



OPEN

Recycling Gene Carrier with High Efficiency and Low Toxicity Mediated by L-Cystine-Bridged Bis(β -cyclodextrin)s

SUBJECT AREAS:

SELF-ASSEMBLY

MOLECULAR SELF-ASSEMBLY

Received
27 October 2014Accepted
18 November 2014Published
15 December 2014Correspondence and
requests for materials
should be addressed toY.L. (yuliu@nankai.
edu.cn)Yu-Hui Zhang¹, Yong Chen¹, Ying-Ming Zhang¹, Yang Yang¹, Jia-Tong Chen² & Yu Liu¹

¹Department of Chemistry, State Key Laboratory of Elemento-Organic Chemistry, Collaborative Innovation Center of Chemical Science and Engineering (Tianjin), Nankai University, Tianjin 300071, P. R. China, ²Department of Biochemistry and Molecular Biology, College of Life Sciences, Nankai University, Tianjin 300071, P. R. China.

Constructing safe and effective gene delivery carriers is becoming highly desirable for gene therapy. Herein, a series of supramolecular crosslinking system were prepared through host-guest binding of adamantyl-modified low molecular weight of polyethyleneimine with L-cystine-bridged bis(β -cyclodextrin)s and characterized by ¹H NMR titration, electron microscopy, zeta potential, dynamic light-scattering, gel electrophoresis, flow cytometry and confocal fluorescence microscopy. The results showed that these nanometersized supramolecular crosslinking systems exhibited higher DNA transfection efficiencies and lower cytotoxicity than the commercial DNA carrier gold standard (25 kDa bPEI) for both normal cells and cancer cells, giving a very high DNA transfection efficiency up to 54% for 293T cells. Significantly, this type of supramolecular crosslinking system possesses a number of enzyme-responsive disulfide bonds, which can be cleaved by reductive enzyme to promote the DNA release but recovered by oxidative enzyme to make the carrier renewable. These results demonstrate that these supramolecular crosslinking systems can be used as promising gene carriers.

Gene therapy as a potential treatment means has drawn more and more attention in recent years, because of its great promise to cure various diseases^{1,2}. However, the design of safe and efficient gene carriers is challenging for gene therapy^{3,4}. In contrast to viral carriers, non-viral carriers have low immunogenicity⁵, good biocompatibility⁶ and satisfactory DNA loading capability⁷. Therefore non-viral gene carriers such as liposomes⁸, polymers⁹ and dendrimers¹⁰ have been widely studied in recent years^{11–15}. Among the mentioned non-viral gene carriers, polyethylenimine (PEI) with molecular weight of 25 kDa has been considered especially as the gold standard of gene transfection¹⁶, mainly due to its high buffer capacity that could protect DNA from the degradation of nuclease¹⁷. It is noteworthy that, although PEI with high molecular weight possesses high transfection efficiency, it also induces higher toxicity in the biological process on account of their non-biodegradability¹⁸. Recently, the crosslinking of PEI with low molecular weight is widely regarded as an effective way to reduce the cytotoxicity at high gene expression level, and various covalently crosslinked PEI bearing biodegradable linkers such as disulfide bond, ester linkage and amido bond were synthesized^{19–21}. In this work, we wish to report the supramolecular crosslinking of PEI mediated by L-cystine-bridged bis(β -cyclodextrin)s (LCD). That is, as a consequence of the noncovalent association of the adamantyl groups grafted to PEI with the CD cavities in LCD, PEI with low molecular weight eventually self-assembled to biocleavable nanosupramolecular crosslinking system (Fig. 1). There are several inherent advantages for choosing LCD as a non-covalent and biodegradable linker: (1) cyclodextrin (CD), a series of cyclic oligosaccharides, is water-soluble, nontoxic, commercially available at low cost; (2) CD can bind various organic/biological/drug molecules and ions in its hydrophobic cavity in aqueous solution, and thus widely used as convenient building blocks for constructing nanostructured drug and gene carriers^{22–34}; (3) the disulfide bond in LCD is reported to be rapidly responsive to a redox environment³⁵, and thus can be cleaved with the trigger of glutathione (GSH) in cytosol to release DNA (pDNA) because the concentration of intracellular GSH is several times higher than that of extracellular one³⁶; (4) Significantly, after releasing DNA, this DNA carrier can be recycled via the treatment with an oxidant such as horseradish peroxidase (HRP). Therefore, this strategy not only avoids the complicated synthesis/separation steps that only involve in the covalent modification of PEI, but also provides a recycling non-viral gene carrier with stronger gene condensation affinity, higher gene transfection efficiency, lower cellular toxicity than 25 kDa bPEI, which will energize the

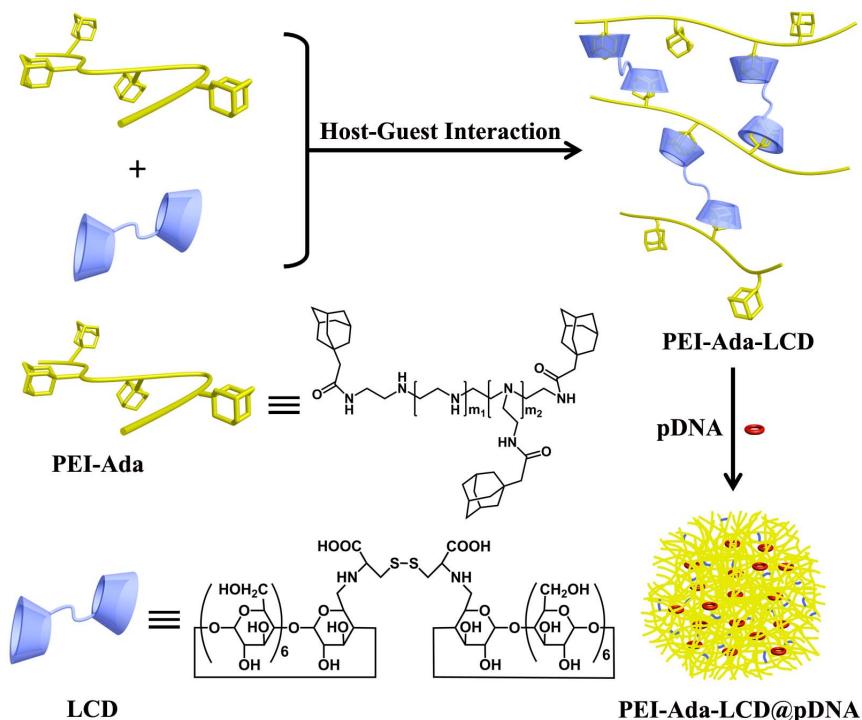


Figure 1 | Chemical structure and construction of PEI-Ada-LCD@pDNA complexes. Complexes were constructed by electrostatic interaction.

potential use of CD-based bioactive supramolecular nanostructures in the construction of safe and highly efficient gene carriers.

