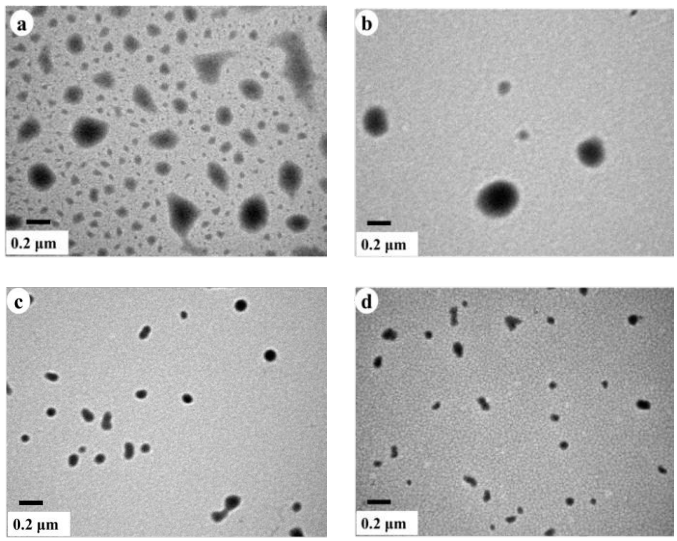


Figure 3 Typical TEM images of CDR/DNA (a), CDR/Az-I-Dex-0.7/DNA at C/P ratio of 2.5 (b), 5 (c) and 10 (d) in 20 mM HEPEs buffer solution (pH=7.4, 20 mM NaCl). The scale bars indicated 200 nm.

Subsequently, the DNA binding capability of supramolecular polycations was examined by agarose gel electrophoresis using CDR as a control. As shown in Figure 4, all polycations/pDNA polyplexes at C/P ratios ranging from 0.25 to 1.75 were electrophoresed separately in agarose gel. No fluorescent bands of free DNA were observed for CDR/pDNA (Figure 2a) above the C/P ratio of 0.75, suggesting that the migration of DNA in agarose gel was completely retarded. A similar phenomenon was observed for CDR/Az-I-Dex-0.4/pDNA, while the migration of DNA in agarose gel for CDR/Az-I-Dex-0.7/pDNA was completely retarded above the C/P ratio of 1. These results suggested that the high CD-incorporating level had some influence of DNA condensation, which was consistent with our previous study.[40]

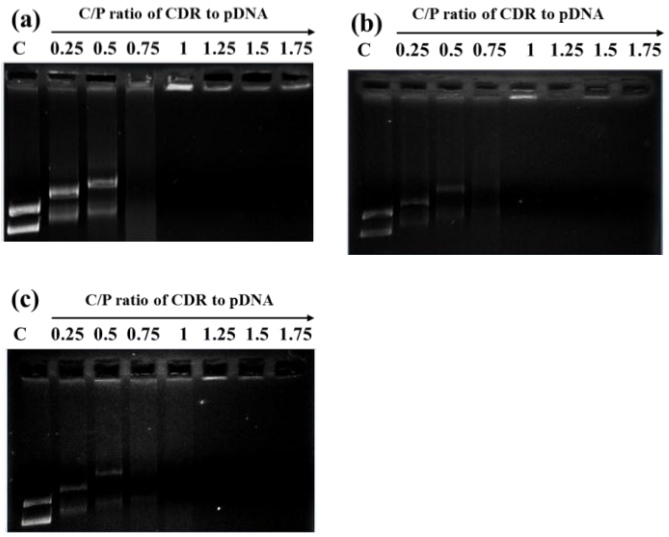


Figure 4 Agarose gel electrophoresis retardation assay of (a) CDR/pDNA, (b) CDR/Az-I-Dex-0.4/pDNA, and (c)CDR/Az-I-Dex-0.7/pDNA.

