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Highly efficient photocontrolled targeted delivery of siRNA by a cyclodextrin-based supramolecular nanoassembly†

Feng-Qing Li,[ab] Qi-Lin Yu,[ac] Yao-Hua Liu,[a] Hua-Jiang Yu,[a] Yong Chen[ab] and Yu Liu*[ab]

In this study, we have constructed a binary supramolecular nanoassembly composed of α-cyclodextrin-modified hyaluronic acid and an azobenzene-modified diphenylalanine derivative with a positively charged imidazole group. This nanoassembly can bind with siRNA through electrostatic interactions and efficiently delivered them into cancer cells and inhibited their growth.

Cyclodextrins (CDs) are a class of biocompatible cyclic oligosaccharides with a hydrophilic outer periphery and a well-defined hydrophobic cavity that can bind various organic molecules,[1] including biologically relevant compounds.[2] CD-based supramolecular nanocarriers exhibit great potential for cancer treatment owing to their excellent biocompatibility, low toxicity to healthy cells, and ability to reliably deliver and release therapeutic drugs to the desired targets.[3] Recently, grafting CDs onto hyaluronic acid (HA), a naturally occurring hydrophilic linear polysaccharide that strongly binds to HA receptors (such as CD44 and RHAMM receptors) on cancer cell membranes,[4] has attracted much attention from supramolecular chemists.[5] The grafted CDs not only improve the hydrophilicity of HA but also provide binding sites for biologically active guest molecules, and such constructs have potential utility as stimulus-responsive nanocarriers.[6]

Recently diphenylalanine (FF), the shortest peptide to form a variety of nanoassemblies with good biocompatibility,[7] has attracted much attention from scientists due to their wide applications in biomedicine, optoelectronics, and materials science.[8] The modification of FF at the amino or carboxyl terminus has been shown to produce assemblies with new performance characteristics[9] and/or interesting active guest molecules.[10] However, FF derivatives and assemblies containing them have rarely been used in biomedical applications and gene therapy in particular, owing to the lack of functional groups that participate in multivalent interactions.[11] Therefore, the development of a supramolecular strategy for functionalizing FF to improve its affinity for biomacromolecules and/or gene drugs for the purpose of fabricating smart vectors for genes would be highly desirable.

Varieties of stimuli-responsive chemical groups to the host or guest molecules facilitated to form nanoassemblies with controllable assembly/disassembly ability.[12] Among external stimuli (including pH-change, temperature-change, ions, enzymes and gases), light is a remarkable attractive external stimulus due to its noninvasive, clean, efficiency, handiness and mild conditions.[13] Photo-responsive macrocyclic host–guest recognitions have been widely used to achieve the smart control of supramolecular assemblies including photoswitchable supramolecular hydrogels[14] and photoresponsive artificial nanocarriers for drug delivery[15] and artificial light harvesting.[16] To the best of our knowledge, supramolecular nanocarriers based on FF derivatives through macrocyclic host–guest recognitions have not yet been reported.

Previously, we have reported an FF derivative bearing an N-terminal azobenzene group that combine with α-CD to form supramolecular assemblies which can be controlled by both photochemical and chemical stimuli; specifically, irradiation of the assemblies at various wavelengths resulted in reversible interconversion between two different morphologies.[17] Herein, we report a new supramolecular gene nanocarrier composed of α-CD-modified HA (HA-α-CD) and an azobenzene-modified FF derivative with a positively charged imidazole group (designated *trans*-G), which self-assembled into a binary complex owing to the strong binding affinity between the azobenzene moiety and the hydrophobic cavity of α-CD (Scheme 1). In addition, as a result of the good biocompatibility and targeting capacity of HA, a ternary supramolecular nanoassembly (*trans*-G/HA-α-CD/siRNA) showed excellent ability to target cancer cells, together with low cytotoxicity to healthy cells. Upon isomerization of the azobenzene double bond by means of UV irradiation (365 nm), the ternary assembly disassembled and released its siRNA cargo, which then showed