Results and Discussion

Synthesis. A series of polycationic PEI derivatives (PEI-Ada), incorporating 8.2–13.7 adamantyl groups grafted to a PEI chain ($M_w = 10000$), were synthesized via an amide condensation reaction between 1-adamantaneacetic acid and PEI in ethanol and characterized by NMR spectroscopy. The number of grafted adamantyl groups on PEI chain was controlled by changing the feed ratio of 1-adamantaneacetic acid to PEI. As shown in Supplementary Fig. S1, ^1H NMR spectra of PEI-Ada showed the characteristic proton signals of adamantyl group at ca. 1.6–1.71 ppm and 2 ppm, and the proton signals at 2.8 ppm to 3.4 ppm were assigned to PEI and amido bond. Through a comparative analysis of the integral peak area of the protons of adamantyl group and the protons of PEI, we could define the degree of substitution (DS) of adamantyl group per PEI chain. Moreover, two additional PEI derivatives with higher DS values, i.e. PEI-Ada-15.5 and PEI-Ada-33, were also synthesized and showed relatively low water solubility (Supplementary Fig. S2). Therefore, only PEI-Ada-8.2 to PEI-Ada-13.7 were used in the further crosslinking with LCD. The DS value and molecular weight of PEI-Ada were summarized in Supplementary Table S1.

Enzyme-responsive supramolecular crosslinking of PEI-Ada with LCD. Benefiting from the strong binding between β -CD cavity and adamantane derivatives ($K_s = 10^4 \text{ M}^{-1}$)^{37,38}, PEI-Ada-LCD supramolecular crosslinking system could be prepared easily by mixing LCD with PEI-Ada. Subsequently, the binding constants between β -CD and 1-adamantaneacetic acid sodium salt (AdAA) were determined in saline (Supplementary Fig. S3) and 5% glucose solution (Supplementary Fig. S4) using ^1H NMR titration method to determine the binding stability in physiological environment. By analyzing the sequential changes of chemical shifts of AdAA in the presence of varying concentrations of β -CD using a nonlinear least-squares curve-fitting method, the binding constants ($\log K_s$) could be calculated to be $2.21 \times 10^4 \text{ M}^{-1}$ and $1.42 \times 10^4 \text{ M}^{-1}$, respectively.

These results indicate that PEI-Ada-LCD supramolecular crosslinking system can keep stable in physiological environment, which is conducive to their biological applications.

The direct morphological information of the supramolecular crosslinking system comes from the high-resolution transmission electron microscopy (HR-TEM). As shown in Supplementary Fig. S5, PEI-Ada existed as spherical particles with an average diameter of ca. 30 nm, probably due to the amphiphilic aggregation of PEI-Ada. After LCD was added, the resultant PEI-Ada-LCD also existed as spherical particles, but their average diameter significantly enlarged to ca. 80 nm. These results demonstrated that the host-guest binding between the LCD and PEI-Ada greatly promoted the supramolecular crosslinking, which leads to the formation of large nanoparticles.

In addition, the enzyme-responsive assembly/disassembly behaviour of the supramolecular crosslinking system was also investigated. Therein, DL-dithiothreitol (DTT) was chosen as a model reductant to cleave the disulfide bond, and horseradish peroxidase (HRP), an extensively studied oxidation-catalyzing enzyme^{39,40}, was chosen as a model oxidant to recover the disulfide bond. With the addition of DTT, the originally spherical particles of PEI-Ada-LCD totally disassembled to amorphous structures. However, after treated by HRP, these amorphous structures could be recovered to spherical nanoparticles like the case of PEI-Ada-LCD (Supplementary Fig. S5). This result undoubtedly demonstrated the potential of PEI-Ada-LCD as an enzyme-responsive recycling carrier.

DNA condensation and release of PEI-Ada-LCD. After verifying the recycling property, it is also important to investigate the gene condensation ability of PEI-Ada-LCD supramolecular crosslinking system, because a very important characteristic feature of a gene carrier is its ability to facilitate the cellular uptake⁴¹. The condensation ability of PEI-Ada-LCD to pDNA was measured by analyzing the electrophoretic mobility at various N/P ratios on agarose gel. As shown in Fig. 2, both PEI-Ada and PEI-Ada-LCD could condense pDNA efficiently at low N/P ratios. Due to the relatively low DS value, PEI-Ada-8.2 showed the quite similar DNA condensation ability to its supramolecular crosslinking system (Fig. 2a, 2b).



Significantly, PEI-Ada-10-LCD, PEI-Ada-12.2-LCD and PEI-Ada-13.7-LCD could condense pDNA completely at N/P ratios of 4, 6, 6, respectively, which was lower than the corresponding values necessary for parent PEI-Ada-10 (N/P ratio = 5), PEI-Ada-12.2 (N/P ratio = 8) and PEI-Ada-13.7 condense pDNA (N/P ratio = 10). This means that the supramolecular crosslinking with LCD can improve the DNA condensation ability. Among the supramolecular crosslinking systems examined, PEI-Ada-13.7-LCD showed the highest enhancement of the DNA condensation ability as compared with its parent PEI-Ada. Therefore, PEI-Ada-13.7-LCD was mainly applied in the following experiments.

