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ARTICLE

A facile modular approach toward multifunctional supramolecular polyplexes for targeting gene delivery

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Jia Liu[a,b,c], Wim E. Hennink[b], Mies J. van Steenbergen[b], Renxi Zhuo[a], Xulin Jiang*[,a]

Extensive efforts and numerous methodologies have been explored to develop safe and efficient gene carriers that are capable of overcoming the multiple extra- and intracellular barriers during transfection process. However, it is a great challenge to fabricate a gene carrier system containing multiple functional components. In this work，we propose a facile modular approach to design targeted multifunctional gene delivery systems, which are based on building blocks with pre-defined functions to tackle specific barriers. These building blocks can be rationally combined and self-assemble to an integrated gene delivery system by host-guest interactions. As proof of concept, three adamantly-terminated targeting guest modules, adamantyl terminated RGD peptide (Ad-RGD), folate and lactobionic acid terminated PEGs (FA-PEG-Ad and LA-PEG-Ad), were synthesized, characterized, and employed for post-functionalizing of adamantly decorated cationic PEI based supramolecular polyplexes (PEI-Ad_4/PCD/DNA) through the free CD units of poly(β-cyclodextran) (PCD) host module. These multifunctional targeting supramolecular polyplexes exhibited enhanced cellular uptake and excellent transfection activity in receptor-positive cells. By modulating the functional components of the supramolecular platform, we can customize the gene carriers for further research on different tissues or cells *in vitro* or *in vivo*.

Introduction

Gene therapy is considered as a potential modality to treat patients with acquired and inherited genetic diseases.[1-4] Since nucleic acid-based drugs (such as free oligonucleotides and plasmid DNA) are rapidly degraded by nucleases present in extracellular matrices and show limited cellular uptake, the design of efficient and safe gene delivery systems, which can condense the nucleotides and efficiently transport them to the target cells, is the key of successful gene therapy.[5-8] Extensive efforts have been made to increase gene transfection efficiency and to lower toxicity through rational design of degradable and multifunctional polymeric vectors, which have to overcome multiple extra- and intracellular barriers in delivering of polynucleotides.[9-14] For instance, biodegradable cationic polymers with low cytotoxicity have been synthesized to complex nucleic acids to yield nano-sized particles,[6,14] referred to as polyplexes.[15] Neutral hydrophilic polymers (e.g. PEG) were conjugated for shielding of nanoparticles to prevent nonspecific interactions, avoid the clearance by reticuloendothelial system (RES) and prolong the circulation time in blood.[16-19] Antibodies and various targeting ligands were introduced to the polymeric vectors to promote receptor-mediated uptake by specific cells.[20] However, it is very challenging to produce polyplexes with all these functionalities.[21,22]

Recently, supramolecular chemistry has been exploited to develop non-viral gene vectors.[23,24] Among others, cyclodextrins (CDs) based host-guest interactions provide a feasible approach for preparation of gene vectors by self-assembly of different building blocks.[25-31] Wang et al.[32] and Xu et al[33-35] designed various kinds of β-CD derivate polycations to fabricate multifunctional supramolecular gene carriers through combining versatile guest molecules via host-guest interactions. In our previous study, a versatile supramolecular gene delivery system based on adamantyl-functionalized polymers (PEI-Ad and Ad-PEG) and poly(β-cyclodextrin) (PCD) was reported.[36] Low molecular weight PEI-Ad_4 (1.8 kDa) complexed with PCD through host-guest interactions can compact nuclei acid into nanosized polyplexes via electrostatic interactions. Additionally, it was shown that the free CD units of PCD could be used for PEGylation using adamantyl-terminated PEG. In the present study, we use our previous system and post-decorate it with targeting ligands based on individual building blocks with pre-defined functions for rendering the polyplexes cell specific identification to overcome multi-obstacles in transfection process. These different building blocks can be rationally combined and self-assemble to an integrated gene delivery system by host-guest interactions.

It is well known that the tri-peptide Arg-Gly-Asp (RGD) binds to the $\alpha_v\beta_3$ integrin receptor which is overexpressed on tumor endothelium.[37] Moreover folate (FA) is used to target the folate receptor (FR) which is frequently overexpressed on cancer cells,[38]

[a] Key Laboratory of Biomedical Polymers of Ministry of Education and Department of Chemistry, Wuhan University, Wuhan 430072, P. R. China. Tel.: +86-27-68755200; Fax: +86-27-68754509. E-mail address: xljiang@whu.edu.cn.
[b] Department of Pharmaceutics, Utrecht Institute for Pharmaceutical Sciences, Utrecht University, 3584 CG Utrecht, The Netherlands.
[c] Research Center for Tissue Engineering and Regenerative Medicine, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430022, P. R. China.

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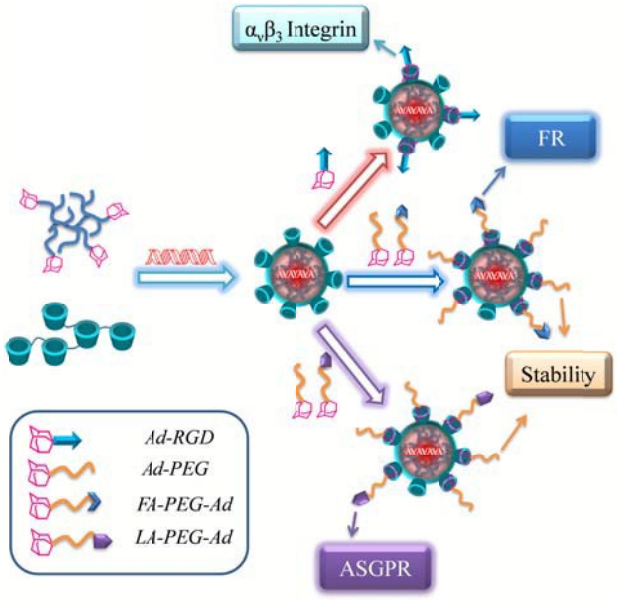