pH-sensitivity of natural polysaccharide shelled polyplexes

pH gradients are known to exist in tumour microenvironments and intracellular endolysosomal compartments. For example, inherent pH of endosomes is ranging from 5.0~6.5,[45] which has been widely used to design pH-responsive nanocarriers by acid-labile linkers (e.g., hydrazone and imine).[39] To investigate the pH-sensitive behaviour, the stability of polycations/DNA polyplexes in physiological condition (150 mM NaCl) was investigated with treatment of acetate buffer (pH~5.4), which represent the environment of endosomes. PEI/DNA, PLL/DNA (N/P = 10) and CDR/DNA polyplexes were used as control. As shown in Figure 5, it clearly demonstrated that PEI/DNA, PLL/DNA and CDR/DNA (C/P 10) polyplexes formed large aggregates rapidly with sizes of nearly or over 1 μm under physiological conditions for 1 h. It was interestingly found that the particle size of CPP-based polyplexes shelled with dextran only increased from around 200 to 500 nm, which suggested that dextran incorporation significantly improved the stability in physiological condition. The reason may be due to the sugar effect of high weight dextran (MW40 000), which was consistent with the previous study.[20] The results of stability in culture media with 10% and 20% FBS (see Figure S6) also supported the above conclusion. However, after incubation in acetate buffer (pH~5.4) at 37 ℃ for 3h, the particle size of CDR/Az-I-Dex/DNA polyplexes (C/P 10) increased to over 1μm. The reason was attributed to the rupture of acid-labile

imine bond, which resulted in the detachment of dextran shell. The pH-sensitivity might be beneficial for DNA release.[20]

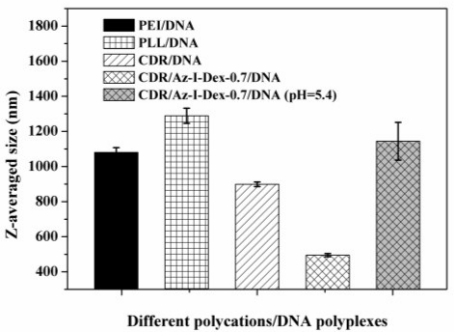


Figure 5 Size change of various polyplexes at C/P ratio of 10 in the presence of 150mM NaCl for 1 h. pH=5.4 indicates that the polycations were treated in pH=5.4 for 3 h at 37 °C before addition of salt.

Cell cytotoxicity study

The cell cytotoxicity of CDR, CDR/Az-I-Dex-0.4, and CDR/Az-I-Dex-0.7 was investigated by MTT assay in HEK293T and Cos7 cells. PEI and PLL as polypeptide were used as a control. Here, the CDR and CDR/Az-I-Dex were kept at the same weight concentration of CDR. As shown in Figure 6, the cell cytotoxicity of all polycations was dependent on the polymer concentration. Specifically, the cellular viability for PEI at 20 μg mL^{-1} decreased even below 30% for HEK293T cells and 40% for Cos7 cells. The results indicated that PEI showed its well-known severe cytotoxicity when the concentration increased to 20 μg mL^{-1}. In terms of PLL, the cell viability decreased to around 20% for HEK293T cells and 60% for Cos7 cells at PLL concentration of 80μg mL^{-1}. It was due to their high charge density and molecular weight. However, for CDR or CDR/Az-I-Dex polymers, regardless of C/A ratios, the cellular viability was over 80% for both cell lines even when the CDR concentration increased to 100 μg mL^{-1}. Although the introduction of CDR on Az-I-Dex increased the charge density, the cytotoxicity of CDR/Az-I-Dex was marginal. It was attributed to two factors. One is the well-known biocompatibility of dextran and CD. Numerous reports have been found that polysaccharide (e.g. CD, dextran, hyaluronic acid, etc.) modification can reduce cytotoxicity of cationic polymers. [16, 46, 47] Another factor may be due to the pH-induced degradability.

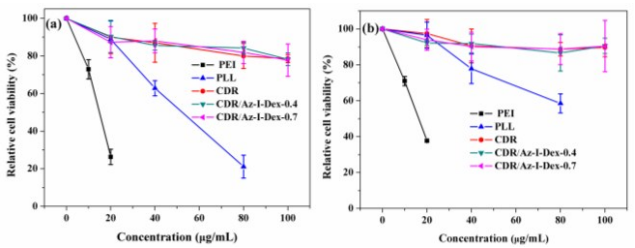


Figure 6 Relative cell viability of HEK293T (a) and Cos7 (b) cells exposed to different polycations at various concentrations. The data are presented as mean ± SD (n=6).

Cellular uptake of CDR/Az-I-Dex/DNA polyplexes

Endocytosis of polyplexes is one of the barriers that polyplexes must overcome for transfection. Here, different polyplexes complexed with Cy3-DNA were exposed to HEK293T cells or Cos7 cells for 5 h and evaluated by flow cytometry. PEI/DNA and PLL/DNA polyplexes at N/P ratio of 10 were used the control. The results were shown in Figure 7.