Other information about the DNA condensation by supramolecular crosslinking system comes from DLS, zeta potential, HR-TEM and atomic force microscopy (AFM) experiments. The DLS results (Supplementary Fig. S6a) showed that both PEI-Ada-13.7 and PEI-Ada-13.7-LCD were able to condense DNA to nanoparticles with the diameter varying from 350 to 70 nm. That is, at an N/P ratio of 10, PEI-Ada-13.7-LCD could condense pDNA to nanoparticles with an average diameter of 260 nm, which is more compact than that of PEI-Ada-13.7@pDNA particles (320 nm). This is consistent with the result of gel electrophoresis experiment that PEI-Ada-LCD supramolecular crosslinking system gave the stronger DNA condensation ability than its parent PEI-Ada. With further increasing the N/P ratio over 20, both PEI-Ada-13.7 and PEI-Ada-13.7-LCD could condense DNA to compact nanoparticles with a diameter around 80 nm, and this particle size is reported favourable for gene transfection⁴². Without DNA, PEI-Ada-13.7-LCD showed the similar positive zeta potential (40.65 mV), an important indicator of surface charges, to PEI-Ada-13.7 (39.39 mV), and these positive zeta potentials decreased after being mixed with DNA, because the surface of PEI-Ada-13.7 or PEI-Ada-13.7-LCD was covered by negatively charged DNA (Supplementary Fig. S7). It is noteworthy that the zeta potential of PEI-Ada-13.7-LCD@pDNA systems (17 to 35 mV) became

lower than that of PEI-Ada-13.7@pDNA systems (27 to 43 mV). This may mean that more negatively charged DNA was associated with PEI-Ada-13.7-LCD than with PEI-Ada-13.7 (Supplementary Fig. S6b). Moreover, PEI-Ada-13.7-LCD@pDNA system also remains a positive surface charge, which will favor its binding with the negatively charged cellular membrane via electrostatic interaction to facilitate the cellular uptake.

In addition, AFM images showed that the free DNA existed as loose strands with a height of 2.0 nm (a typical diameter of DNA). When PEI-Ada-LCD was added at an N/P ratio of 20, loose DNA strands could not be observed, and spherical particles were formed with a diameter of 60 nm (Fig. 3). Similar phenomenon also was observed by TEM (Supplementary Fig. S8). At a relatively low N/P ratio of 10, DNA was condensed to loose aggregates by PEI-Ada-13.7, accompanied by the existence of a little linear structures assigned to self-aggregated DNA. This indicates that there may be a competition in solution between the PEI-Ada/DNA aggregation and the self-aggregation of DNA. At a relatively low N/P ratio, PEI-Ada/DNA aggregation predominates. However, no similar self-aggregated DNA could be observed in the TEM image of PEI-Ada-LCD@pDNA system at the same N/P ratio, and all free DNA were condensed to large aggregates, verifying the stronger DNA condensation ability of PEI-Ada-LCD supramolecular crosslinking system. When the N/P ratio increased to 20, both PEI-Ada-13.7 and PEI-Ada-13.7-LCD could condense DNA to tight spherical particles with an average diameter of 50 nm, which is consistent with the results of DLS and AFM experiments.

After verifying the DNA condensation ability, we subsequently investigated the DNA release behavior of supramolecular crosslinking system using DTT as a reductant, because the LCD moiety of supramolecular crosslinking system possesses a disulfide bond that can be cleaved in a reducing environment. From the DLS data, we could see that the particle size of PEI-Ada-13.7-LCD@pDNA system enlarged, indicating the compacted PEI-Ada-13.7-LCD@pDNA aggregate became loose in the presence of DTT (Supplementary Fig. S6a). Moreover, the zeta potential of PEI-Ada-13.7-LCD@pDNA increased with the addition of DTT (Supplementary Fig. S6b), maybe indicating the dissociation of some negatively charged DNA from the PEI-Ada-13.7-LCD. In addition, gel electrophoresis experiments showed that the DNA mobility increased in the presence of DTT (Supplementary Fig. S9). On the other hand, DNA can be released in the presence of negatively charged substances, such as heparin, a sulfated sugar that is always used as a model polyanion molecular to induce the pDNA release from the system in extracellular environment⁴³. As shown in Supplementary Fig. S10, the addition of heparin ($\geq 400 \mu\text{g/mL}$) could trigger the DNA release from PEI-Ada-13.7-LCD@pDNA or PEI-Ada-13.7@pDNA system. However, this triggered concentration of heparin decreased to 60 $\mu\text{g/mL}$ in the case of PEI-Ada-13.7-LCD@pDNA/DTT system. These phenomena jointly demonstrated that a reductive environment, such as DTT or intracellular glutathione, is favourable to the DNA release, and the DNA release could be further promoted via the exchange with negatively charged macromolecules such as mRNA and sulfated sugars that widely existed in cells⁴⁴.