[a] *College of Chemistry, State Key Laboratory of Elemento-Organic Chemistry, Nankai University, Tianjin 300071, China. E-mail: yuliu@nankai.edu.cn*
[b] *Collaborative Innovation Center of Chemical Science and Engineering (Tianjin), Tianjin 300071, China*
[c] *College of Life Sciences, Nankai University, Tianjin 300071, China*

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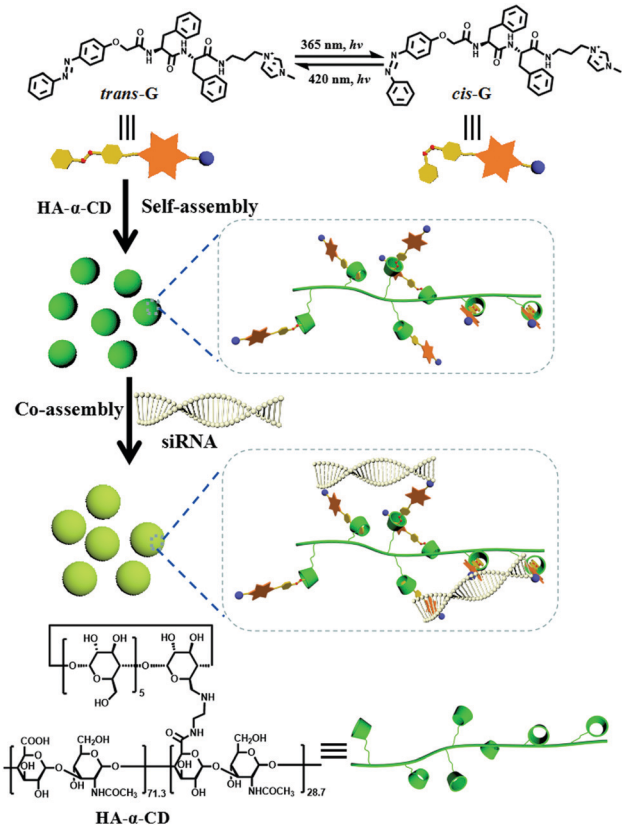
excellent cytotoxicity against the cancer cells. This approach can be expected to overcome the disadvantages of gene transfection reagents with a high density of positive charges, which can damage the membranes and organelles of normal cells, and thus provides a promising method for gene delivery.

HA-α-CD was synthesized according to the reported procedure[18] from HA with a molecular weight of approximately 40 kDa. The degree of substitution of α-CD units grafted on HA-α-CD was 28.7%, as determined from the ^{1}H NMR spectrum of HA-α-CD (Fig. S4, ESI†). FF derivative (*trans*-G) was prepared in 65% yield *via* the condensation reaction of AZO-FF and Im-NH_2 and then was purified using prep-HPLC (ESI† 1.1).

As shown in Scheme 1, the azobenzene group at one end of *trans*-G formed an inclusion complex with the hydrophobic cavities of the α-CD moieties of HA-α-CD, whereas the imidazole group at the other end of the *trans*-G remained exposed and could thus improve the water solubility of the supramolecular assembly and also interact with negatively charged siRNA. The host–guest interaction between α-CD and the azobenzene group is well understood. Herein, we used ^{1}H NMR and UV/Vis absorption spectroscopy to elucidate the binding behaviors of HA-α-CD with *trans*-G and *cis*-G, and we compared these behaviors with those of α-CD as a reference host. The ^{1}H NMR spectrum of *trans*-G was recorded in aqueous solution at a concentration of 2.5×10^{-3} mol L^{-1}, and the spectrum was compared with that of *trans*-G in the presence of α-CD (Fig. S5, ESI†).



Scheme 1 Schematic illustration of the *trans*-G/HA-α-CD/siRNA ternary supramolecular nanoassembly.

