Electronic Supplementary Information

Light-Regulated Host-Guest Interaction as a New Strategy for Intracellular PEG-Detachable Polyplexes to Facilitate Nuclear Entry

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Materials

Branched polyethylenimine (PEI, 25kDa) was obtained from Sigma-Aldrich. 4-Phenylazbenzoyl chloride (AzCOCl) was purchased from Tokyo Chemical Industry (Shanghai, China) Development Co., Ltd. Methoxypolyoxyethylene amine (mPEG-NH_2, 5kDa) was obtained from Aladdin (Shanghai, China). 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. Plasmid pEGFP (4733bp) as transfection reagent was purchased from Clonetech. 3-(4, 5-dimethylthiAzl-2-yl)-2, 5-diphenyltetrAzlium bromide (MTT) was obtained from Bio Basic Inc. Loading buffer was purchased from TakaRa Biotechnology (Dalian, China) Co.Ltd. 0.5×TBE buffer was diluted from 4× TBE buffer (0.36M tris-boric acid, 8mM EDTA).

Synthesis of PEI-CD and Az-PEG

PEI-CD was prepared according to the previous study.[1, 2] Briefly, 6-deoxy-(p-toluenesulfonyl) -β-CD (6-OTs-β-CD) was firstly synthesized by reaction of 4-toluene sulfonyl chloride with β-CD. Then PEI-CD was prepared by reaction of 6-OTs-β-CD with the amines of PEI. Az-PEG was synthesized according to the method reported by Yui.[3] Briefly, 0.40 g of mPEG-NH_2 (0.08 mmol) was dissolved in 20 ml of anhydrous N, N-dimethylformamide (DMF). 0.20 g of AzCOCl (0.8 mmol) and excess amount of MgO were added into the above solution and stirred for 24 h under nitrogen environment at room temperature. Then the product was filtrated, precipitate from ether, washed repeatedly with ether and dialyzed for 4 days. The product was analyzed by ^{1}H NMR (300MHz, Varian Spectrometer, USA) and Ultraviolet-visible spectrophotometer (UV-2550, Shimadzu, Japan). The spectra of ^{1}H NMR of PEI-CD and Az-PEG were shown in Fig. S1. The results indicated that the CD-grafting level was 2.5%, which mean that every PEI chain had about 15 CD molecules. For Az-PEG, the proton peaks of benzene appeared at 7.0-8.0 ppm and methylene at 3.2-3.5 ppm. UV spectrum (dissolved in H_2O) of the product showed that the absorption peak were at 325 nm, which was consistent with the report in the literature.[3] These results indicated that Az was actually introduced at the terminal of PEG. The functionality of Az groups in PEG was found 0.9 by integration according to ^{1}H NMR spectrum.

