Drug/siRNA Co-Delivery

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Cationic Vesicles Based on Amphiphilic Pillar[5]arene Capped with Ferrocenium: A Redox-Responsive System for Drug/siRNA Co-Delivery**

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Abstract: *A novel ferrocenium capped amphiphilic pillar[5]-arene (FCAP) was synthesized and self-assembled to cationic vesicles in aqueous solution. The cationic vesicles, displaying low cytotoxicity and significant redox-responsive behavior due to the redox equilibrium between ferrocenium cations and ferrocenyl groups, allow building an ideal glutathione (GSH)-responsive drug/siRNA co-delivery system for rapid drug release and gene transfection in cancer cells in which higher GSH concentration exists. This is the first report of redox-responsive vesicles assembled from pillararenes for drug/siRNA co-delivery; besides enhancing the bioavailability of drugs for cancer cells and reducing the adverse side effects for normal cells, these systems can also overcome the drug resistance of cancer cells. This work presents a good example of rational design for an effective stimuli-responsive drug/siRNA co-delivery system.*

Drug resistance and similar cytotoxicity in both cancerous and healthy cells are major problems in chemotherapy.[1] One promising way to overcome these obstacles is the development of stimuli-responsive co-delivery systems of drug/siRNA (small interfering RNA), which possess the ability to recognize and respond to the specific microenvironmental changes (such as pH,[2] temperature,[3] and glutathione (GSH) concentration)[4] associated with neoplastic diseases.[5] Due to the specific stimulus, the nanocarriers disassemble after entering cancer cells, leading to a rapid release of encapsulated siRNA and drug. siRNA can knock down drug resistance genes and the drug sensitivity of cells will be restored to enable effective chemotherapy. Meanwhile, due to the absence of the specific stimulus, drug release does not occur or occurs slowly in healthy cells, thus efficiently avoiding severe side effects.

The indispensable structural features of a material used for such systems are: 1) positive charges, which allow effective loading of polyanionic siRNA by electrostatic interaction,[6] and 2) units that respond to intracellular stimuli. So far, most of the reported siRNA cationic systems are limited to the protonation of amino groups under acidic conditions[7] and the stimuli-responsive structural units reported include amino groups, hydrazone linkages, disulfide bonds (-S–S-),[4,8] and diselenide bonds (-Se–Se-).[9] Considering that the concentration of GSH in tumor cells is higher than that in normal cells,[10] it is ideal and ubiquitous as a trigger for redox-sensitive system to accomplish rapid drug release in cancer cells.[8] Nevertheless, few GSH-responsive nanocarriers for the drug/siRNA co-delivery have been reported up to date.[11]

Pillar[*n*]arenes[12] are a new class of macrocyclic compounds which possess a hydrophobic core sandwiched between two functional rims. In the past few years, they have attracted considerable attention in the fabrication of nanocarriers for biological applications, because of their unique rigid pillar architecture and facile chemical modification. Among various drug nanocarriers,[13] vesicles formed from amphiphilic pillararenes (APs)[14] have shown great potential in drug delivery due to an efficient drug encapsulation in their cavities. To the best of our knowledge, cationic vesicles assembled with APs for drug/siRNA co-delivery have not yet been reported.

The ferrocenium cation, sensitive to GSH,[15] undergoes a polarity shift (a driving force of assembly and disassembly for constructing vesicles)[16] when reduced to ferrocene (a known biocompatible building block).[17] More importantly, in comparison to other typically exploited redox-responsive bonds, the positive charge of ferrocenium makes possible for the loading of negatively charged siRNA onto nanocarriers.[4,18] Therefore, it is a potential structural unit for the construction of GSH-responsive drug/siRNA co-delivery systems. We envision that the conjugation of ferrocenium cation head groups to pillararenes will lead to an AP with positive charges as well as redox-active structural units. This AP will, possibly, be able to self-assemble into cationic vesicles in aqueous solution and thus be applied in drug/siRNA co-delivery.