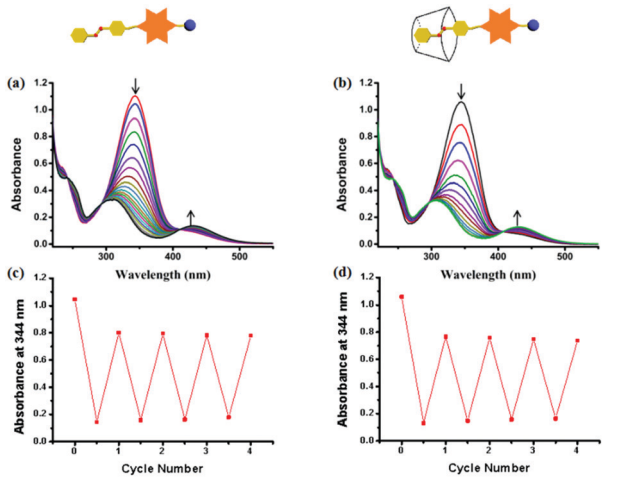


Fig. 1 UV/Vis absorption spectra showing the photoisomerization of (a) free *trans*-G (5×10^{-5} mol L^{-1}) and (b) *trans*-G/α-CD in water at 25 °C. Variation of UV/Vis absorption intensities of (c) free G and (d) G/α-CD at 344 nm observed upon alternating irradiation with UV (365 nm) and visible (450 nm) light.

In the latter spectrum, the signals of the aromatic protons of the azobenzene group were shifted downfield relative to those of free *trans*-G, whereas the protons of the FF and imidazole moieties remained essentially unchanged. Upon irradiation of *trans*-G/α-CD with UV light (365 nm) for 10 min, approximately 90% of the *trans*-G was converted to *cis*-G; all of the aromatic protons in the spectrum of *cis*-G + α-CD were consistent with those of free *cis*-G. Moreover, *cis*-G could be transformed back into *trans*-G either by means of visible-light irradiation (420 nm) for 30 min or by heating at 80 °C for 1 h.

Furthermore, UV/Vis absorption spectroscopy suggested that the association of *trans*-G with α-CD accelerated the isomerization of the azobenzene moiety in *trans*-G (Fig. 1). When a solution of *trans*-G was irradiated at 365 nm, the intensity of the absorption band around 344 nm dramatically decreased, and simultaneously a new absorption band appeared around 431 nm, along with a clear isosbestic point at 410 nm. These changes were attributed to the isomerization of *trans*-G to *cis*-G, which was calculated to have a rate constant (*kt*) of 0.058 s^{-1} (Fig. S6a, ESI†). In contrast, when a solution containing equimolar α-CD and *trans*-G was irradiated at 365 nm, up to approximately 90% of the *trans*-G was converted to *cis*-G within 50 s; the rate constant for this process was calculated to be 0.088 s^{-1}, which is 1.5 times larger than that for isomerization of free *trans*-G (Fig. S6b, ESI†). The irradiation-induced photoisomerization of the azobenzene moiety could be repeated tens of times with no obvious change in absorption at 344 nm, demonstrating the good reversibility of the isomerization process.

The morphological conversion involved in the *trans*-G/HA-α-CD/siRNA assembly/disassembly process was characterized by transmission electron microscopy (Fig. 2 and Fig. S7, ESI†) and scanning electron microscopy (Fig. S8, ESI†). In aqueous solution, free *trans*-G formed long nanofibers with a diameter of *ca.* 10 nm, and irradiation of the *trans*-G solution at 365 nm

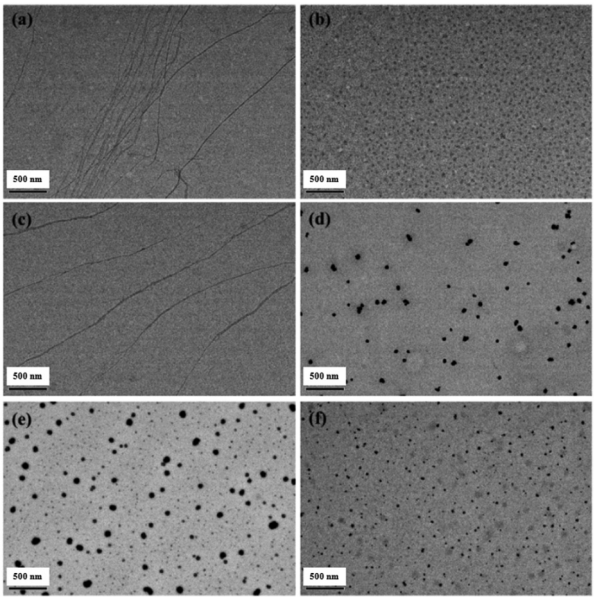


Fig. 2 Transmission electron microscopy images of (a) free *trans*-G, (b) free *cis*-G, (c) *trans*-G/α-CD, (d) *trans*-G/HA-α-CD, (e) *trans*-G/HA-α-CD/siRNA, and (f) *cis*-G/HA-α-CD/siRNA.