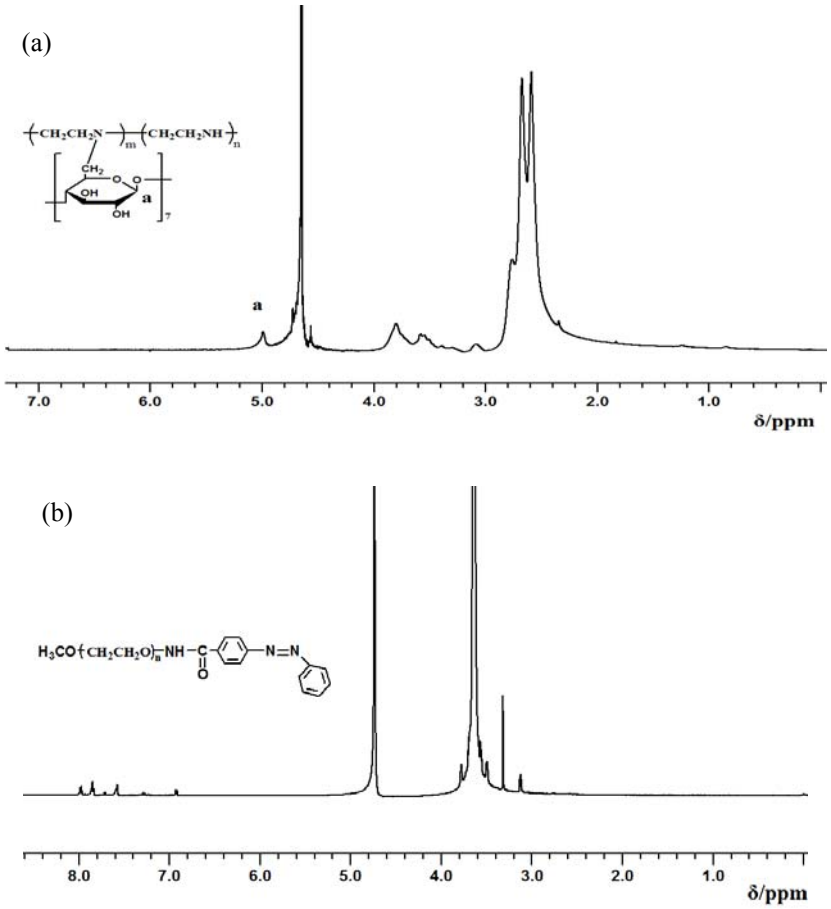


Fig. S1 ^{1}H NMR spectra of PEI-CD (a) and Az-PEG (b).

Inclusion complexation of Az-PEG and PEI-CD

Inclusion complexation process of Az-PEG and PEI-CD: The inclusion complexation process of Az-PEG and PEI-CD was monitored the UV absorption of Az-PEG in the presence of PEI-CD with ultrasonic time increasing. The absorbance difference increased over time. Then $(A_0-A_t)/(A_0-A_{eq})$ was plotted against ultrasonic time. Here, A_0 represents the absorbance of Az-PEG before addition of PEI-CD. A_t and A_{eq} denotes the absorbance at ultrasonic time of t and the equilibrium of inclusion complexation. As shown in Fig. S2 (see the Electronic Supplementary Information), it was found that Az-PEG/PEI-CD inclusion could be obtained within ultrasonic time of 25 min.

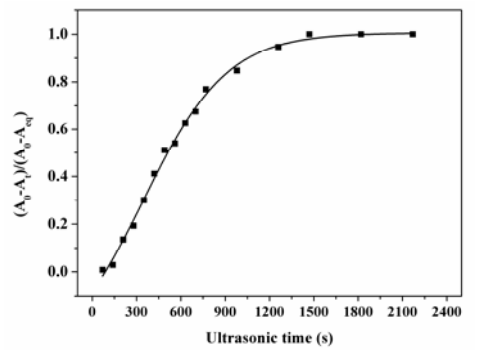


Fig. S2 UV absorption changes of Az-PEG/PEI-CD inclusion with ultrasonic time increasing. The concentration of Az-PEG was 1.2×10^{-4} M.

Proof of Az on Az-PEG chains within CD on PEI-CD chains: UV spectra were investigated to confirm the inclusion formation. As shown in Fig. S3, it was found that the absorption of trans-Az-PEG at 325 nm is enhanced after association with β-CD in PEI chains, as shown in the above figure. According to modified Benesi-Hildebrand equation, it can be calculated that the molar extinction coefficients of trans-Az-PEG in the absence and presence of PEI-CD were 8.65×10^{3} and 2.76×10^{4} $M^{-1}cm^{-1}$, respectively. Therefore, the enhanced absorption should be caused by the enhanced molar extinction coefficient after association. Moreover, the absorption peak of Az-PEG (325 nm) was shifted to longer wavelength (about 330 nm) after the addition of PEI-CD. These results indicated that Az on Az-PEG chains formed an inclusion complex with β-CD on PEI-CD chains.[3, 4]