Thus, we synthesized a novel ferrocenium-capped amphiphilic pillar[5]arene (FCAP), which was further applied to construct nanoscale cationic vesicles for drug/siRNA co-delivery as depicted in Scheme 1. This is the first report of redox-responsive cationic vesicles for drug/siRNA co-delivery and it is the first use of ferrocenium as GSH-responsive structural unit in a drug delivery system. The cytotoxicity, redox-responsive behavior, drug encapsulation and release rate, and bioavailability of the cationic vesicles were studied. Moreover, as a proof-of-concept, doxorubicin hydrochloride (DOX) and drug resistance gene silencing siRNA (MRP1

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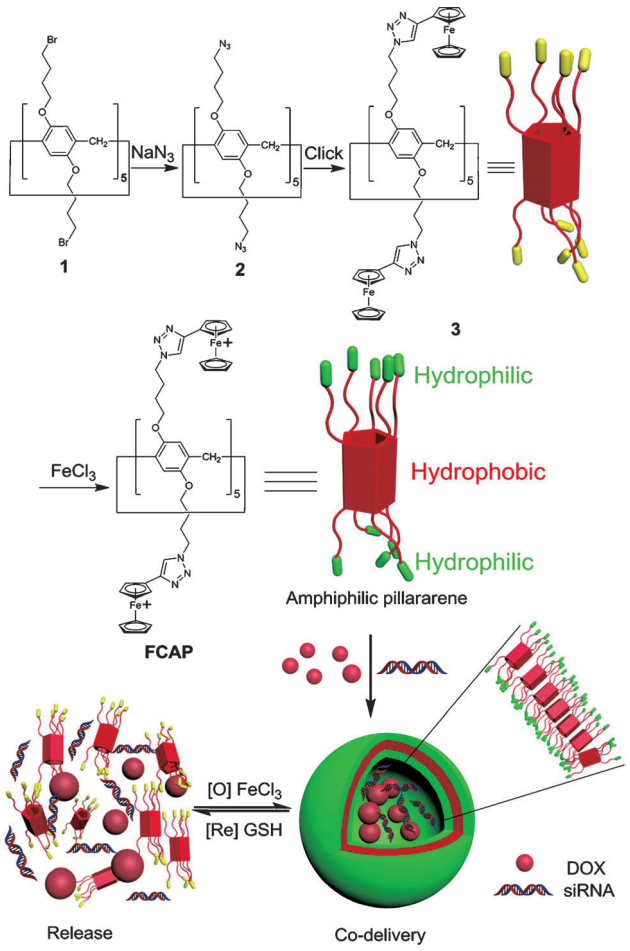

Scheme 1. Illustration of the synthesis of FCAP, formation of cationic vesicles, and their redox-responsive drug/siRNA release.

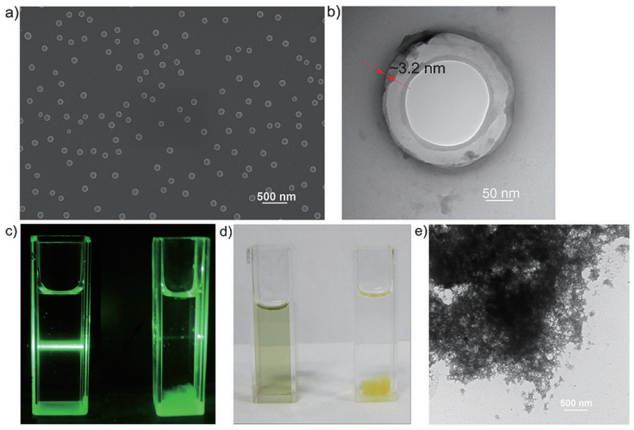

Figure 1. a) SEM image of the cationic vesicles formed from FCAP. b) TEM image. c) Tyndall effect of FCAP aggregation (left) and precipitates formed in the presence of excess GSH (right). d) The view of samples in Figure 1 c under normal light. e) TEM image of FCAP in the presence of GSH.

siRNA) were selected as the anticancer drug/siRNA pair for a co-delivery study in human ovarian SKOV-3 cancer.[1b,c]

To synthesize FCAP, **1**[19] was first functionalized with sodium azide. Azide-modified pillar[5]arene **2** was obtained in 92 % yield, and subjected to copper-catalyzed azide-alkyne cycloaddition (CuAAC) to form pillar[5]arene **3** in a yield of 88 % (Scheme 1). Oxidation of the ferrocenyl groups on **3** by $FeCl_3$ gave FCAP stoichiometrically, accompanying a polarity shift from hydrophobicity of **3** to amphipathy of FCAP which could lead to regular aggregates in water. For details of the synthesis and characterization of **1**–**3** and FCAP, see the Supporting Information (SI).