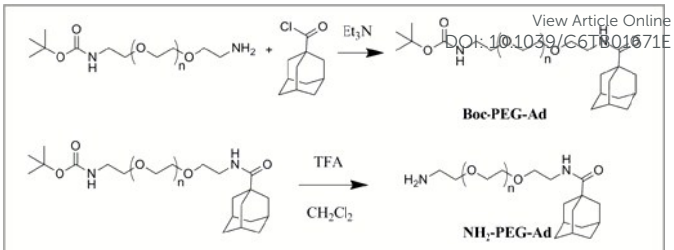
Scheme 1. Schematic illustration of the formation procedures of multifunctional supramolecular polyplexes for targeted gene delivery.

and galactose can specifically bind to asialoglycoprotein receptors (ASGPR) on liver cells.[39] Thus, to demonstrate this modular concept, three adamantyl functionalized targeting guest blocks, adamantyl terminated RGD peptide (Ad-RGD, mainly for in vitro test), folate and lactobionic acid terminated PEGs (FA-PEG-Ad and LA-PEG-Ad), were employed for post-functionalization of adamantly decorated cationic PEI based supramolecular polyplexes (PEI-Ad_4/PCD/DNA) through the free CD units of poly(β-cyclodextran) (PCD) host module, as shown in **Scheme 1**. The specific cellular uptake and transfection of these supramolecular polyplexes were studied in several types of cell lines which overexpress these targeting receptors.

Experimental

Materials

1-Adamantanecarbonylchloride, 1-adamantane carboxylic acid, trifluoroacetic acid (TFA), N, N'-dicyclohexylcarbodiimide (DCC), N-hydroxysuccinimide (NHS), folic acid, triethylamine and anhydrous dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich. Lactobionic acid (LA) and N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC) were purchased Aladdin Industrial Inc (China). Fmoc-L-Gly-OH, Fmoc-L-Arg(Pbf)-OH, Fmoc-L-Asp(tBu)-OH, Triisopropylsilane (TIS), 1-hydroxybenzotriazole (HOBt), 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronoium hexafluorphosphate (HBTU), diisopropylethylamine (DIEA) and Wang resin were purchased from GL Biochem Ltd (China). PD-10 desalting column was obtained from Amersham Biosciences. Boc-PEG-NH_2 (M_w: 3400 Da) was obtained from Shearwater Polymers.



Scheme 2. Synthesis of NH_2-PEG-Ad.

Adamantly functionalized PEG (M_n: 4700 Da, PDI: 1.13) and PEI (Ad-PEG and PEI-Ad_4) and poly(β-cyclodextrin) (PCD, Mw: 60.4 kDa, PDI: 2.02) were synthesized as described in our previous work.[36]

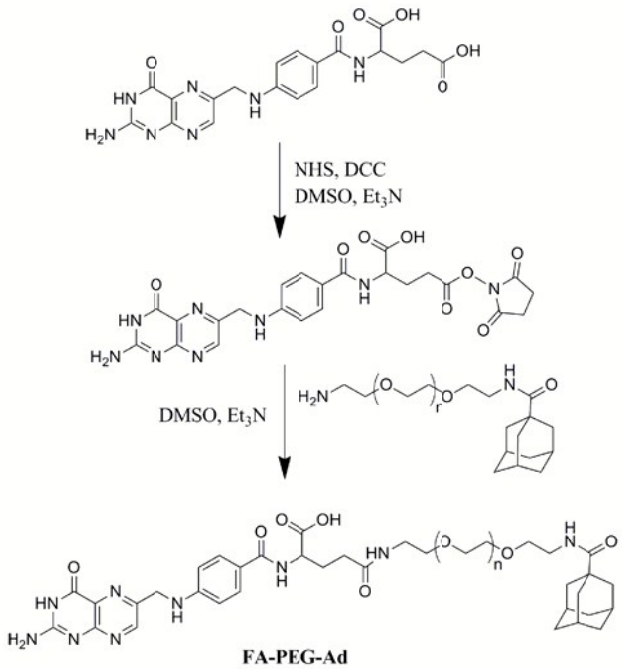
Synthesis of Ad-RGD

The synthesis of adamantyl terminated RGD (Ad-Arg-Gly-Asp) peptide (see the structure of this conjugate in **Fig. S1**) was carried out manually using standard Fmoc coupling protocols.[40] The RGD peptide was conjugated on Wang resin (1.0 mmol/g, 1 equiv.) in a stepwise way. The coupling of amino acids was carried out using 4 equiv. of Fomc-protected amino acids, HBTU, HOBt and DIEA in a DMF solution at room temperature for 3 h. The protecting Fmoc groups were removed using 20% piperidine/DMF (v/v) solution. The coupling efficacy of each step was confirmed via the Kaiser test.[41] Finally, 1-adamantanecarboxylic acid was conjugated to the amine group of the peptide. The cleavage of the Ad-RGD was performed using a mixture of TFA, deionized water and TIS in the volume ratio of 95:2.5:2.5 for 2 h. The obtained solution was collected and dropped in cold diethyl ether to precipitate the product. Next, the product was dissolved in deionized water and lyophilized. The molecular weight of peptide-Ad conjugate was determined by electrospray ionization mass spectrometry (Finnigan LCQ advantage).

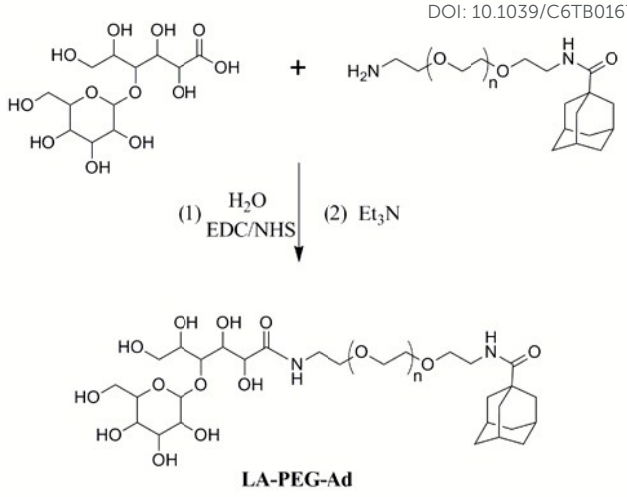
Synthesis of Adamantyl-functionlized PEG (NH_2-PEG-Ad).