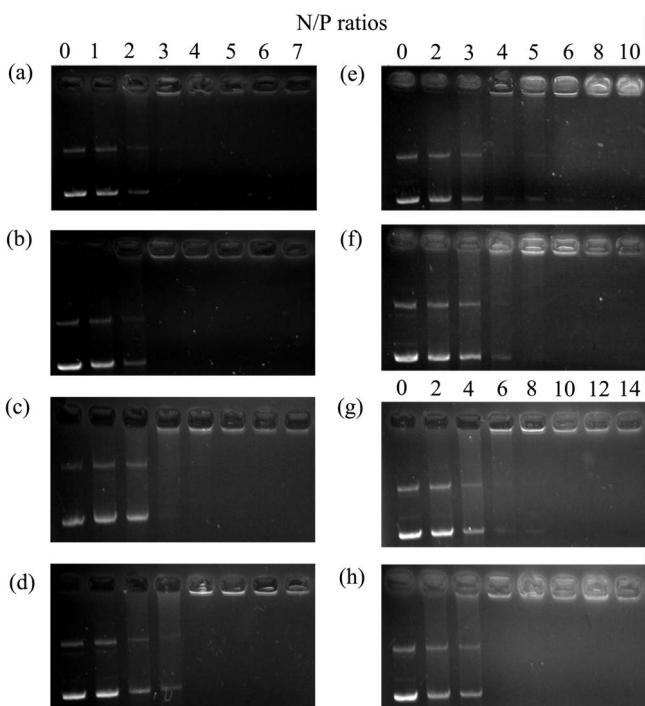


Figure 2 | Agarose gel electrophoresis of a series of polycations with pDNA. (a) PEI-Ada-8.2, (b) PEI-Ada-8.2-LCD, (c) PEI-Ada-10, (d) PEI-Ada-10-LCD at N/P ratios of 0, 1:1, 2:1, 3:1, 4:1, 5:1, 6:1, 7:1. (e) PEI-Ada-12.2, (f) PEI-Ada-12.2-LCD at N/P ratios of 0, 2:1, 3:1, 4:1, 5:1, 6:1, 8:1, 10:1. (g) PEI-Ada-13.7, (h) PEI-Ada-13.7-LCD at N/P ratios of 0, 2:1, 4:1, 6:1, 8:1, 10:1, 12:1, 14:1.

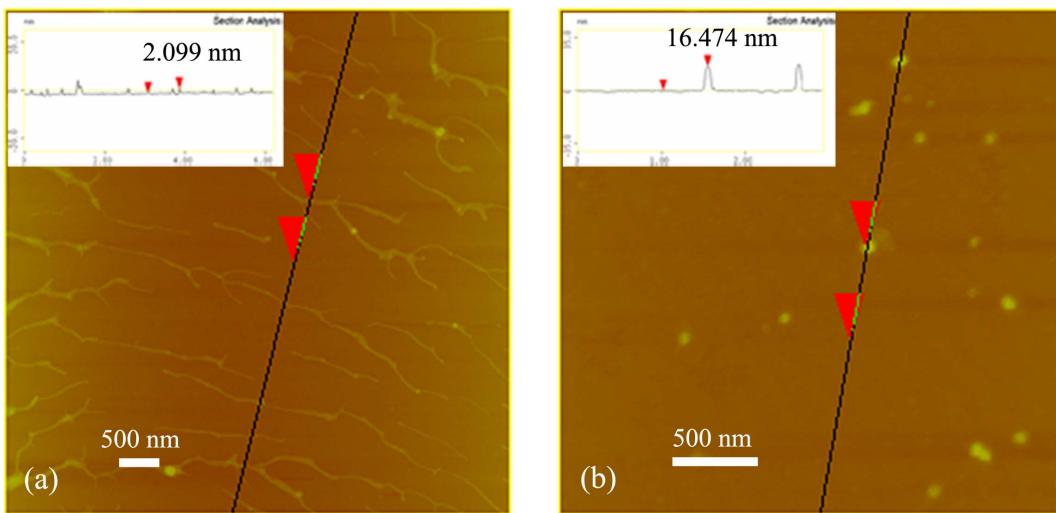


Figure 3 | AFM images of pDNA (2 ng/μL). (a) in the absence of and **(b)** in the presence of PEI-Ada-LCD at an N/P ratio of 20.

adamantane are biocompatible components, and the substitution of the amino groups can reduce the charge density to decrease cytotoxicity⁴⁵.

DNA transfection. The gene transfection efficiencies of supramolecular crosslinking system *in vitro* were evaluated with

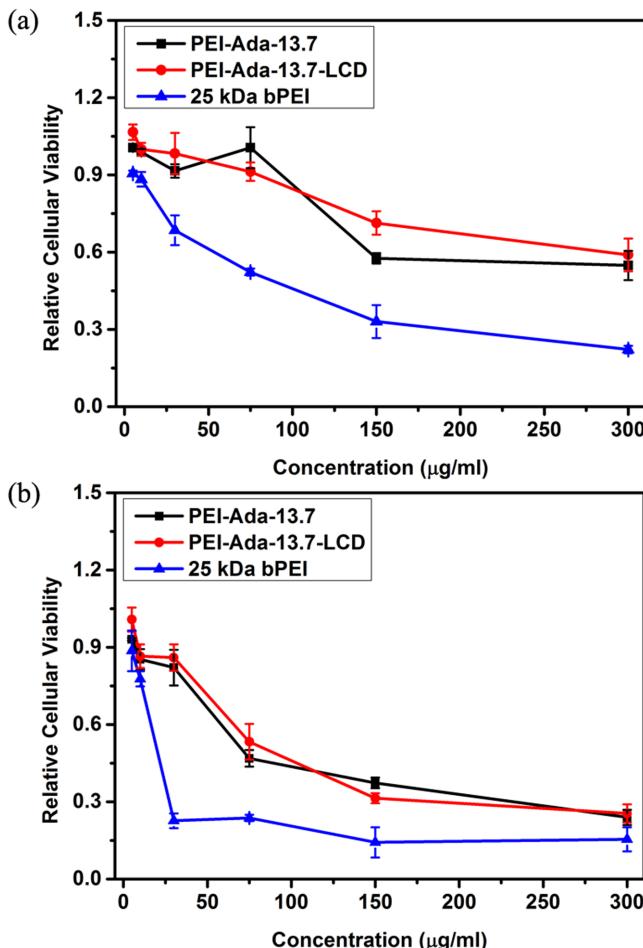


Figure 4 | Cytotoxicity experiments in vitro in 24 h. Relative cellular viability of (a) 293T and (b) HeLa cell lines after 24 h of treatment with different concentrations of PEI-Ada-13.7, PEI-Ada-13.7-LCD, 25 kDa bPEI.

EGFP gene as a reporter gene in 293T and HeLa cells. Fig. 5 showed the typical fluorescent pictures of visible GFP expression of PEI-Ada-13.7, PEI-Ada-13.7-LCD and 25 kDa bPEI at various N/P ratios. When PEI-Ada-13.7-LCD@pDNA aggregates at various N/P ratios were added to cells, significant gene expression was observed. These images suggested that the supramolecular crosslinking system was able to act as a gene carrier to transfer the GFP information into cells, and its transfection efficiency was dependent on the N/P ratio. At an N/P ratio of 20, PEI-Ada-13.7-LCD exhibited higher gene transfection efficiency than PEI-Ada-13.7 and even 25 kDa bPEI in serum.