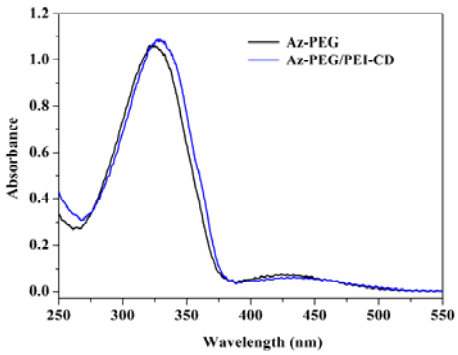


Fig. S3 UV spectra of Az-PEG and Az-PEG/PEI-CD at the same concentration of Az-PEG

Association constant between Az-PEG and PEI-CD: The association constant between Az-PEG and PEI-CD in tri-distilled water was determined by monitoring the UV absorption changes at 325 nm. The concentration of Az-PEG was fixed at 2×10^{-5} M. As shown in Fig.S4, the absorption of Az-PEG increased significantly with addition of PEI-CD. With an assumption of a 1:1 stoichiometry, the inclusion complexation of CD (G) on PEI-CD chains with trans-Az (H) on Az-PEG chains is expressed by the following equation:

$$H + G \overset{K_a}{\longleftrightarrow} H\bullet G$$

We employed the usual double reciprocal plot according to the modified Hidebrand-Benesi equation:

$$\frac{1}{\Delta A\varepsilon} = \frac{1}{K_a\Delta\varepsilon[H][G]} + \frac{1}{\Delta\varepsilon[H]}$$

Where Ka, [H], [G] represents association constant, the concentration of CD (host) on PEI-CD chains and Az (gest) on Az-PEG chains, respectively. ΔA represents the absorbance difference before and after inclusion forming. Δε denotes the change of the molar extinction coefficient before and after inclusion complexation at the same wavelength. The association constant K_a was calculated by the equation:

$$K_a = \frac{1}{k\Delta\varepsilon[H]} = 1.43\times10^3\ M^{-1}$$

Here k is the slope value of line plot in Fig. S4. At the same time, we investigated the association constant in the presence of sodium chloride (NaCl, 150 mM). It was found that addition of NaCl did not affect the association constant between Az-PEG and PEI-CD. Therefore, we concluded that NaCl could not make PEG detach form the inclusion of Az-PEG into PEI-CD.

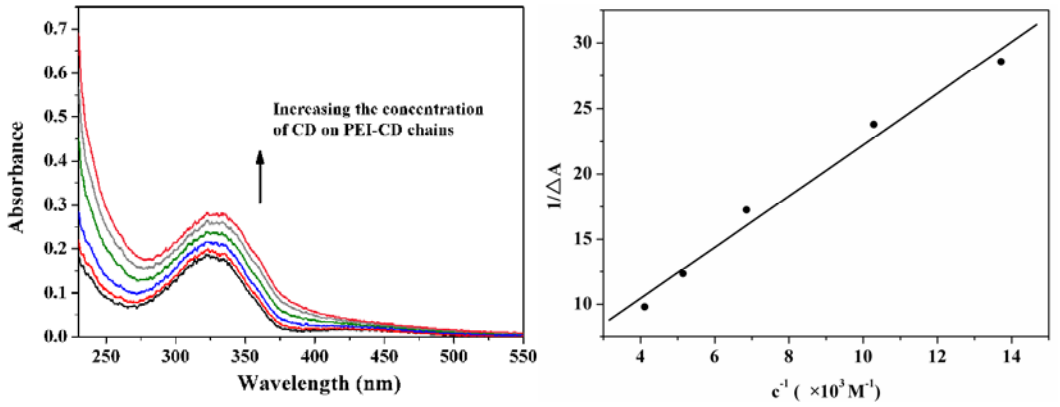


Fig. S4 The UV absorption of Az-PEG upon addition of PEI-CD. The concentration of Az-PEG keeps at 2×10^{-5} M.