1-Adamantanecarbonylchloride (100 mg, 0.50 mmol) was dissolved in 10 mL anhydrous dichloromethane (DCM) and keep in an ice bath. Next, Boc-PEG-NH_2 (250 mg, 0.074 mmol, Mw 3400 Da) and triethylamine (105 µL) solution in 10 mL DCM was added dropwise within 1 h. After the mixture solution was stirred at room temperature overnight, the solvent was removed under reduced pressure and the obtained residue was dissolved in 5 mL water. The solution was centrifuged at 5100 rpm for 40 min to remove 1-adamantane carboxylic acid (precipitate). The supernatant was dialyzed against water for 24 h at 4°C (MWCO: 1000 Da), and BOC-PEG-Ad was obtained after freeze-drying with a yield of 92% and was characterized by ^{1}H nuclear magnetic resonance (NMR).

Boc-PEG-Ad (200 mg) was dissolved in 50% TFA/DCM (v/v) solution and stirred at room temperature for 2 h. Next, TFA and DCM were removed under vacuum, and the obtained polymer was dissolved in 1 mL DCM and then precipitated in 40 mL diethyl ether. The polymer (NH2-PEG-Ad, yield 88%) was characterized by ^{1}H nuclear magnetic resonance (NMR).



Scheme 3. Synthesis of FA-PEG-Ad.



Scheme 4. Synthesis of LA-PEG-Ad.

Synthesis of FA-PEG-Ad

Synthesis of folate N-hydroxysuccinimidyl ester (FA-NHS). FA-NHS was synthesized as described previously.[42] In brief, folic acid (0.50 g, 1.13 mmol) and triethylamine (0.25 mL) were dissolved in anhydrous DMSO (20 mL) for 1 h. Then, *N*-hydroxysuccinimide (NHS, 0.26 g, 2.26 mmol) and dicyclohexylcarbodiimide (DCC, 0.25 g 1.21 mmol) were added, and the mixture was stirred for overnight in dark. Subsequently, the mixture was filtered and used directly for the next reaction.

NH_2-PEG-Ad (170 mg, 48 μmol) and triethylamine (20 μL) were dissolved in 1 mL anhydrous DMSO and 2.7 mL FA-NHS solution (151 μmol) was subsequently added. The resulting solution was stirred at room temperature for 24 h. After the reaction, DMSO was removed under vacuum, the residue was dissolved in 2.5 mL deionized H_2O and centrifuged at 11,000 rpm for 30 min to remove insoluble impurities. The product was further purified by gel-filtration using PD-10 columns (Sephadex G-25) to remove unreacted folic acid with 0.1 M $NaHCO_3$ as eluent, and desalted with water as eluent. After freeze-drying, a yellow solid (FA-PEG-Ad) was obtained with a yield of 59% and was analyzed by ^{1}H NMR, UV-Vis and HPLC.

Synthesis of LA-PEG-Ad

Lactobionic acid (LA, 0.126 g, 0.35 mmol), N-hydroxysuccinimide (NHS, 0.162 g, 1.41 mmol) and N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC, 0.27 g, 1.41 mmol) were dissolved in 5 mL deionized H_2O and reacted for 10 minutes (the pH was 5.8). Subsequently, NH_2-PEG-Ad (75 mg, 0.021 mmol) was added and the solution was stirred at room temperature for 24 hours. Then, 200 μL triethylamine was added and the solution was stirred 24 hours. Subsequently, the solution was dialyzed against water for 4 days (MWCO: 1000 Da), and LA-PEG-Ad was obtained by freeze-drying with a yield of 79%. The product was characterized by HPLC analysis.

Characterization

^{1}H NMR and ^{13}C NMR spectra were measured with a Mercury 300 MHz spectrometer (Varian Associates Inc. NMR instruments, Palo Alto, CA) using $CDCl_3$ or D_2O as solvents. Nuclear Overhouser effect spectroscopy (NOESY) spectra were recorded with a Mercury 300 MHz spectrometer using D_2O as solvent for 14 h.

The folate PEG conjugate (FA-PEG-Ad) was analyzed by a Waters HPLC system that consisted of a Waters 2695 separations module, a Waters 2487 Dual λ absorption detector (UV) and a Waters 2414 refractive index detector (RI). Two chromatographic columns (2 × Shodex OHpak SB-804 HQ) with a pre-column (Shodex SB-G) were used in series. A sodium chloride (NaCl, 5 mM) solution was used as eluent at a flow rate of 1 mL/min. Both the UV absorption at 280 nm and 363 nm were used to detect the folate group. Free folic acid and NH_2-PEG-Ad were used as references. Furthermore, the folate content of FA-PEG-Ad was determined using UV-vis spectrometer (Lambda Bio40, Perkin-Elmer). Folic acid and FA-PEG-Ad were separately dissolved in DMSO. The absorbance of folic acid solution (0.01 mg/mL to 0.05 mg/mL) at 363 nm was recorded, which exhibited a linear correlation with folic acid concentration. The folate content in FA-PEG-LA was calculated using the calibration curve of folic acid.

The lactobionic acid terminated PEG (LA-PEG-Ad) was analyzed by a CoM 6000 HPLC system that consisted of a CoMetro 6000 LDI-100 separations module and a Shodex RI-101 refractive index detector (RI). A chromatographic column (Shodex OHpak SB-804 HQ) and a pre-column (Shodex SB-G) were used in series. A sodium chloride (NaCl, 5 mM) solution was used as eluent at a flow rate of 1 mL/min. NH_2-PEG-Ad was used as a reference.

Cell culture

Human cervix carcinoma (HeLa) cells, human liver hepatocellular carcinoma (HepG2) cells and SV-40 transformed African green

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monkey kidney (COS-7) cells were incubated in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS), 100 units/mL penicillin and 100 g/mL streptomycin at 37°C under a humidified atmosphere of 95% air and 5% CO_2.

Formulation of multifunctional polyplexes

PCD, PEI-Ad_4, Ad-PEG, Ad-RGD, FA-PEG-Ad and LA-PEG-Ad were dissolved in Hepes buffer (20 mM. pH 7.4), respectively. PCD and PEI-Ad_4 were diluted to proper concentrations in Hepes and mixed with 150 µL of pCMV_Luc solution (50 ng/µL, 20 mM Hepes) in sequence, and incubated 30 min at room temperature to obtain PEI-Ad_4/PCD/DNA polyplexes. The feed ratio of $Ad_{(PEI\text{-}Ad4)}/CD_{(PCD)}$ was 1:2, because this ratio showed highest transfection efficiency in our previous paper.[43] The PEI-Ad_4/PCD based supramolecular polyplexes are further termed as PPS.