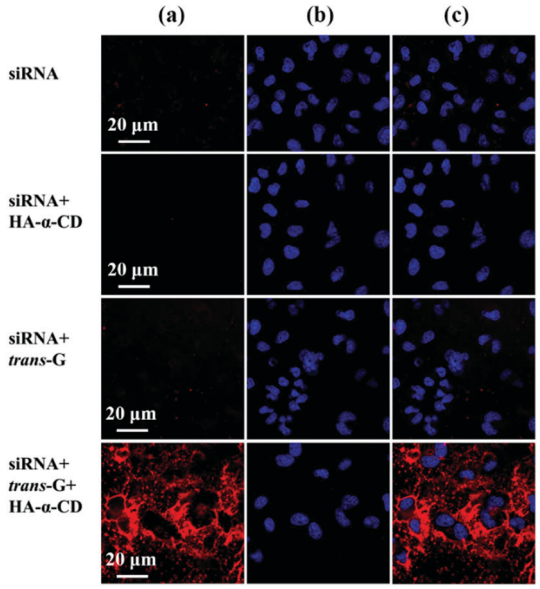


Fig. 3 Confocal laser fluorescence microscope images of human lung adenocarcinoma cells (A549) cultured with free siRNA, siRNA + HA-α-CD, siRNA + *trans*-G, and *trans*-G/HA-α-CD/siRNA: (a) fluorescence emitted by siRNA in the cytoplasm (red), (b) fluorescence of cell nuclei stained with 4′,6-diamidino-2-phenylindole (blue) and (c) merged images.

for 3 min resulted in conversion of the nanofibers to nanospheres with a diameter of *ca.* 30 nm. In contrast, a mixture of α-CD and *trans*-G formed slightly thicker nanofibers with an average diameter of 15 nm, and a mixture of *trans*-G and HA-α-CD formed nanoparticles with an average diameter of *ca.* 50 nm, which can be attributed to the formation of inclusion complexes between *trans*-G and HA-α-CD. When siRNA was added to a freshly prepared solution of *trans*-G/HA-α-CD, much larger nanoparticles (average diameter *ca.* 60 nm) formed; and upon irradiation of these nanoparticles (*trans*-G/HA-α-CD/siRNA) at 365 nm, they disassembled into small nanoparticles and nanosheets, which were similar to those formed upon irradiation of *trans*-G and *trans*-G/HA-α-CD. Taken together, these results indicate that the *trans*-G/H-α-CD/siRNA ternary supramolecular nanoassembly responded to UV light by undergoing morphological transformation and subsequently releasing siRNA.

Having demonstrated the formation of the *trans*-G/HA-α-CD/siRNA ternary supramolecular nanoassembly, we carried out cell viability experiments with an assembly containing synthetic siRNA (GAPDH-homo). First, we confirmed that the ternary assembly could deliver siRNA into cancer cells, by means of confocal laser scanning microscopy. Specifically, human lung adenocarcinoma cells (A549) were cultured with *trans*-G/HA-α-CD/siRNA, as well as with free siRNA and mixtures of *trans*-G + siRNA and HA-α-CD + siRNA as controls (Fig. 3). Upon excitation at 550 nm, the red fluorescence of the siRNA was clearly observed in the cytoplasm of cells treated with *trans*-G/HA-α-CD/siRNA. In contrast, in cells cultured with either free siRNA or with HA-α-CD + siRNA, no red fluorescence was observed. A little red fluorescence assigned to siRNA was observed in the cytoplasm of cells treated with free *trans*-G, a result that we attributed to the good biocompatibility of FF and the positive charge of the imidazole moiety. These confocal laser scanning microscopy experiments indicate that the *trans*-G/HA-α-CD nanoassembly effectively delivered siRNA into cancer cells. In addition, we also tested the 293T cell lines (human embryonic kidney cell) that lack the HA-receptor, and the result showed that the *trans*-G/HA-α-CD displayed no appreciable siRNA delivery ability under the same conditions.