By subjecting a solution of FCAP to sonication for 30 min, spherical vesicles were formed and confirmed by scanning electron microscopy (SEM, Figure 1 a). Negative-stained transmission electron microscopy (TEM) images suggested that the thickness of the vesicles was about 3.2 nm from the distinguishably dark periphery to the light central part (Figure 1 b), which is close to the molecular length of **3** [2.9 nm calculated with the energy-minimized structure (Figure S10)]. This indicates that the vesicles were assembled by FCAP in a monolayer packing mode. Further analysis with dynamic light scattering (DLS, Figure S11) showed that the average diameter and polydispersity index of the vesicles were 91.9 nm and 0.298, respectively. The spherical assemblies were stable in solution at 300 K for one week, implying the vesicles had a good stability. The critical aggregation concentration (CAC) of FCAP was found to be 58 μM by the water surface tension method[14a](Figure S12).

The redox-responsiveness of the vesicles formed from FCAP was investigated by exposing the solution to the reductant GSH or the oxidant $FeCl_3$. As shown in Figure 1 c, a clear Tyndall effect was observed with FCAP aqueous solution (60 μM), indicating the existence of abundant nanoparticles. However, accompanied by the disappearance of the Tyndall effect, yellow precipitates formed gradually from the transparent light green solution of FCAP upon addition of GSH (Figure 1 d). TEM studies, as expected, found no vesicles accordingly (Figure 1 e). All these results suggested that the vesicles were disassembled when exposed to GSH, corresponding to the reduction of the light green ferrocenium cation to the yellow ferrocenyl groups. Further studies using UV/Vis spectroscopy confirmed that the reversible transformation between ferrocenium cations and ferrocenyl groups upon the presence of reductant (GSH) or oxidant ($FeCl_3$; Figure S13). Therefore, the vesicles based on FCAP can be used as redox-responsive nanocarriers capable responding to GSH.

Cytotoxicity of unloaded vesicles to normal cells was evaluated by the methyl thiazole tetrazolium (MTT) cell-survival assay. As shown in Figure S14, the relative cell viability of 293T cells (normal cells) incubated with unloaded vesicles was over 85 % in the concentrations of 10 μM FCAP after 24 h, indicating that the cationic vesicles based on FCAP developed in this work had low cytotoxicity to normal cells, which was essential for the design of drug nanocarriers.

To study the drug release behavior of the redox-responsive cationic vesicles, doxorubicin hydrochloride (DOX), a fluorescence dye and anticancer drug molecules, was selected as model drug.[20] SEM disclosed that the surface of DOX-loaded vesicles (see SI for preparation in details) became quite rough (Figure S15 a). DLS analysis showed that

the DOX-loaded vesicles had an average diameter of 79 nm and a polydispersity index of 0.232 (Figure S15b). UV/Vis spectra of the DOX-loaded vesicular solution confirmed that DOX molecules were successfully encapsulated in the vesicles. In addition, the color of the unloaded vesicular solution changed from light green to brown upon DOX-loading into the vesicles (inset of Figure S15c). From UV/Vis absorption spectra, the DOX encapsulation and loading efficiency were calculated to be 67.0% and 9.1%, respectively, indicating a good drug-loading capability of the cationic vesicles.

DOX release profiles from DOX-loaded vesicles with or without exposure to GSH in water were shown in Figure S16. 10 mM GSH was used to trigger the responsive release of DOX, which was relevant to the concentration of GSH in cancer cells (1–11 mM).[21] The amount of DOX release reached a maximum of 92% with 10 mM GSH, resulting from the disassembly of DOX-loaded vesicles when the ferrocenium cations were reduced to ferrocenyl groups by GSH. In contrast, the DOX-loaded vesicles without GSH exposure remained relatively stable and only 39% of DOX was released in the same period. The rapid release behavior resulting from the redox-responsive DOX-loaded vesicles responding to GSH makes the DOX-loaded vesicles ideal nanocarriers for drug delivery.