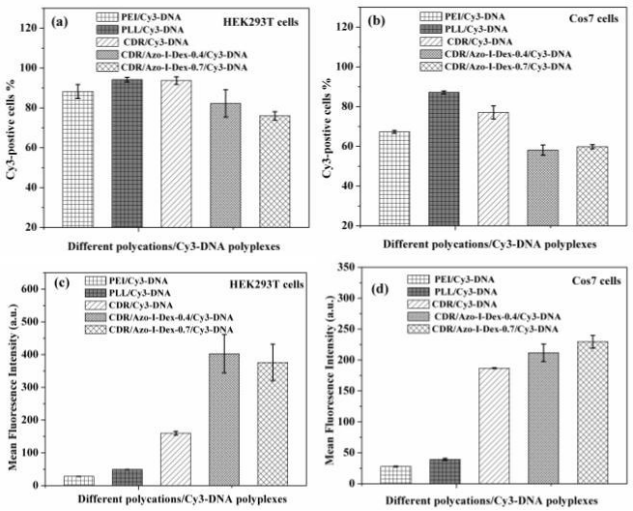


Figure 7 Cellular uptake mediated by polycations/DNA polyplexes at C/P ratios of 10 exposed to HEK293T cells (a, c) and Cos7 cells (b, d). The data are presented as mean ± SD (n=3).

As shown in Figure 7 (a, b), it was found that all the polyplexes had over 60% uptake efficiency for both cell lines and the uptake efficiency of CDR/Az-I-Dex/DNA polyplexes showed slight decrease, which may be due to shielding effect of dextran. However, for the mean fluorescence intensity (MFI) of uptake cells exposed to the polyplexes (Figure 7 (c, d)), the results suggested that CDR/Az-I-Dex/DNA polyplexes showed higher MFI than PEI/DNA and PLL/DNA polyplexes in HEK293T cells (Figure 7(c)), with the similar trend observed in Cos7 cells (Figure 7(d)). For example, in HEK293T cells, the fluorescence intensity of CDR/Az-I-Dex/DNA polyplexes with arginine was around 3-fold higher than that of PEI/DNA polyplexes. The different trends for CDR/cy3-DNA polyplexes for HEK293T and Cos7 cells may be due to the cell types. These results suggested that all CDR/Az-I-Dex/DNA polyplexes with octa-arginine had high cellular uptake efficiency, which was due to the well-known cell penetrating ability of arginine.[48]

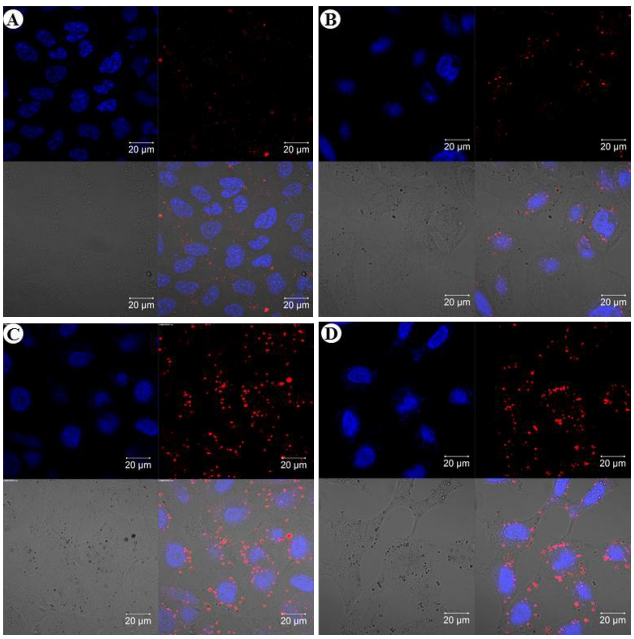


Figure 8 Intracellular distribution of pDNA polyplexed with PEI at N/P ratio of 10 (A), CDR (B), and CDR/Az-I-Dex-0.4 (C), and CDR/Az-I-Dex-0.7 (D) at C/P ratio of 10. The polyplexes with Cy3-labeled pDNA (red) were incubated with HEK293T cells for 5 h. The cells were post-incubated for another 10 h. The CLSM observation was performed using a 63× objective. The nuclei were stained with DAPI (blue). The scale bar represents 20 μm.