The quantitative measurement of the EGFP gene transfection efficiencies of PEI-Ada-13.7, PEI-Ada-13.7-LCD and 25 kDa bPEI comes from the flow cytometry (Supplementary Fig. S11, S12). At various N/P ratios of 10, 20, 30, PEI-Ada-13.7-LCD showed higher gene transfection efficiencies for 293T cells than the corresponding value by PEI-Ada-13.7. Significantly, PEI-Ada-13.7-LCD presented a very high gene transfection efficiency up to 54%, which is nearly two times higher than that of 25 kDa bPEI. On the other hand, the gene transfection efficiency of the non-crosslinked system PEI-Ada was measured to be 44%, which was lower than that of the supramolecular crosslinking system PEI-Ada-13.7-LCD. For HeLa cells, it was also found that the supramolecular crosslinking system still maintained better transfection efficiencies than 25 kDa bPEI and PEI-Ada-13.7 (Supplementary Fig. S13, S14), but its gene transfection efficiencies towards HeLa cells were somewhat lower than towards 293T cells, which may be due to the different biological activities of cell lines⁴⁶. This phenomenon, along with the results of the gel electrophoresis, DLS and TEM experiments where the supramolecular crosslinking system PEI-Ada-13.7-LCD exhibited higher DNA condensation ability than the non-crosslinked system PEI-Ada, jointly demonstrated the advantage of the PEI-Ada-LCD crosslinking system. It is well known that serum albumin is a major component of the blood, which has been identified as a crucial factor that destabilizes the gene vector⁴⁷. Among the various reported non-viral gene delivery vectors, only few of them could transfect gene efficiently without the disturbance from serum^{48–51}. Thus we compared the gene transfection ability of the supramolecular crosslinking system in the absence and presence of serum. As shown in Supplementary Fig. S15–S18, the gene transfection efficiencies without serum was comparable to the ones with serum in the cases of both 293T and HeLa cells lines. This result indicated that the transfection efficiency of the supramolecular crosslinking system is not disturbed by serum.

To gain more insight into the efficient cellular uptake of the supramolecular crosslinking system PEI-Ada-13.7-LCD, the fluorescent

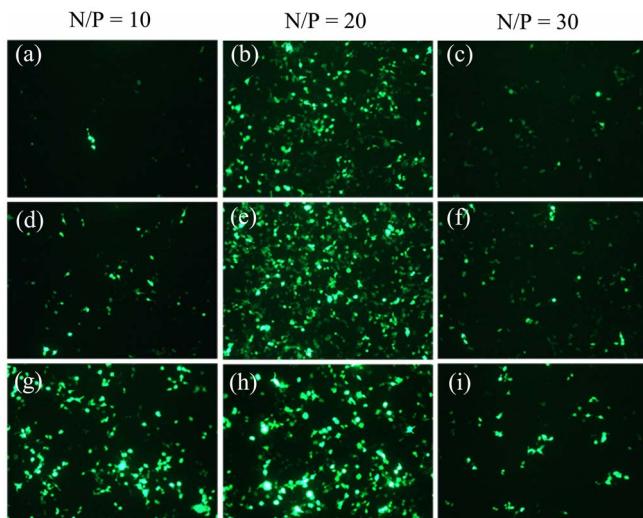


Figure 5 | Transfection efficiencies in 293T cells. Fluorescence microscopy images of 293T cells transfected by (a)–(c) PEI-Ada-13.7; (d)–(f) PEI-Ada-13.7-LCD; (g)–(i) 25 kDa bPEI after 48 h at the N/P ratios 10, 20, 30 in the presence of serum.

confocal images using rhodamine (RDM)-labeled pDNA (red) with HeLa cells as the model cell line was performed to study the intracellular localization, and DAPI (blue) was used to stain nucleus to observe the localization of carriers. As shown in Fig. 6, the cells treated by PEI-Ada-13.7-LCD exhibited stronger red fluorescence than those treated by PEI-Ada-13.7, indicating a higher DNA translocation efficiency of PEI-Ada-13.7-LCD. It should be noteworthy that the rhodamine-labeled pDNA mainly located around the nucleus, which would be favourable to the transport of DNA through the karyotheca.

Furthermore, the redox-responsive DNA release behavior of supramolecular crosslinking system was investigated in 293T cells, where the supramolecular crosslinking system PEI-Ada-13.7-LCD was incubated with 5 equiv. of DTT before complexation with EGFP gene. The fluorescence microscopy images showed that the transfection efficiency of PEI-Ada-13.7-LCD pre-treated by DTT obviously decreased (Figure S19 in the supporting information), indicating that the cleavage of disulfide bond triggered by the GSH in cytosol to release DNA is important to the enhanced gene transfection efficiency.

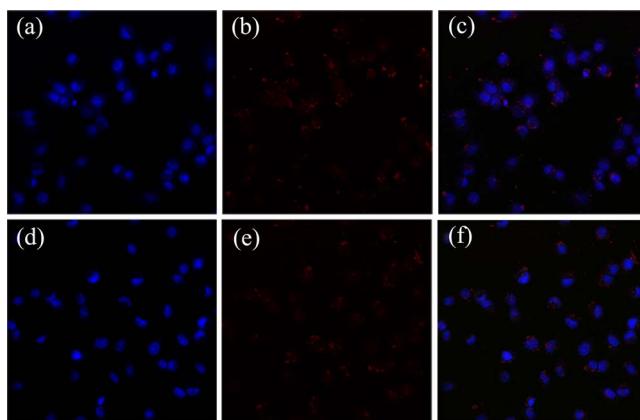


Figure 6 | Confocal fluorescence images of HeLa cells transfected with complexes for 24 h in the presence of serum. (a)–(c) cells were incubated with PEI-Ada-13.7@RDM-labeled DNA; (d)–(f) cells were incubated with PEI-Ada-13.7-LCD@RDM-labeled DNA. The plasmid DNA was labelled with rhodamine (RDM) and the nucleus were stained by DAPI (N/P = 20).