The cellular uptake of DOX-loaded vesicles and free DOX was investigated by confocal laser scanning microscope (CLSM) for comparison. Red fluorescence could be observed clearly in the nuclei of HeLa cells after 1 h incubation with DOX-loaded vesicles (Figure S17a), whereas the red fluorescence of HeLa cells incubated with free DOX was weaker (Figure S17) under the same conditions, indicating that DOX loaded by vesicles could enter the cells more rapidly than free DOX.

To evaluate the anticancer efficiency of DOX-loaded vesicles, 293T cells and HeLa cells were incubated with DOX-loaded vesicles and free DOX for 24, 48, and 72 h, respectively. For 293T cells (Figure 2a), the relative viabilities after incubation with DOX-loaded vesicles at all three tested time periods were visibly higher than those with free DOX (especially at 24 h). In contrast, HeLa cells under the same conditions showed lower relative viabilities after incubation with DOX-loaded vesicles than with free DOX (Figure 2b). This is also supported by the images of normal (293T) and cancer (HeLa) cells treated with DOX-loaded vesicle for 24 h (Figure S18), which showed better growth of 293T cells compared to HeLa cells.[22] In addition, the enhanced apoptotic effects of DOX-loaded vesicles on cancer cells were also confirmed in the cases of SKOV-3 and KM-12 cells (Figure S19). All these results suggested that the encapsulation of DOX by the vesicles could enhance the drug's efficiency against cancer cells while effectively reducing its cytotoxicity to normal cells.

Synthetic siRNAs fail to cross biological membranes by passive diffusion because of their high molecular weight and polyanionic nature; therefore, they generally require delivery systems to be positively charged for loading of siRNA by electrostatic interactions.[6] FCAP synthesized in this work has ten positive charges due to the ten capped ferrocenium cations in its molecular structure. When mixed with FAM-siRNA (a fluorescent labeled siRNA) at a ferrocenium cation/P ratio of 5:1 (defined as the molar ratio of ferrocenium cation units in the cationic vesicles to phosphate units in siRNA), the cationic vesicles self-assemble to FAM-siRNA/cationic vesicle complexes through electrostatic interactions. The SEM image of the complexes showed nanoparticles with an average diameter of 60 nm (Figure 3a). The cellular uptake and intracellular distribution of the FAM-siRNA/cationic vesicle complexes were studied by flow cytometry and CLSM. As shown in Figure 3b, HeLa cells transduced with FAM-siRNA/cationic vesicle complexes showed ca. 54% cellular internalization, a similar result to Lipofectamine 2000 (58%) which had been used as siRNA transfection agent.[23] The results were further confirmed by confocal microscopy. Strong green fluorescence was found

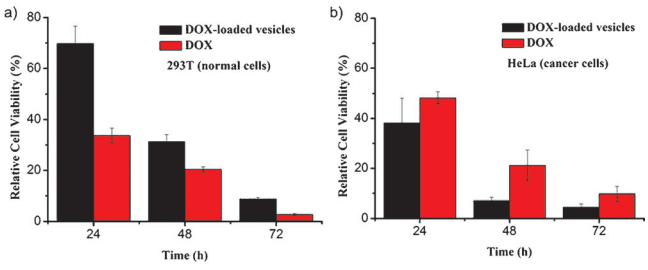

Figure 2. Comparison of DOX and DOX-loaded vesicles on viabilities of 293T cells (a) and HeLa cells (b) at different times.