Formulation and characterization of polyplexes

Formulation of PEI-CD/DNA and Az-PEG/PEI-CD/DNA polyplexes: PEI-CD/DNA polyplexes were prepared by vortexing equal volume of PEI-CD solution with DNA solution (100 μg/ml dissolve in 20 mM HEPEs buffer solution) at the appropriate N/P ratio. Az-PEG/PEI-CD/DNA polyplexes were prepared as follows: Az-PEG solution and PEI-CD solution were ultrasonically agitated for 30 min and incubated for another 1 h. The molar ratio of Az to CD was controlled at 4:1. 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.

Particle size and zeta (ζ-) potential measurements: Particle size of PEI-CD/DNA and Az-PEG/PEI-CD/DNA polyplexes was determined by Dynamic Light Scattering (DLS). Measurement was performed on 90plus/BI-MAS particle size analyzer (Brookhaven, Holtsville, NY, USA). Samples were carried out in 4 serial measurements at 90° angle and determined at 25℃. The ζ-potential of PEI-CD/DNA and Az-PEG/PEI-CD/DNA polyplexes was examined by Zetasizer 3000HS (Malvern, UK) at room temperature. All experiments were performed in triplicate.

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) , 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. Then the gel was immersed in ethidium bromide solution (0.5 μg/ml) for 30 min, observed by UV illuminator (Gel Doc, Bio-Rad, USA). As shown in Fig. S5, when pDNA was mixed with supramolecular polymer, the free DNA band disappeared above the N/P ratio of 2, indicating complete polyplex formation between pDNA and the supramolecular polymer. By contrast, the same phenomenon was observed when PEI-CD was used to condense DNA.

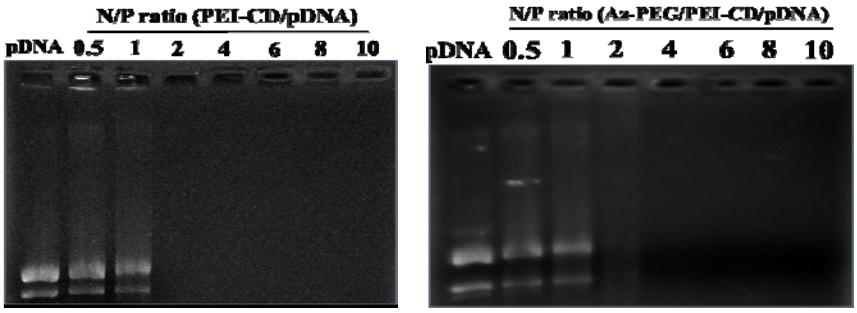
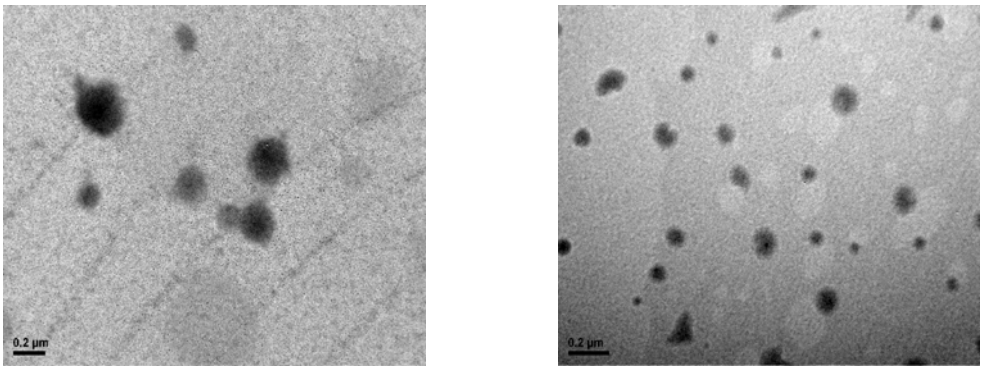


Fig.S5 Agarose gel electrophoresis of PEI-CD/DNA and Az-PEG/PEI-CD/DNA polyplexes at various N/P ratios.