RGD functionalized polyplexes were prepared by mixing PPS with Ad-RGD followed by incubation for 30 min at room temperature. The feed ratio of $Ad_{(PEI\text{-}Ad4)}/CD_{(PCD)}/Ad_{(Ad\text{-}RGD)}$ was 1:2:0.5, and the polyplexes are termed as RGD-PPS.

PEGylated polyplexes were prepared by mixing PPS and Ad-PEG followed by incubation for 30 min at room temperature. The feed ratio of $Ad_{(PEI\text{-}Ad4)}/CD_{(PCD)}/Ad_{(Ad\text{-}PEG)}$ was 1:2:0.5, and the polyplexes are termed as PEG-PPS. Folate and lactobionic acid decorated polyplexes were fabricated with same method by incubating PPS with Ad-PEG and FA-PEG-Ad (or LA-PEG-Ad) for 30 min at room temperature. The feed ratio of $Ad_{(PEI\text{-}Ad4)}/CD_{(PCD)}/Ad_{(Ad\text{-}PEG)}/Ad_{(FA\text{-}PEG\text{-}Ad\ or\ LA\text{-}PEG\text{-}Ad)}$ was 1:2:0.3:0.2, and these polyplexes are termed as FA-PEG-PPS and LA-PEG-PPS, respectively. The obtained polyplex dispersions were adjusted to 20 mM Hepes, 130 mM NaCl (HBS) by a 20 mM Hepes, 1.95 M NaCl solution (pH 7.4) before use.

The stability of polyplexes in physiological salt.

The polyplexes with/without PEG coating at N/P 10 were prepared in 20 mM Hepes as described above, and subsequently a 20 mM Hepes, 1.95 M NaCl, pH 7.4 solution was added to yield 20 mM Hepes and 130 mM NaCl. Next, the particle size of the polyplexes was monitored over time by Dynamic Light Scattering (DLS) on an ALV CGS-3 system (Malvern Instruments, Malvern, UK) equipped with a JDS Uniphase 22 mW He-Ne laser operating at 632.8 nm. The morphology of PEI-Ad_4/PCD/DNA polyplexes (PPS) and PEI-Ad_4/PCD/DNA/PEG (PEG-PPS) prepared at N/P ratio of 10 was observed by transmission electron microscope (TEM). The polyplex solution was dropped on a copper grid and stained with 0.2% (w/v) phosphotungstic acid solution. The copper grid was dried and performed on a HITACHI H-7650 TEM.

***In vitro* cytotoxicity assay**

Cytotoxicity experiments were performed with HeLa and HepG2 cells using MTT assay. In detail, the cells were seeded in a 96-well plate at a density of 8000 cells/well and incubated at 37°C for 24 h. Next, the medium of each well was replaced with 100 µL fresh medium (DMEM with 10% FBS), and PEI-Ad_4/PCD based polyplex dispersions (prepared as described above) were added. After incubation for 48 h, the medium was replaced by 200 µL fresh medium and MTT reagent (20 µL in PBS, 5.0 mg/mL) was added followed by 4 h incubation at 37°C. Then, the medium was removed and DMSO (150 µL) was added to dissolve the formed formazan crystals. The absorbance at 570 nm was determined using a Microplate Reader (BIO-RAD 550) and the cell viability was calculated as follows:

$$\textbf{Relative cell viability}\ (\%) = \frac{\boldsymbol{OD_{sample} - OD_{blank}}}{\boldsymbol{OD_{control} - OD_{blank}}} \times \mathbf{100}\%$$

where OD_{sample} was the solution absorbance for cells exposed to the different polyplex dispersions, $OD_{control}$ was the absorbance for untreated cells (with HBS), OD_{blank} was the absorbance without cells.

***In vitro* transfection activity assay**

Transfection activity of the different polyplexes was determined in HeLa, HepG2 and COS-7 cells using pCMV_Luc plasmid DNA as report gene. The cells were seeded in a 96-well plate at a density of 8000 cells/well and incubated for 24 h before transfection. The medium of each well was replaced by fresh medium (100 µL DMEM with 10% FBS) and the polyplexes (in HBS; 0.25 µg DNA per well) were added and incubated with the cells for 48 h at 37 °C. Subsequently, the cells were washed with PBS and lysed with 50 µL 1× Reporter Lysis buffer (Promega) according to manufacturer's protocols. The luciferase expression was determined with chemiluminometer (Lumat LB9507, EG&G Berthold, Germany). The total protein concentration in the cell lysates was measured using a Micro BCA Protein Assay kit (Pierce). Luciferase activity is expressed as RLU/mg protein (mean ± SD, n=3).

Cellular internalization assay

Live cell confocal microscopy was used to study the internalization of the different polyplexes in HeLa and HepG2 cells. The pCMV_Luc was labelled with YOYO-1 (green molecular probe, Invitrogen) and the nuclei were stained with Hoechst 33258 (blue molecular probe, Sigma-Aldrich). HeLa (or HepG2) cells were seeded in a 6-well plate at a density of 60,000 (80,000) cells/well and incubated for 24 h at 37°C under a humidified atmosphere of 95% air and 5% CO_2. Subsequently, the medium was replaced by 0.9 mL fresh DMEM with 10% FBS, and 100 µL polyplexes dispersion (1 µg pCMV_Luc, N/P 20) was added. After 4 h incubation at 37 °C, the medium was removed, and the cells were washed with PBS for 3 times followed by fixing with 4% paraformaldehyde for 30 minutes at 4 °C. After that, the cells were washed with PBS for 3 times and 1 mL Hoechst 33258 solution (0.01 mg/mL) was added and incubated with the cells for 20 minutes. Finally, the cells were washed with PBS for 3 times, and 1 mL PBS was added. The cells were imaged using a confocal laser scanning microscopy (Nikon C1-si TE2000, Japan). The quantitative fluorescence analysis of confocal images was performed by ImageJ software, the data are shown as mean fluorescence intensity per cell.