Conclusion

In this work, we successfully constructed a supramolecular cross-linking system, composed of adamantyl-modified polyethylenimine and L-cystine-bridged bis(β -CD)s, through the host-guest interaction as a bioavailable recycling DNA carrier, which showed better DNA condensation and transfection ability as well as lower cytotoxicity than the gold standard, i.e. 25 kDa bPEI. Significantly, the disulfide bond in this carrier can be cleaved by reductive enzyme to promote the DNA release but recovered by oxidative enzyme to make the carrier renewable. These advantages, along with the easy preparation, good biocompatibility, biodegradable and serum stability, jointly enable the great application potential of this supramolecular crosslinking system in gene therapy.

Methods

Reagents. All chemicals were reagent grade unless noted. β -CD was recrystallized twice from water and dried in vacuo at 90°C for 24 h before use. L-cystine, 1-adamantanecarboxylic acid, branched polyethylenimine ($M_w = 10000$), 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC), N-hydroxysuccinimide (NHS), DL-dithiothreitol (DTT), horseradish peroxidase (HRP), heparin sodium salt were purchased from commercial sources and used as received. Branched PEI with a molecular weight of 25 kDa (25 kDa bPEI) was purchased from Sigma-Aldrich. Mono-(6-O-p-tolylsulfonyl)- β -CD⁵², mono-(6-deoxyl-6-iodo)- β -CD⁵³, L-cystine-bridged bis(β -CD)s⁵⁴ were prepared according to the reported methods.

Preparation of PEI-Ada-LCD assembly. L-cystine-bridged bis(β -CD)s (0.15 mg, 0.06 μ mol) was added into a solution of PEI-Ada-13.7 (24.65 mg, 1.99 μ mol) in deionized water (5 mL), and then the mixture was ultrasonicated for 5 min. The resultant PEI-Ada-LCD solution was stored at 4°C and diluted to desired concentration with deionized water before use.

Redox sensitivity. DL-dithiothreitol (DTT) and horseradish peroxidase (HRP) were chosen as the model reductant and oxidant, respectively. First, the PEI-Ada-LCD solution was incubated with DTT at 37°C for 30 min, then incubated with HRP in phosphate buffer (pH = 8.5, 0.01 M) at 25°C for 4 h (the final concentration of DTT and HRP were 10 mM and 50 U mL⁻¹, separately). The obtained solution was examined by TEM.

pDNA condensation. In order to evaluate pDNA condensation ability of PEI-Ada and PEI-Ada-LCD, the agarose gel electrophoresis was used. PEI-Ada-LCD at different concentration in 5 μ L loading buffer was mixed with 120 ng of plasmid DNA in 5 μ L loading buffer, and the mixture with various N/P ratios were incubated at room temperature for 30 min. The mixture was diluted with 2 μ L of 5 \times loading buffer and run on 1% (w/v) agarose gel in 0.1 \times Tris-acetate-EDTA (TAE) buffer (pH = 8.0) at 60 V for 1 h. After put the agarose gel into TAE solution containing 0.5 μ g/mL ethidium bromide for 15 min, and the DNA bands could be seen at $\lambda = 302$ nm.

Stability. The heparin competition stability experiment was evaluated by agarose gel electrophoresis. Firstly, 2.5 μ L of PEI-Ada-13.7 or PEI-Ada-LCD was mixed with equal volume of pDNA solution (240 ng) with an N/P ratio of 20 for 30 min at room temperature. Then various amounts of heparin sodium solutions were added till the final concentrations of heparin sodium reached 0, 20, 60, 100, 400, 700, 1000, 1300 μ g/mL. The mixture was incubated at 37°C for 30 min. In addition, the polyanion competition stability experiment in the presence of 10 mM DTT was also conducted. PEI-Ada-LCD@pDNA complexes was incubated with 10 mM DTT at 37°C for 90 min, then incubated with heparin at 37°C for 30 min. After that the mixture were performed on agarose gel electrophoresis as described earlier.

Transfection in vitro. 293T cells and HeLa cells were seeded in 24-well plates (1 \times 10⁵ cells mL⁻¹, 1 mL per well) for 24 h at 37°C in 5% CO₂. The cells were incubated with PEI-Ada-13.7@pDNA, PEI-Ada-LCD@pDNA and 25 kDa bPEI@pDNA complexes at N/P ratios of 10, 20, 30 for 4 h (1.6 μ g DNA per well). Then the medium in each well was replaced with 1 mL of fresh DMEM medium with 10% FBS. The cells were further incubated for 44 h. The complexes were prepared by adding polycations into pDNA solutions, and the complexes solutions were incubated for 30 min at room temperature.

- Cavazzana-Calvo, M. et al. Gene therapy of human severe combined immunodeficiency (SCID)-X1 disease. *Science* **288**, 669–672 (2000).
- Kanasty, R., Dorkin, J. R., Vegas, A. & Anderson, D. Delivery materials for siRNA therapeutics. *Nat. Mater.* **12**, 967–977 (2013).
- Verma, I. M. & Somia, N. Gene therapy-promises, problems and prospects. *Nature* **389**, 239–242 (1997).
- Green, J. J., Langer, R. & Anderson, D. G. A combinatorial polymer library approach yields insight into nonviral gene delivery. *Acc. Chem. Res.* **41**, 749–759 (2008).