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 PEI-CD/DNA and Az-PEG/PEI-CD/DNA polyplexes prepared as described above was deposited onto 200-mesh carbon-coated copper grid. The excess aqueous solution was blotted with filter paper. In order to obtain enough particles on the grid, the above processes were repeated three times. Here, PEI-CD/DNA and Az-PEG/PEI-CD/DNA polyplexes at N/P ratio of 10 were applied. As shown in Fig.S6, it was easy to find the particles with compacted-spherical structure.



(a) (b)

Fig.S6 TEM images of PEI-CD/DNA-10 (a) and Az-PEG/PEI-CD/DNA-10 (b) polyplexes.

The stability of Az-PEG/PEI-CD/DNA polyplexes

Aggregation stability: The polyplexes at appropriate N/P ratio were prepared as mentioned above. Then the salt concentration was adjusted to 150 mM. The polyplexes were incubated for different time. The stability profile was detected by DLS and shown in Fig.S7. The results indicated that the stability of polyplexes showed some reliance on N/P ratio. At N/P ratios of 4 and 6, the particle size of Az-PEG/PEI-CD/DNA polyplexes was still increased and aggregated gradually. By contrast, at N/P ratio of 8 and 10, the polyplexes were stable enough in physiological salt condition.

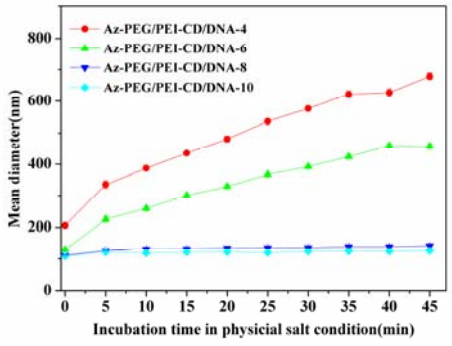


Fig.S7 The aggregation stability of different polyplexes in physiological salt condition detected by DLS (0 min means at prepared condition). Error bars represent means±SD for n=3.

Competition stability assay： Complexation stability was detected by gel retardation assay. The polyplexes containing 300 ng pDNA were prepared as described above at N/P ratio of 10, then mixed with heparin at final concentration of 10, 20, 30, 40, 80 and 100 μg/mL, respectively. The mixture was incubated for 20 min and then gel electrophoresis gel was performed as the above method**.**

Light-regulated PEG-detachment study

UV source: Here, a low-intensity 8 W long wave UV light lamp (365 nm, Spectroline, Model NO. EN-180L/FE, USA) offers a relatively low UV-A dose of 1600 μW/cm^2 at the peak intensity of 365 nm and used according to the manufacture's protocol. The extent of UV damage is determined by the wavelength of illumination, the light intensity and illumination time. It is well known that typical UV sterilization uses high-intensity (>10 000 μW/cm^2) UV-C light at wavelength of 264 nm. However, 365 nm UV-A light was widely used in the biological study. The lamp used in Lee's work offered a relatively low UV-A dose of 1100 μW/cm^2 at the peak intensity of 365 nm in cell culture experiment.[5] Here, the lamp used in this work offers a relatively low UV-A intensity of 1600 μW/cm^2 at the peak intensity of 365 nm, when kept distance between lamp and sample of 15 cm as reported by the manufacturer. In this study, the distance between the lamp and sample was kept at approximately about 20 cm.

The influence of light irradiation on the ζ-potential of PEGylated polyplexes: Az-PEG/PEI-CD/DNA polyplexes were prepared as described above. Then the influence of irradiation time on the PEG detachment was investigated by ζ-potential measurement. At every time interval of irradiation, the ζ-potential of Az-PEG/PEI-CD/DNA polyplexes was determined by Zetasizer 3000HS.