Statistical analysis

All results were exhibited as mean ± standard deviation (SD) with at least three tests. The statistical analysis was performed by student t-test. *P<0.05 was considered to be significant; *P<0.01 was considered to be highly significant; N.S. was considered to be not significant.

Results and discussion

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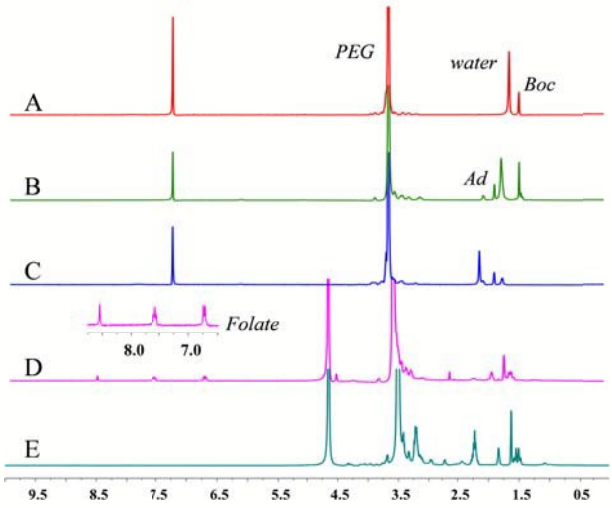

Fig. 1 ^{1}H NMR spectra of Boc-PEG-NH_2 (A), Boc-PEG-Ad (B) and Ad-PEG-NH_2 (C) in $CDCl_3$, and FA-PEG-Ad (D) and LA-PEG-Ad （E） in D_2O.

Synthesis and characterization of adamantyl functionalized targeting ligands

The gene delivery vehicle studied in the present paper is composed of three building blocks: (1) adamantyl functionalized polycation (PEI-Ad_4), (2) water-soluble and neutral poly(β-cyclodextrin) (PCD) and (3) adamantyl functionalized shielding and/or targeting conjugates to decorate the polyplexes. PEI-Ad_4, Ad-PEG and PCD polymers were synthesized as described in our previous paper.[36] The adamantyl functionalized guest targeting ligands Ad-RGD, FA-PEG-Ad and LA-PEG-Ad were synthesized and characterized in detail in the Experimental section.

Adamantyl terminated arginine-glycine-aspartic acid (Ad-RGD) peptide was synthesized by a standard Fmoc chemistry through a solid phase peptide synthesis method, and the molecular weight of this peptide was determined to be 509.63 $[M+H]^+$ by ESI-MS (**Fig. S1A**), indicating its successful synthesis. The ^{1}H NMR spectrum further confirmed the structure of Ad-RGD (**Fig. S1B**).

Polyethylene glycol modified with adamantyl and folate terminal groups (FA-PEG-Ad) was synthesized by several steps as shown in **Scheme 2 and 3**. First, Boc-PEG-NH_2 was reacted with excess 1-adamantanecarbonylchloride to obtain Boc-PEG-Ad. The NMR spectrum of Boc-PEG-Ad (**Fig. 1B** and **S2B**) demonstrated that the reaction between the terminal amine group of Boc-PEG-NH_2 with acyl chloride was almost quantitative. Next, the t-butyloxy carbonyl group was removed by trifluoroacetic acid (TFA), and NMR analysis showed that the Boc group was completely removed and thus Ad-PEG-NH_2 was obtained (**Fig. 1C, S2C and S2F**). Folic acid was reacted with *N*-hydroxysuccinimide (NHS) to yield folate *N*-hydroxysuccinimide ester (FA-NHS) and this product was reacted with the amine of Ad-PEG-NH_2 to obtain FA-PEG-Ad. NMR analysis of the product showed the presence of three proton peaks of folate at 6.5 to 9.0 ppm (**Fig. 1D)** and carbon peaks of folate (**Fig. S3A**), and the modification degree of folate was calculated to be over 90% using NMR integrals (**Fig. S2D**), which indicated successful folate conjugation. To investigate the conjugating efficiency and purity of FA-PEG-Ad, HPLC was employed with RI and UV detection. **Fig. S4** showed that, as expected, no UV signal of Ad-PEG-NH_2 was detected at both 280 nm and 363 nm, whereas its RI trace showed a peak at 19-23 min. After conjugation with folate, the RI trace of FA-PEG-Ad shifted and the retention time was 15-19 min, and the corresponding UV signals (both at 280 nm and 363 nm) also appeared at 15-19 min. The HPLC results demonstrated that the folate modification of Ad-PEG-NH_2 was almost quantitative. Furthermore, the folate content of the obtained FA-PEG-Ad was determined to be 10.0 ± 0.1 wt% by UV-Vis, based on the folic acid calibration curve (**Fig. S5)**, which is close to the expected value (10.7 wt%). It is therefore concluded that based on NMR, HPLC and UV-Vis analysis, the PEG with adamantyl and folate terminal groups was obtained with good purity.

Polyethylene glycol with adamantyl and lactobionic acid terminal groups (LA-PEG-Ad) was synthesized as shown in **Scheme 4**. No obvious characteristic peaks of lactobionic acid moiety could be observed in the NMR spectrum of LA-PEG-Ad in D_2O (**Fig. 1E, S2E** and **S3B**) due to the weak signal from the glucose end-group overlapped with the signal of PEG backbone. It is also difficult to determine the lactobionic acid content in LA-PEG-Ad by UV-Vis analysis because it does not possess any structural elements absorbing in the commonly applied UV region. Fortunately, separation method of PEGs and amino-terminated PEGs by HPLC under near critical conditions has been reported very recently in our group[44]. The modification efficiency and purity of LA-PEG-Ad