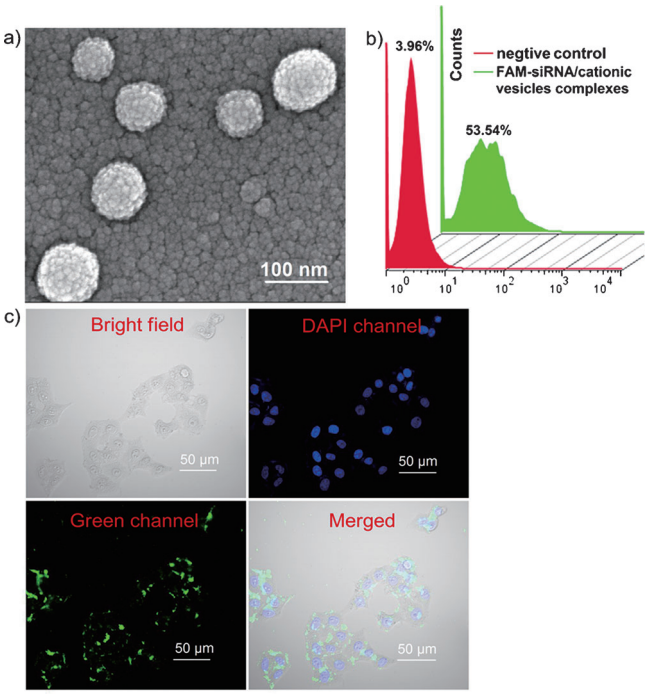

Figure 3. a) SEM image of FAM-siRNA/cationic vesicle complexes. b) Flow cytometry analyses of HeLa cells after incubation with FAM-siRNA/cationic vesicle complexes at ferrocenium cation/P ratio of 5:1. c) CLSM images of HeLa cells, after being treated with FAM-siRNA/cationic vesicles complexes; scale bar 50 μm.

in the cytoplasm of the cells incubated with FAM-siRNA/cationic vesicle complexes, demonstrating successful delivery (Figure 3c).

As a proof-of-concept, multidrug-resistant protein siRNA (MRP1 siRNA) was selected to fabricate DOX-loaded MRP1 siRNA/cationic vesicle complexes, which were used for the co-delivery study to assess the drug resistance gene silencing of SKOV-3 cells.[1b,c] A negative siRNA was used as the control. For siRNA loading, freshly prepared DOX-loaded vesicles were first diluted to the desired ferrocenium cation/P ratio, followed by the addition of the siRNA solution. The mixture was allowed to stand for 20 min before use. The internalization of MRP1 siRNA into SKOV-3 cells is shown in Figure 4a. After incubating SKOV-3 cells with DOX-loaded FAM-MRP1 siRNA/vesicles complexes for 6 h, both green and red fluorescence were observed in the cells. This suggested that both DOX and FAM-MRP1 siRNA were internalized into the cells by the cationic vesicles (Figure 4a). The gene silencing efficiency was investigated at both mRNA and protein level. The transcriptional MRP1 mRNA was detected by quantitative real-time polymerase chain reaction (RT-PCR) (Figure 4b) and MRP1 protein was determined by ELISA (Figure 4c). The DOX-MRP1 siRNA/vesicles (DOX-loaded MRP1 siRNA/cationic vesicles complexes) showed marked down-regulation of MRP1 mRNA and a decrease in the MRP1 expression compared to the negative control (DOX-loaded negative siRNA/cationic vesicle complexes). Then the annexin V conjugate staining assay was performed to evaluate the cytotoxicity of the DOX-loaded MRP1 siRNA/cationic vesicle complexes. As shown in Figure 4d, the cell death and apoptosis of cells incubated with DOX-loaded negative siRNA/cationic vesicle complexes was 37.9%, whereas that with DOX-loaded MRP1 siRNA/cationic vesicles complexes reached 52.8%. By the MTT method, the IC_{50} value in SKOV-3 after 48 h incubation with DOX-loaded MRP1 siRNA/cationic vesicles complexes was calculated to be 2.1 μM, whereas it was 4.9 μM with DOX-loaded negative siRNA/cationic vesicles complexes (Figure S20).[24] These results suggested that the DOX resistance of the cancer cells was re-sensitized after being transfected with DOX-loaded MRP1 siRNA/cationic vesicles complexes, and chemotherapeutic efficacy was enhanced to a certain extent by the synergistic effects of MRP1 siRNA and DOX.