Photoisomerization of azobenzene on Az-PEG/PEI-CD/DNA polyplexes: In order to check the photoisomerization of Az, polyplexes were exposed to 365 nm light for 15 min and then examined by UV spectrophotometer. The results were shown in Fig.S8. The UV spectrum of PEI-CD/DNA polyplexes did not change before and after

irradiation. However, in terms of Az-PEG/PEI-CD/DNA polyplexes, the absorbance at 325 nm significantly declined and the absorbance at about 428 nm increased slightly. The above photoisomerization provided the possibility of PEG detachment.

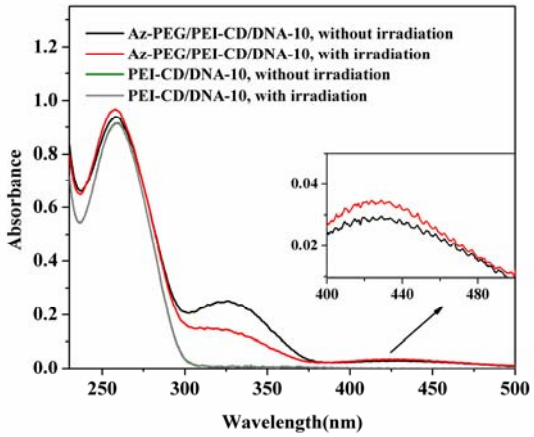


Fig.S8 The UV spectra of PEI-CD/DNA and Az-PEG/PEI-CD/DNA polyplexes with or without 365 nm light irradiation.

The influence of light irradiation on competition stability of PEGylated polyplexes: The competition stability of Az-PEG/PEI-CD/DNA polyplexes after 15 min light irradiation was investigated. After mixing PEGylated polyplexes at N/P ratio of 10 and heparin, the mixture was exposed with 365 nm UV light for 15 min. The results were shown in Fig.S9. Ethidium bromide (EtBr) is weakly fluorescent but exhibits strong fluorescence upon intercalation into the DNA duplex. So DNA bands would be revealed under UV illuminator when polyplexes become loose or dissociate to release free DNA. The result in Fig.S9 showed that after 15 min light irradiation, fluorescent intensity of DNA bands was significantly improved in the agarose gel chamber. It indicated that the tight complexes changed to loose structure after light irradiation. Combining the result of ζ-potential measurement, it should be contributed to the partial PEG detachment.