Having confirmed that *trans*-G/HA-α-CD co-assembled with siRNA and that the resulting *trans*-G/HA-α-CD/siRNA ternary supramolecular nanoassembly could be efficiently delivered into A549 cancer cells, we performed gene-silencing experiments with A549 cells to evaluate the cytotoxicity of the nanoassembly *in vitro*. A549 cells cultured with free *trans*-G, free HA-α-CD, the *trans*-G/HA-α-CD binary nanoassembly, and *trans*-G + siRNA and HA-α-CD + siRNA mixtures served as controls (Fig. 4). The concentration of siRNA in each sample was 1 μM. In the absence of UV irradiation, none of the samples showed obvious toxicity to the cancer cells (data not shown). Upon irradiation at 365 nm, the *trans*-G/HA-α-CD/siRNA nanoassembly showed much greater gene-silencing efficiency than the controls, and the cytotoxicity of the ternary assembly increased as the concentration was increased. It is noteworthy that the gene delivery efficiency of the nanoassembly was similar to the case reported by Ravoo *et al.*[19] and Pei *et al.*[20] Moreover, HA-α-CD alone showed little cytotoxicity to the cancer cells. In addition, the ternary assembly showed no cytotoxicity toward normal cells (data not provided). In the supramolecular assembly, the *trans*-G played an indispensable role as a bridge connecting HA-α-CD and siRNA so that the latter could exert its biological activity. At a concentration of 320 μM, more than 80% cancer cell growth was inhibited by the *trans*-G/HA-α-CD/siRNA nanoassembly,

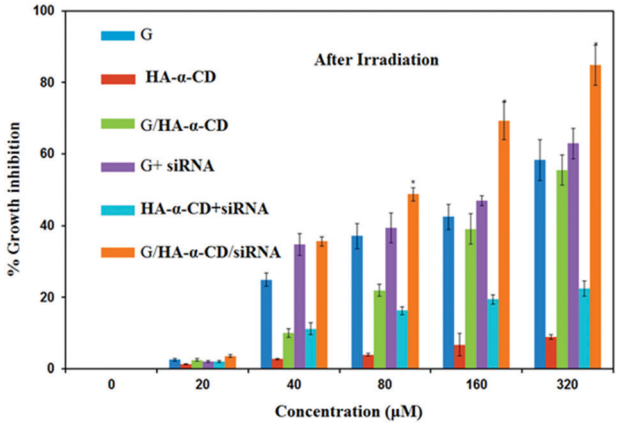


Fig. 4 Growth inhibition to cancer cells induced *in vitro* at various concentrations after UV irradiation (365 nm).

owing to the joint effects of photo-triggered release of siRNA and the ability of HA-α-CD to target cancer cells. We also performed an experiment to detect the expression levels of GAPDH, and found that the nanoassembly exhibited the highest gene silencing efficiency of *ca.* 55% among the tested agents (Fig. S9, ESI†).

In conclusion, a supramolecular nanoassembly for targeted delivery of siRNA was constructed from CD-modified HA and a cationic azobenzene-modified FF derivative. The binding behaviours of the *trans*-G/HA-α-CD assembly and *cis*-G + HA-α-CD to combine with siRNA were analyzed by transmission electron microscopy, scanning electron microscopy, UV/Vis absorption spectroscopy, and NMR spectroscopy. Interestingly, the *trans*-G/HA-α-CD/siRNA ternary supramolecular assembly could transport siRNA into cancer cells, where its release could be triggered by UV irradiation. The strategy described herein has great potential to be an attractive one for targeted, controlled gene delivery.

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Conflicts of interest

There are no conflicts to declare.

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