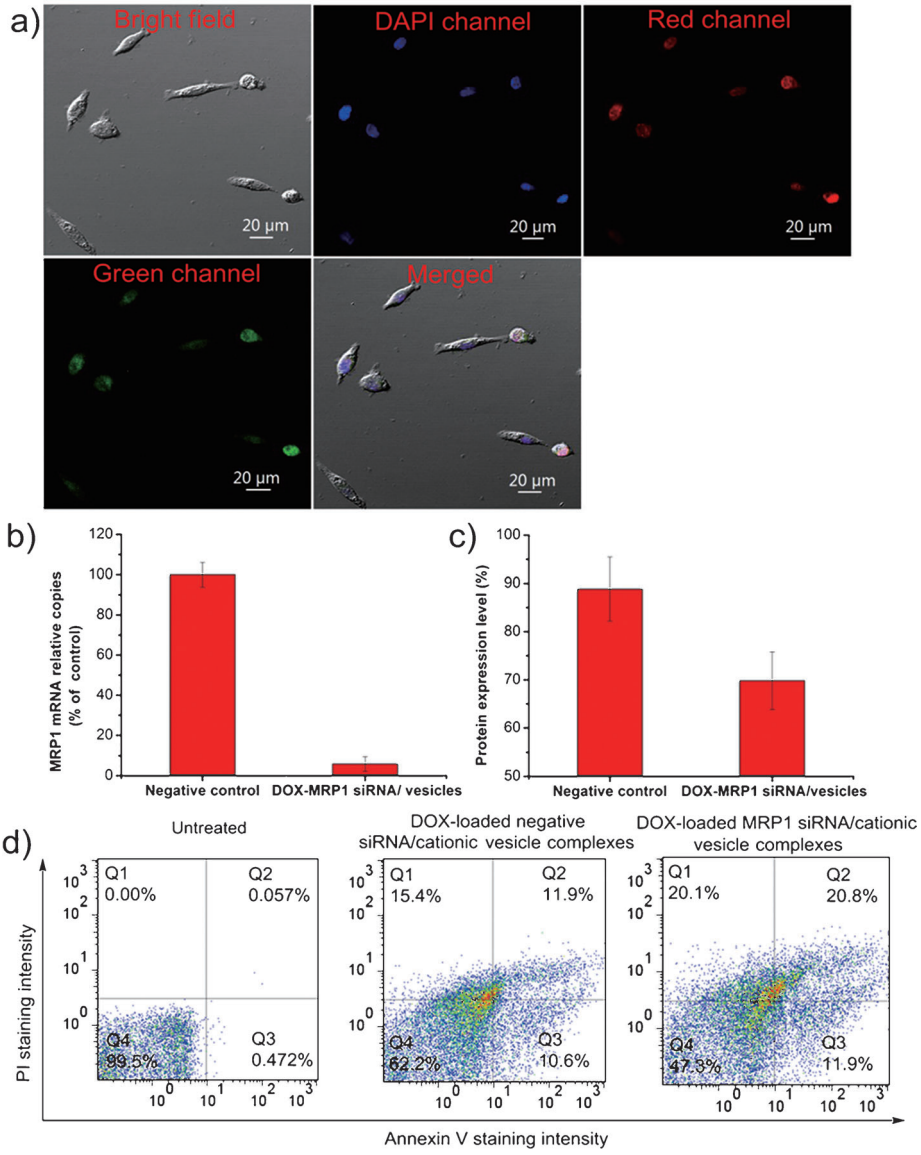

Figure 4. a) CLSM images of SKOV-3 cells after being treated with DOX-loaded FAM-siRNA/vesicles complexes for 6 h; scale bar 50 μm. b) Gene silencing efficiency of SKOV-3 cells at the mRNA level determined by RT-PCR. c) Gene silencing efficiency of SKOV-3 cells at the MRP1 protein expression level determined by ELISA. d) The cell death and apoptosis of SKOV-3 cells after staining with annexin V–FITC and propidium iodide (PI) by flow cytometry. Untreated SKOV-3 cells were used as a control. Cells that were negative for both annexin V–FITC and PI staining were classified as alive, whereas cells stained positively for annexin V–FITC and negatively for PI were classified as apoptotic. Cells that stained positively for PI were classified as necrotic.

In summary, novel cationic vesicles resulting from the self-assembly of FCAP have been developed for GSH-triggered drug and siRNA co-delivery. Drug release studies in vitro demonstrated that DOX-loaded cationic vesicles can improve the inhibition of cancer cell growth resulting from subsequent GSH-triggered rapid drug release in cancer cells. Furthermore, DOX-loaded vesicles are markedly less toxic to normal cells than free DOX. More importantly, the co-delivery of

siRNA and DOX into cancer cells by cationic vesicles has been achieved, where drug resistance gene silencing siRNA (MRP1 siRNA) was used and SKOV-3 cells with DOX resistance were re-sensitized, resulting in a clear synergistic inhibition of SKOV-3 cells. This work thus provides a good example of rational design for effective stimuli-responsive drug/siRNA co-delivery systems.

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