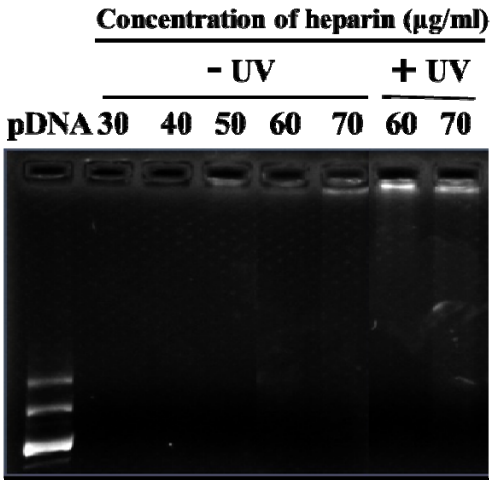


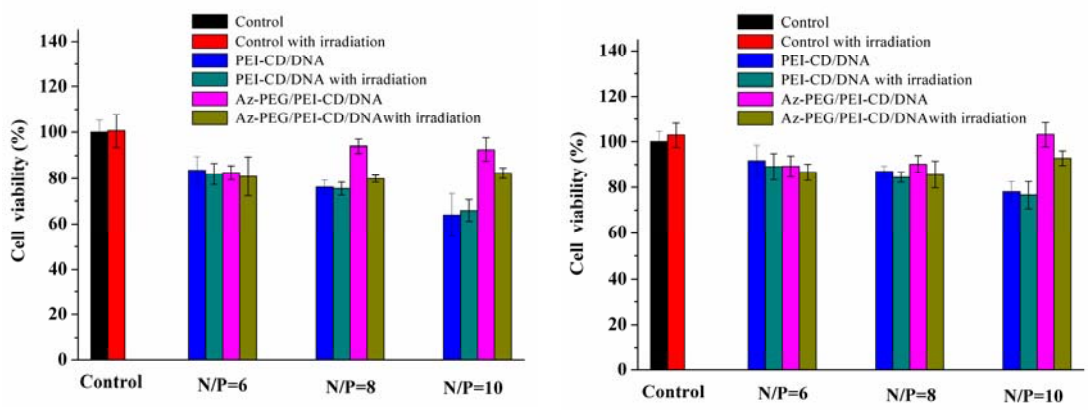
Fig. S9 Agarose gel electrophoresis of Az-PEG/PEI-CD/pDNA in the presence of heparin with and without 365 nm UV light irradiation.

Cell culture experiment

HEK293T cells (Human embryonic kidney cell line) and HepG2 cells (Human Hepatoblastoma cell line) were culture in DMEM with 10% FBS and 1% penicillin-streptomycin. The cells were maintained under humidified air containing 5% CO_2 at 37 °C.

Cell cytotoxicity assay: HEK293T and HepG2 cells were seeded into 96-well plates at a density of 1×10^4 cells/well and 6×10^3 cells/well, respectively. The cells were cultured for 24 h. The medium was replaced with 100 μl of fresh medium. PEI-CD/DNA and Az-PEG/PEI-CD/DNA polyplexes containing 1 μg DNA were added into the cells. After incubation for 4.5 h, cells were exposed to 365 nm light for 15 min. Followed by incubation for another 31.5 h, 20 μl of MTT (5 mg/ml, dissolved in PBS) and 100 μl of fresh DMEM were added. The cells were incubated for 4 h at 37 °C. Then the medium was removed, 200 μl of DMSO was added and incubated for additional 15 min at 37 °C. The absorbance of 100 μl of the above mixture at 570 nm was measured by microplate reader (550, Bio-Rad, USA). All experiments were performed in quintuplicate. As shown in Fig.S10, the cytotoxicity assay illustrated that Az-PEG/PEI-CD/DNA polyplexes had lower cytotoxicity than PEI-CD/DNA polyplexes especially at N/P ratio of 10. It was probably due to the charge-shielding

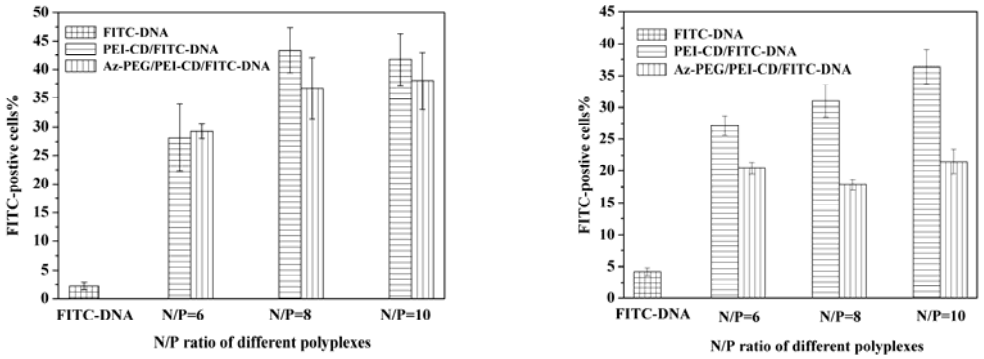
effect of PEG shell reduced the disturbance of the cell membrane. Moreover, light irradiation did not affect the cell toxicity of blank cells and cells treated with PEI-CD/DNA polyplexes. However, the cell viability of cells exposed to Az-PEG/PEI-CD/DNA polyplexes decreased slightly when cells with light irradiation. It was contributed to the PEG detachment caused by light irradiation.