Intracellular trafficking of CDR/Az-I-Dex/DNA polyplexes

Next, to gain more insight into the cell penetrating ability, intracellular trafficking of DNA was observed using a confocal laser scanning microscope (CLSM). CDR or CDR/Az-I-Dex complexed with Cy3-DNA (red) at C/P ratio of 10 were prepared as above and exposed to HEK293T cells for 5 h. Nuclei were stained with DAPI (blue). As shown in Figure 8A, HEK293T cells were observed with weak red fluorescence of PEI/DNA polyplexes, and the polyplexes were mainly distributed in cytoplasm. Similar phenomenon was also observed for CDR/DNA polyplexes (Figure 8B), which may be due to large particle size and low ζ-potential. However, strong red fluorescence of CDR/Az-I-Dex/DNA polyplexes was observed by most of HEK293T cells. The fluorescence intensity of CDR/Az-I-Dex/DNA polyplexes was much stronger than that of PEI/DNA and CDR/DNA polyplexes, which was consistent with cellular uptake profile detected by flow cytometer as mean fluorescence for analysis (Figure 7 (c)). These results indicated that the octa-arginine significantly enhanced the cellular uptake of the CDR/Az-I-Dex/DNA polyplexes, which was consistent with the previous reports.[38, 49-51]

Transfection experiments *in vitro*

The *in vitro* transfection efficiency of polyplexes were evaluated in HEK293T and Cos7 cells using luciferase report gene (pGl-3). PLL as common polypeptide has been widely used for DNA delivery. Herein, PLL/DNA, PEI/DNA at N/P ratio of 10 and CDR/DNA polyplexes were used as a control. Figure 9a showed the results of HEK293T cells in the presence of serum. The transfection efficiency was significantly dependent on the C/P ratios (weight mass ratio of CDR to DNA) and molar C/A ratios (C/A ratios (CD on CDR to Az on Az-I-Dex), despite they were lower than that of PEI/DNA polyplexes. For example, the transfection of CDR/Az-I-Dex-0.4/DNA (C/A=0.4) polyplexes were about 10, 160, and 300 times higher than that of CDR/DNA at C/P ratios of 5, 10, and 20, respectively. CDR/Az-I-Dex-0.7/DNA polyplexes showed about 6~67 times higher transfection efficiency than CDR/DNA polyplexes. Importantly, the CDR/Az-I-Dex/DNA polyplexes had similar transfection efficiency with PLL/DNA polyplexes, and CDR/Az-I-Dex/DNA-0.4 even had higher transfection efficiency than PLL/DNA. However, for Cos7 cells (Figure 9b), the transfection of CDR/Az-I-Dex-0.4/DNA were about 6~17 times higher than that of CDR/DNA polyplexes, and CDR/Az-I-Dex-0.7/DNA had around 30~40 times higher transfection than CDR/DNA polyplexes. The transfection was not obviously dependent on C/P ratios for Cos7 cells, which may be due to the different cell types. These results suggested that arginine-incorporated to polysaccharide *via* host-guest interaction could greatly increase the transfection efficiency, which was consistent with the results of the traditional chemical graft strategy.[49] Although CDR/Az-I-Dex/DNA polyplexes had higher cell uptake efficiency than PEI/DNA polyplexes, they had lower transfection efficiency than PEI/DNA polyplexes. The reason might be due to the different types (e.g. liner and branch) of the polycations.[52] Dou also found that star-PAsp-PDMAEMA showed higher transfection efficiency than liner-PAsp-PDMAEMA.[28] Importantly, CDR/Az-I-Dex showed improved stability and much higher cellular viability even when the CDR concentration increased to 100 μg mL^{-1}, which might be more suitable for *in vivo* application.