5. Merdan, T., Kopeček, J. & Kissel, T. Prospects for cationic polymers in gene and oligonucleotide therapy against cancer. *Adv. Drug. Deliver. Rev.* **54**, 715–758 (2002).
6. Richardson, S. C. W., Kolbe, H. V. J. & Duncan, R. Potential of low molecular mass chitosan as a DNA delivery system: biocompatibility, body distribution and ability to complex and protect DNA. *Int. J. Pharm.* **178**, 231–243 (1999).
7. Pack, D. W., Hoffman, A. S., Pun, S. & Stayton, P. S. Design and development of polymers for gene delivery. *Nat. Rev. Drug. Discov.* **4**, 581–593 (2005).
8. Liu, Y. *et al.* Factors influencing the efficiency of cationic liposome-mediated intravenous gene delivery. *Nat. Biotechnol.* **15**, 167–173 (1997).
9. Ooya, T. *et al.* Biodegradable polyrotaxane-plasmid DNA polyplex for Enhanced gene delivery. *J. Am. Chem. Soc.* **128**, 3852–3853 (2006).
10. Wang, H. *et al.* A rapid pathway toward a superb gene delivery system: programming structural and functional diversity into a supramolecular nanoparticle library. *ACS Nano* **4**, 6235–6243 (2010).
11. Mintzer, M. A. & Simanek, E. E. Nonviral vectors for gene delivery. *Chem. Rev.* **109**, 259–302 (2009).
12. Dandekar, P. *et al.* Cellular delivery of polynucleotides by cationic cyclodextrin polyrotaxanes. *J. Controlled Release* **164**, 387–393 (2012).
13. Li, L.-L. *et al.* Core-shell supramolecular gelatin nanoparticles for adaptive and “on-demand” antibiotic delivery. *ACS Nano* **8**, 4975–4983 (2014).
14. Yi, W.-J. *et al.* TACN-based oligomers with aromatic backbones for efficient nucleic acid delivery. *Chem. Commun.* **50**, 6454–6457 (2014).
15. Wang, M., Liu, H., Li, L. & Cheng, Y. A fluorinated dendrimer achieves excellent gene transfection efficacy at extremely low nitrogen to phosphorous ratios. *Nat. Commun.* **5**, 3053 (2014).
16. Read, M. L. *et al.* A versatile reducible polycation-based system for efficient delivery of a broad range of nucleic acids. *Nucleic Acids Res.* **33**, e86 (2005).
17. Mao, H.-Q. *et al.* Chitosan-DNA nanoparticles as gene carriers: synthesis, characterization and transfection efficiency. *J. Controlled Release* **70**, 399–421 (2001).
18. Koo, H. *et al.* Biodegradable branched poly(ethylenimine sulfide) for gene delivery. *Biomaterials* **31**, 988–997 (2010).
19. Yamashita, A. *et al.* Synthesis of a biodegradable polyrotaxane-plasmid DNA (pDNA) polyplex and its use for the rapid nonviral delivery of pDNA to cell nuclei. *Nat. Protoc.* **1**, 2861–2869 (2006).
20. Breunig, M., Lungwitz, U., Liebl, R. & Goepferich, A. Breaking up the correlation between efficacy and toxicity for nonviral gene delivery. *Proc. Natl. Acad. Sci. U. S. A.* **104**, 14454–14459 (2007).
21. Liu, H., Wang, H., Yang, W. & Cheng, Y. Disulfide cross-linked low generation dendrimers with high gene transfection efficacy, low cytotoxicity, and low cost. *J. Am. Chem. Soc.* **134**, 17680–17687 (2012).
22. Davis, M. E. & Brewster, M. E. Cyclodextrin-based pharmaceuticals: past, present and future. *Nat. Rev. Drug. Discov.* **3**, 1023–1035 (2004).
23. Liu, Y. *et al.* Inclusion complexation and solubilization of paclitaxel by bridged bis(β-cyclodextrin)s containing a tetraethylenepentaamino spacer. *J. Med. Chem.* **46**, 4634–4637 (2003).
24. Wang, H., Chen, Y., Li, X.-Y. & Liu, Y. Synthesis of oligo (ethylenediamino)-β-cyclodextrin modified gold nanoparticle as a DNA concentrator. *Mol. Pharm.* **4**, 189–198 (2007).
25. Mellet, C. O., Fernández, J. M. G. & Benito, J. M. Cyclodextrin-based gene delivery systems. *Chem. Soc. Rev.* **40**, 1586–1608 (2011).
26. Yang, Y., Zhang, Y.-M., Chen, Y., Chen, J.-T. & Liu, Y. Targeted polysaccharide nanoparticle for adamplatin prodrug delivery. *J. Med. Chem.* **56**, 9725–9736 (2013).
27. Kulkarni, A. *et al.* Cationic α-cyclodextrin: poly (ethylene glycol) polyrotaxanes for siRNA delivery. *Mol. Pharm.* **10**, 1299–135 (2013).
28. Uekama, K., Hirayama, F. & Irie, T. Cyclodextrin drug carrier systems. *Chem. Rev.* **98**, 2045–2076 (1998).
29. Nalluri, S. K. M., Voskuhl, J., Bultema, J. B., Boekema, E. J. & Ravoo, B. J. Light-responsive capture and release of DNA in a ternary supramolecular complex. *Angew. Chem. Int. Ed.* **50**, 9747–9751 (2011).
30. Hu, Y. *et al.* Supramolecular pseudo-block gene carriers based on bioreducible star polycations. *Biomaterials* **34**, 5411–5422 (2013).
31. Li, W. *et al.* Multifunctional nanoparticles via host-guest interactions: a universal platform for targeted imaging and light-regulated gene delivery. *Chem. Commun.* **50**, 1579–1581 (2014).
32. Bartlett, D. W. *et al.* Impact of tumor-specific targeting on the biodistribution and efficacy of siRNA nanoparticles measured by multimodality *in vivo* imaging. *Proc. Natl. Acad. Sci. U. S. A.* **104**, 15549–15554 (2007).
33. Davis, M. E. The first targeted delivery of siRNA in humans via a self-assembling, cyclodextrin polymer-based nanoparticle: from concept to clinic. *Mol. Pharm.* **6**, 659–668 (2009).
34. Davis, M. E. *et al.* Evidence of RNAi in humans from systemically administered siRNA via targeted nanoparticles. *Nature* **464**, 1067–1070 (2010).
35. Son, S., Namgung, R., Kim, J., Singha, K. & Kim, W. Bioreducible polymers for gene silencing and delivery. *Acc. Chem. Res.* **45**, 1100–1112 (2012).
36. Saito, G., Swanson, J. A. & Lee, K.-D. Drug delivery strategy utilizing conjugation via reversible disulfide linkages: role and site of cellular reducing activities. *Adv. Drug Delivery Rev.* **55**, 199–215 (2003).
37. Zhang, B. & Breslow, R. Enthalpic domination of the chelate effect in cyclodextrin dimers. *J. Am. Chem. Soc.* **115**, 9353–9354 (1993).
38. Zhang, Y.-H., Zhang, Y.-M., Chen, Y., Yang, Y. & Liu, Y. Phenanthroline bridged bis(β-cyclodextrin)s/adamantane-carboxylic acid supramolecular complex as an efficient fluorescence sensor to Zn²⁺. *Org. Chem. Front.* **1**, 355–360 (2014).
39. Singh, S., Topuz, F., Hahn, K., Albrecht, K. & Groll, J. Embedding of active proteins and living cells in redox-sensitive hydrogels and nanogels through enzymatic cross-linking. *Angew. Chem. Int. Ed.* **52**, 3000–3003 (2013).
40. Li, Y. *et al.* Surface coating-dependent cytotoxicity and degradation of graphene derivatives: towards the design of non-toxic, degradable nano-graphene. *Small.* **10**, 1544–1554 (2014).
41. Ping, Y., Hu, Q., Tang, G. & Li, J. FGFR-targeted gene delivery mediated by supramolecular assembly between β-cyclodextrin-crosslinked PEI and redox-sensitive PEG. *Biomaterials* **34**, 6482–6494 (2013).
42. Prabha, S., Zhou, W.-Z., Panyam, J. & Labhasetwar, V. Size-dependency of nanoparticle-mediated gene transfection: studies with fractionated nanoparticles. *Int. J. Pharm.* **244**, 105–115 (2002).
43. Moret, I. *et al.* Stability of PEI-DNA and DOTAP-DNA complexes: effect of alkaline pH, heparin and serum. *J. Controlled Release* **76**, 169–181 (2001).
44. Kang, H. C., Kang, H.-J. & Bae, Y. H. A reducible polycationic gene vector derived from thiolated low molecular weight branched polyethylenimine linked by 2-iminothiolane. *Biomaterials* **32**, 1193–1203 (2011).
45. Ping, Y. *et al.* Chitosan-graft-(PEI-β-cyclodextrin) copolymers and their supramolecular PEGylation for DNA and siRNA delivery. *Biomaterials* **32**, 8328–8341 (2011).
46. Hsu, C. Y. M. & Uludag, H. Cellular uptake pathways of lipid-modified cationic polymers in gene delivery to primary cells. *Biomaterials* **33**, 7834–7848 (2012).
47. Tseng, W.-C. & Jong, C.-M. Improved stability of polycationic vector by dextran-grafted branched polyethylenimine. *Biomacromolecules* **4**, 1277–1284 (2003).
48. Peng, Q., Zhong, Z. & Zhuo, R. Disulfide cross-linked polyethylenimines (PEI) prepared via thiolation of low molecular weight PEI as highly efficient gene vectors. *Bioconjugate Chem.* **19**, 499–506 (2008).
49. Srinivasachari, S., Fichter, K. M. & Reineke, T. M. Polycationic β-cyclodextrin “click clusters”: Monodisperse and versatile scaffolds for nucleic acid delivery. *J. Am. Chem. Soc.* **130**, 4618–4627 (2008).
50. Xu, P., Quick, G. K. & Yeo, Y. Gene delivery through the use of a hyaluronate-associated intracellularly degradable crosslinked polyethylenimine. *Biomaterials* **30**, 5834–5843 (2009).
51. Sawant, R. R. *et al.* Polyethylenimine-lipid conjugate-based pH-sensitive micellar carrier for gene delivery. *Biomaterials* **33**, 3942–3951 (2012).
52. Petter, R. C., Salek, J. S., Sikorski, C. T., Kumaravel, G. & Lin, F.-T. Cooperative binding by aggregated mono-6-(alkylamino)-β-cyclodextrins. *J. Am. Chem. Soc.* **112**, 3860–3868 (1990).
53. Tabushi, I., Kuroda, Y. & Mochizuki, A. The first successful carbonic anhydrase model prepared through a new route to regiospecifically biofunctionalized cyclodextrin. *J. Am. Chem. Soc.* **102**, 1152–1153 (1980).
54. Li, L., Li, X.-Y. & Liu, Y. Synthesis of L-cysteine bridged bis(β-cyclodextrin) and its cooperative binding toward guest molecules. *Chin. Sci. Bull.* **49**, 115–118 (2004).