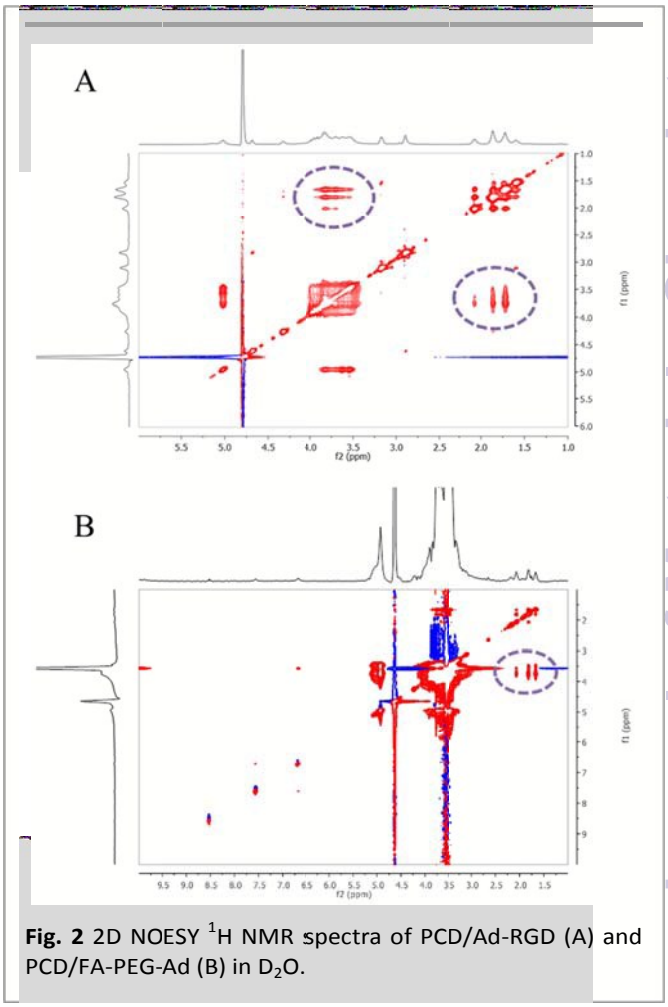

Fig. 2 2D NOESY ^{1}H NMR spectra of PCD/Ad-RGD (A) and PCD/FA-PEG-Ad (B) in D_2O.

were investigated by HPLC (**Fig. S6).** The retention time of starting polymer Ad-PEG-NH_2 was at 9.5-11.7 min. The main signal of the product polymer shifted to 6.8-9.6 min and only a small peak remained at the original position (9.5-11.7 min), which indicated the LA-PEG-Ad was synthesized successfully with the modification degree of LA about 96%, calculated by the HPLC integral area.

Self-assembly and colloidal stability of polyplexes

The polyplexes were prepared through self-assembly of the host polymer (PCD), the guest polymers (PEI-Ad_4, Ad-RGD, Ad-PEG, FA-PEG-Ad or LA-PEG-Ad) and pDNA, via electrostatic interaction and host-guest interactions using the same method described in our previous paper.[36,43] To confirm the host-guest interaction between PCD and adamantyl terminated guest polymers, mixtures of PCD and Ad-RGD or FA-PEG-Ad were analyzed by 2D ^{1}H nuclear Overhouser effect spectroscopy (NOESY) in D_2O. As shown in **Fig. 2**, the NOE cross peaks between the inner protons of PCD (3.5- 4.1 ppm) and the protons of adamantyl (1.6-2.1 ppm) appeared, indicating that PCD indeed forms supramolecular inclusion complexes with these guest polymers in aqueous solution, which is in agreement with our previous results.[36] In the same paper, it was also shown that PCD and adamantly modified PEI can condense pDNA to obtain supramolecular polyplexes (PEI-Ad_4/PCD/DNA, denoted as PPS) and the free CD units of polyplexes could be subsequently post-PEGylated with Ad-PEG via host-guest interaction, which enhanced the colloidal stability of the PEGylated polyplexes under physiological condition. Herein, the morphology of the polyplexes PPS and PEG-PPS was also investigated using transmission electron microscope (TEM), as shown in Fig. S7. The PEGylated polyplexes PEG-PPS formed slightly bigger spherical nanoparticles than the PPS polyplexes without PEGylation. In addition, we investigated the colloidal stability of PPS decorated with various functional ligands (Scheme 1) by dynamic light scattering (DLS). Both PPS and RGD-PPS [(PEI-Ad_4+PCD)/DNA+50%Ad-RGD] aggregated rapidly in saline. On the other hand, the PEGylated polyplexes, FA-PEG-PPS [(PEI-Ad_4+PCD)/DNA+30%Ad-PEG+20%FA-PEG-Ad] and LA-PEG-PPS [(PEI-Ad_4+PCD)/DNA+30%Ad-PEG+20%LA-PEG-Ad] showed excellent stability under physiological conditions (**Fig. S8**), again demonstrating that PEGylation increases the stability of polyplexes in saline.[17,36,45]

***In vitro* cytotoxicity**

HeLa and HepG2 cells were incubated with PPS and PEGylated PEG-PPS, and the cell viability was evaluated by the MTT assay. Fig. 3 shows that commercial transfection reagent ExGen based polyplexes showed high cytotoxicity with cell viability of only 20%. On the other hand, the PPS and PEG-PPS polyplexes showed an excellent cytocompatibility with cell viability higher than 80%. Therefore, this supramolecular gene delivery system designed from 1.8 kDa PEI inherits the advantage of low molecular weight (LMW) PEI with a low cytotoxicity.

Cellular internalization of multifunctionalized supramolecular polyplexes

Various host-guest supramolecular self-assembled nanosized polyplexes (PPS, RGD-PPS, PEG-PPS, FA-PEG-PPS and LA-PEG-PPS) based on PEI-Ad_4, PCD, and targeting ligands such as Ad-RGD, FA-PEG-Ad and LA-PEG-Ad were prepared. The cellular internalization of these polyplexes was evaluated in HeLa cells ($\alpha_v\beta_3$ integrin and folate receptors positive tumor cells) [46, 47] and HepG2 cells

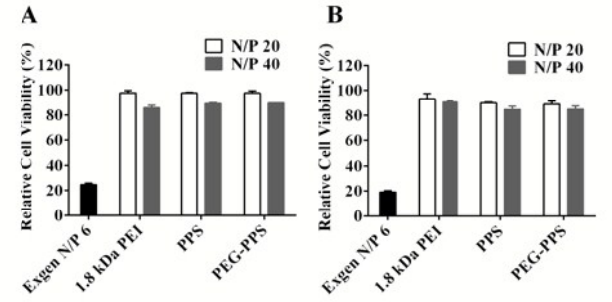

Fig. 3 Relative cell viability of HeLa (A) and HepG2 (B) cells after incubation for 48 h with ExGen/DNA polyplexes (N/P 6), 1.8 kDa PEI/DNA polyplexes, PPS and PEG-PPS.