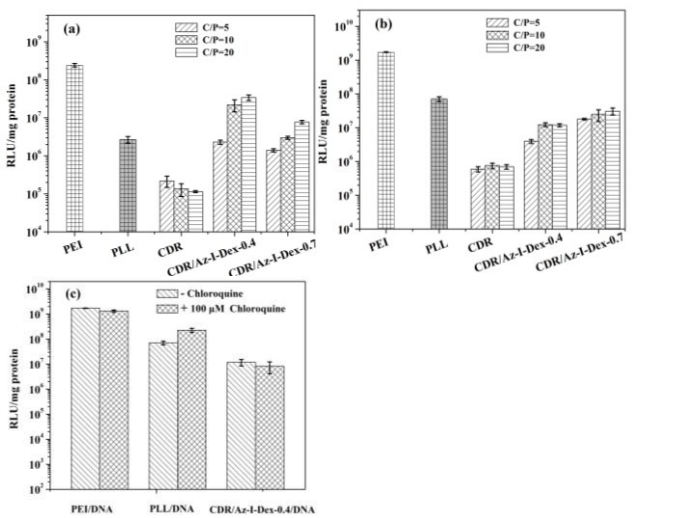


Figure 9 *In vitro* transfection of the luciferase gene into HEK293T (a) and Cos7 cells mediated by different nanoparticles for 48 h; (c) In vitro transfection of the luciferase gene in Cos7 cells with or without 100 μM chloroquine mediated by CDR/Az-I-Dex-0.4/pGl-3 polyplexes at C/P ratio of 20, PEI/DNA and PLL/DNA (N/P =10) polyplexes. The data are presented as mean ± SD (n=3).

To our knowledge, PEI has its high transfection due to its so called "proton sponge effect", which is benefit for the ability of endosome escape.[53] Herein, chloroquine was used for well-known endosome disrupting agent, which can facilitate the release of polyplexes from endosome and lead to enhanced transfection. PEI was used as a control. 100 μM chloroquine was added to the Cos7 cells, further incubated for 5 h in the presence of the polyplexes, and transfected for total 48h. As shown in Figure 9c, the transfection efficiency of PLL/DNA polyplexes was increased by over 3 times. However, that of PEI/DNA and CDR/Az-I-Dex/DNA polyplexes decreased in the presence of chloroquine, which might be due to the cytotoxicity of chloroquine. It was well-known that branched PEI had three different types of amines. Due to the proton sponge effect, it facilitated the polyplexes escape from the endosome. Chloroquine could not increase the transfection efficiency of CDR/Az-I-Dex/DNA polyplexes. Therefore, it was proposed that CDR/Az-I-Dex polycation had its own ability for endosomal escape. The reason may be due to two factors. One is the unique structure of R8, which had three different kinds of amine groups such as primary amine, secondary amine and guanidine groups. The similar results about endosomal escape ability of arginine were also reported by Kim.[49] On the other hand, previous study suggested that dePEGylation facilitated the endosomal escape.[36,54] We inferred that the detachment of dextran caused by cleavage of the imine bonds may increase the osmotic pressure in the endosome, which was favourable for endosomal escape.

Conclusions

In summary, octa-arginine modified dextran gene vector with pH-sensitivity was developed via host-guest interaction. The best advantage of this strategy was that the backbone polymer and host units were all polysaccharide, which showed excellent biocompatibility. The supramolecular polymer CDR/Az-I-Dex could condense DNA into spherical nanoparticles with size about 150 nm. CDR/DNA polyplexes aggregated in the presence of 150 mM NaCl, while the stability of dextran-shelled polyplexes significantly improved in physiological salt condition. After treatment with acetate buffer (pH 5.4), the particle size of the dextran-shelled polyplexes increased to over 1 μm due to the detachment of dextran caused by cleavage of acid-labile imine bonds. In addition, the vector had over 80% cellular viability even at 100 μg mL^{-1} CDR, showing that it can be used for gene delivery system without consideration of cytotoxicity. Moreover, CDR/Az-I-Dex/DNA polyplexes had higher cellular uptake efficiency than PEI/DNA polyplexes, which was confirmed by the intracellular trafficking study. The transfection efficiency of CDR/Az-I-Dex/DNA polyplexes was 6~300 times higher than that of CDR/DNA polyplexes. It was even higher than high molecular weight PLL-based polyplexes by HEK293T cells. Importantly, chloroquine as endosome escape agent could not improve the transfection of CDR/Az-I-Dex/DNA polyplexes. These results suggested that the CPP-based polyplexes shelled with polysaccharide had good endosome escape ability and can be a promising non-viral gene delivery carrier.

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Notes and references

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† Electronic Supplementary Information (ESI) available: ^{1}H NMR spectra of allyl-α-CD (Figure S1), the consumption of thiol group in the system (Figure S2), FT-IR and UV-vis spectra of Dextran and Dex-CHO (Figure S3), and ^{1}H NMR spectrum of Az-I-Dex (Figure S4) are provided. See DOI: 10.1039/b000000x/

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