Acknowledgments

We thank 973 Program (2011CB932502) and NNSFC (91227107, 21432004, 21272125 and 21102075) for financial support.

Author contributions

Z.Y.H. performed the synthesis of PEI-Ada and the preparation of the supramolecular crosslinking system. C.Y. and Z.Y.M. performed the functional analyses of the crosslinking system. Y.Y. and C.J.T. performed the cytotoxicity and gene transfection experiments. Z.Y.H. and C.Y. wrote the main manuscript. All authors discussed the results and commented on the manuscript. L.Y. supervised the work and edited the manuscript.

Additional information

Supplementary information accompanies this paper at <http://www.nature.com/scientificreports/>

Competing financial interests: The authors declare no competing financial interests.

How to cite this article: Zhang, Y.-H. *et al.* Recycling Gene Carrier with High Efficiency and Low Toxicity Mediated by L-Cysteine-Bridged Bis(β-cyclodextrin)s. *Sci. Rep.* **4**, 7471; DOI:10.1038/srep07471 (2014).

This work is licensed under a Creative Commons Attribution-NonCommercial-ShareAlike 4.0 International License. The images or other third party material in this article are included in the article's Creative Commons license, unless indicated otherwise in the credit line; if the material is not included under the Creative Commons license, users will need to obtain permission from the license holder in order to reproduce the material. To view a copy of this license, visit <http://creativecommons.org/licenses/by-nc-sa/4.0/>