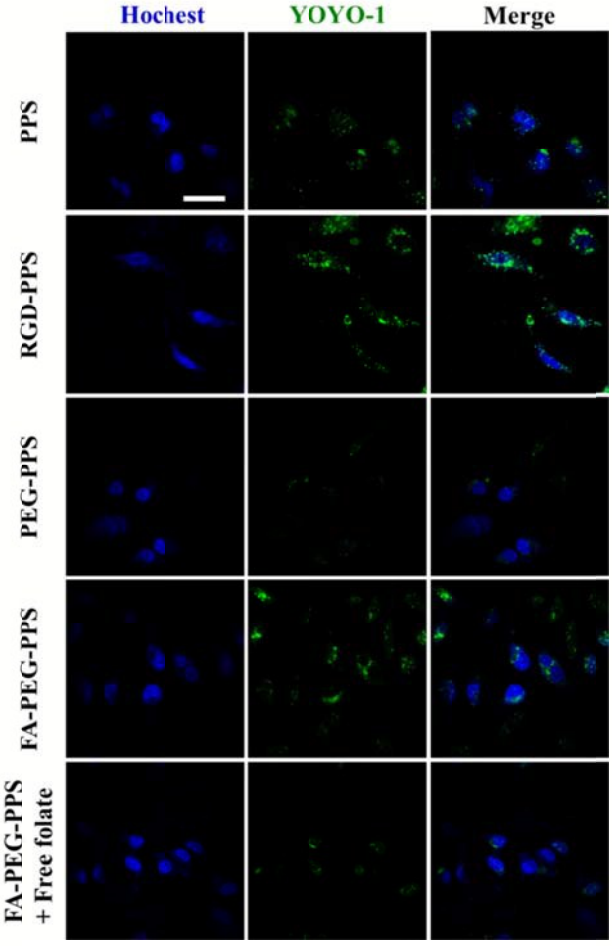

Fig. 4 Confocal laser scanning images of HeLa cells after incubation with YOYO-1 stained pCMV_Luc (green) polyplexes. The following polyplexes were used: PPS, RGD-PPS, PEG-PPS, FA-PEG-PPS in DMEM without folate or with 0.5 mM folate at N/P of 40, respectively. The scale bar is 50 μm.

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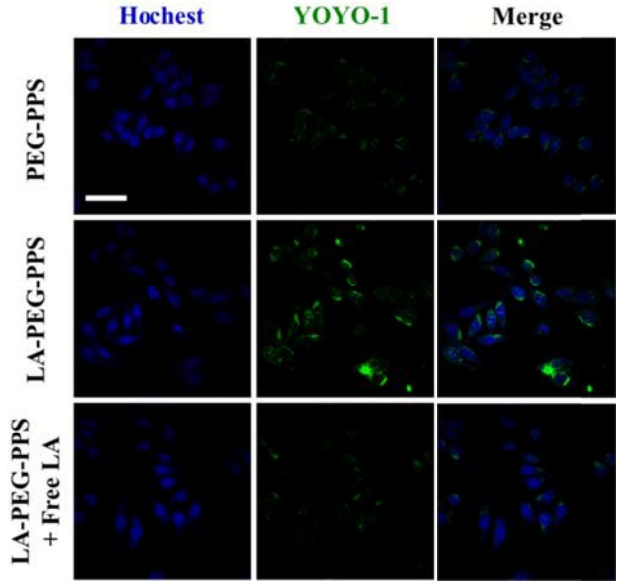

Fig. 5 Confocal laser scanning images of HepG2 cells after incubation with YOYO-1 stained pCMV_Luc (green) polyplexes. The following polyplexes were used: PEG-PPS, LA-PEG-PPS and LA-PEG-PPS in DMEM without or with 10 mM lactobionic acid (LA) at N/P of 40, respectively. The scale bar is 50 μm.

(asialoglycoprotein receptors positive cells)[48] by confocal laser scanning microscopy (CLSM). The cell nuclei were stained with Hoechst 33258 (blue fluorescence), and the plasmid DNA was labelled with YOYO-1 (green fluorescence).

After incubation with the cells for 4 hours, PPS with green fluorescence was observed in HeLa cells, suggesting PEI-Ad$_4$/PCD could deliver pDNA into cells. A higher number of fluorescent dots were detected in HeLa cells incubated with RGD-PPS (no PEG) (**Fig. 4**) and the mean fluorescence intensity (MFI) increased to 2.8 times as compared with that of PPS treated cells (**Fig. S9A**). This indicates that RGD (a ligand for the integrin $\alpha_v\beta_3$ receptor present on HeLa cells) modification on polyplex surface improved the cellular

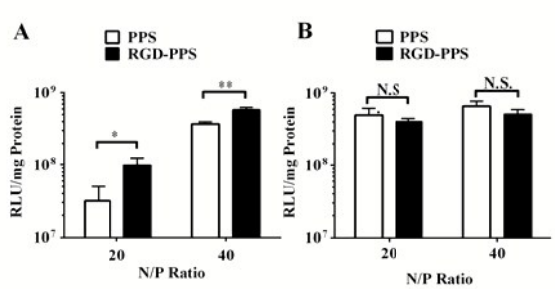

Fig. 6 Luciferase expression in HeLa (A) and COS-7 (B) cells upon incubation with PPS and RGD-PPS polyplexes. Data are shown as mean ± SD (*P<0.05, **P<0.01, n=3).