(a) (b)

Fig.S10 Cell cytotoxicity of PEI-CD/DNA and Az-PEG/PEI-CD/DNA polyplexes exposed to HEK293T cells (a) and HepG 2 cells (b) for 36 h.

The cellular uptake efficiency: The cellular uptake efficiency of polyplexes was performed in HEK293T and HepG2 cells using FITC-DNA which was prepared according to the previous study.[6] 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 polyplexes addition, the medium was replaced with 0.5 ml of fresh media. PEI-CD/FITC-DNA and Az-PEG/PEI-CD/FITC-DNA polyplexes containing 2 μg FITC-DNA were prepared as described above. The polyplexes were added and incubated for 0.5 h. Then the cells were washed three times with PBS in order to detach surface-associated polyplexes, trypsinized and analyzed by flow cytometry. All experiments were performed in triplicate. The results shown in Fig.S11 clearly demonstrated that PEGylated polyplexes via host-guest interaction had lower cellular uptake efficiency by both cell lines, suggesting that PEGylation impede the uptake of unPEGylated polyplexes.



(a) (b)

Fig.S11 Cellular uptake efficiency of PEI-CD/DNA and Az-PEG/PEI-CD/DNA polyplexes exposed to HEK293T cells (a) and HepG 2 cells (b).

In vitro transfection efficiency: HEK293T and HepG2 cells were seeded in 24-well plates at a density of 1×10^5 cells/well and 6×10^4 cells/well respectively and cultured for 24 h in 0.5 ml of medium. PEI-CD/pEGFP and Az-PEG/PEI-CD/pEGFP polyplexes containing 2 μg pEGFP were added and incubated for 4.5 h. Then the cells treated with Az-PEG/PEI-CD/ pEGFP polyplexes were exposed to 365 nm light, with the lamp approximately 15 cm from the cells. After the cells incubated for 48 h total, the transfected cells were observed by fluorescence microscope and transfection efficiency was detected by flow cytometer. All transfection experiments were performed in triplicate.

Intracellular localization of polyplexes: Intracellular localization of polyplexes was investigated in HepG2 cells. The cells were seeded into glass base dishes at a density of 5×10^4 cells/dish and incubated overnight in 1.5 mL of medium. Az-PEG/PEI-CD/FITC-DNA polyplexes at N/P ratio of 10 containing 6 μg FITC-DNA were added and incubated for 4.5 h. Then, the cells were exposed to 365 nm light for 15 min and incubated for another 12 h. After that, the cells were fixed with 4% (w/v) paraformaldehyde for 30 min and stained with DAPI (2.5μg/ml) for 20 min. The intracellular distribution of polyplexes was analyzed by confocal laser scanning microscope (CLSM, Leica TSSP5, Germany)

References

1 W. Y. Li, L. N. Chen, Z. X. Huang, X. F. Wu, Y. F. Zhang, Q. L. Hu and Y. X. Wang, *Org. Biomol. Chem.*, 2011, 7799.

2 M. E. Davis, S. H. Pun, N. C. Bellocq, A. J. Liu, G. Jensen, T. Machemer, E. Quijano, T. Schluep, S. F. Wen, H. Engler and J. Heidel, *Bioconjugate Chem*, 2004, **15**, 831.

3 T. Ikeda, T. Ooya and N. Yui, *Polym J*, 1999, **31**, 658.

4 Y. P. Wang, N. Ma, Z. Q. Wang and X. Zhang, *Angew Chem Int Edit*, 2007, **46**, 2823.

5 C. T. Lee, Y. C. Liu, A. L. M. Le Ny, J. Schmidt, Y. Talmon and B. F. Chmelka, *Langmuir*, 2009, **25**, 5713.

6 Y. X. Wang, Z. X. Xu, R. Zhang, W. Y. Li, L. Yang and Q. L. Hu, *Colloid Surface B*, 2011, **84**, 259.