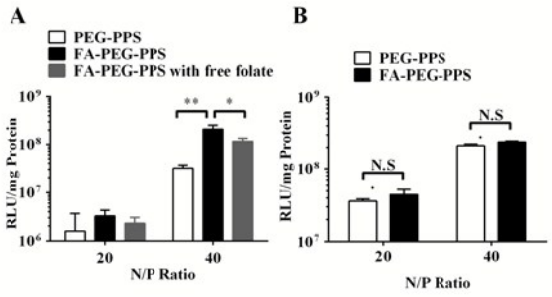

Fig. 7 Luciferase expression of HeLa (A) and COS-7 (B) cells upon incubation with PEG-PPS, and FA-PEG-PPS in DMEM without or with 0.5 mM folic acid. Data are shown as mean ± SD (*P<0.05, **P<0.01, n=3).

internalization of RGD-PPS in HeLa cells. The uptake of PEGylated polyplexes (PEG-PPS) by HeLa cells was significantly less than that of PPS, (mean fluorescence intensity decreased to about 20% in HeLa cells (Fig. 4 and S9A)). Since the PEG shielding decreased the positive ζ -potential of polyplexes,[36] which would inhibit the non-specific interaction between cellular membranes and polyplexes, therefore the cellular uptake decreased due to the stealth effect of PEG.[49] Notably, FA-PEG-PPS polyplexes showed a substantial higher uptake (2.4 fold) than PEG-PPS polyplexes, which points to uptake of folate decorated polyplexes by HeLa cells via the folate receptor. Importantly, the internalization of FA-PEG-PPS polyplexes was knocked down by addition of free folate (0.5 mM) in the medium, which demonstrates the folate-mediated enhanced cellular uptake of folate functionalized polyplexes by FR-positive cells.[50,51]

The targeting ligand lactobionic acid (LA), which is a specific ligand for asialoglycoprotein receptors (ASGPR) on liver cells,[52] was also coupled to the polyplexes via host-guest interactions. **Fig. 5** and **S9B** show that the lactobionic acid decorated polyplexes (LA-PEG-PPS) were internalized by HepG2 cells to a higher extent (2.7 fold higher fluorescence intensity) than PEG-PPS polyplexes. Again, this higher uptake of the targeted polyplexes was knocked down by addition of free LA in the medium, demonstrating receptor mediated uptake of LA-PEG-PPS polyplexes.

Transfection activity of multi-functional supramolecular polyplexes

The transfection activity of the different supramolecular polyplexes was investigated in both receptor-positive and receptor-negative cells (HeLa, HepG2 and COS-7). The transfection activity of RGD-PPS polyplexes in integrin αvβ3 receptor positive cells (HeLa) was significantly higher than that of PPS at N/P of 20 and 40 (**Fig. 6A**). Since the RGD peptide functionalization remarkably enhanced the cellular uptake of the supramolecular polyplexes (RGD-PPS) in receptor-positive cells (**Fig. 4** and **S9A**), the RGD decoration promoted the transfection activity as well. In contrast, no obvious difference in the transfection activity for RGD-PPS and PPS was observed in integrin receptor-devoid COS-7 cells.[53]

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The transfection activity of PEG-PPS in HeLa cells was about 10 times lower than that of PPS (**Fig. 7A**), because the PEG coating of the polyplexes reduced the interaction between the nanoparticles and cellular membrane to result in lower cellular uptake of polyplexes (**Fig. 4** and **S9**) and thus in lower transgene expression, in line with gene expression data of other PEGylated polyplexes.[45,54] **Fig. 7A** shows that the transfection activity of FA-PEG-PPS polyplexes increased significantly in FR-positive HeLa cells as compared with those of PEG-PPS. Less luciferase expression was observed when HeLa cells were incubated with FA-PEG-PPS polyplexes in the presence of 0.5 mM free folic acid (**Fig. 7A**). This can be explained by a lower uptake of the folate decorated polyplexes due to inhibition of their binding to the folate receptor in the presence of free folic acid. While, the transfection activity of FA-PEG-PPS had no significant difference with that of PEG-PPS in FR-negative COS-7 cells (**Fig. 7B**).[55] These data of the competitive inhibition of free folate in medium (**Fig. 7A**) and negligible transfection difference in COS-7 cells (**Fig. 7B**) further confirm that the specific transfection enhancement of FA-PEG-PPS in FR-positive cells is caused by the folate-mediated targeting. The LA-PEG-PPS decorated with LA groups also showed higher luciferase expression in ASGPR-positive HepG2 cells than that of PEG-PPS, but exhibited comparable transfection activity as PEG-PPS in ASGPR-negative COS-7 cells (**Fig. 8**).[56]

Conclusions

To design multifunctional polymeric gene carriers for overcoming the different transfection barriers, we describe in the present paper a supramolecular modular approach for generating customized gene delivery systems via host-guest self-assembly using different building blocks. Three adamantly-terminated guest components, Ad-RGD, FA-PEG-Ad and LA-PEG-Ad were synthesized and utilized as functional components. By rational combining with cationic guest ($PEI\text{-}Ad_4$) and host (PCD) modules, three targeted supramolecular polyplexes were prepared and characterized. It was shown that these polyplexes were internalized into target cells by receptor-mediated uptake and showed great transfection activity. Importantly, by altering the targeting ligands, the supramolecular carrier system could target to specific cells and enhance the transgene expression. The modular approach described in this paper might be a promising and convenient way of customizing multifunctional gene carriers for practical applications.

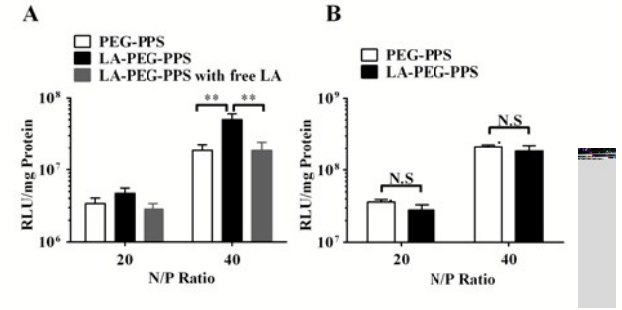

Fig. 8 Luciferase expression of HepG2 (A) and COS-7 (B) cells upon incubation with PEG-PPS, and LA-PEG-PPS in DMEM without or with 10 mM lactobionic acid (LA). Data are shown as mean ± SD (**P<0.01, n=3).

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TOC Graphical abstract:

Convenient modular approach for multifunctional supramolecular self-assembly polyplexes of poly(cyclodextrin) and mono-adamantane-terminated guest polymers, displaying targeting cellular uptake and transfection.