Functional Build-in Template Directed Siliceous Fluorescent Supramolecular Vesicles as Diagnostics

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Abstract

Functional template directed synthesis of hybrid siliceous fluorescent vesicle (HSFV) is fabricated by using fluorescent vesicle as a build-in template. The template vesicle is the ionic self-assembly of an aggregation-induced emission (AIE) fluorogen. Upon depositing folic acid modified silica shell on its surface, the obtained HSFVs display low cytotoxicity, significant fluorescence and targeted drug delivery toward cancer cells. Furthermore, the wall-thickness of the HSFVs can be controlled via altered concentration of silica source. This is the first report of HSFV employing the template vesicle as a build-in fluorescent agent, which represents a good example of rational design for an effective diagnostics, and may open up a new avenue for precision medicine.

Keyword: hybrid self-assembly, aggregation-induced emission, template, fluorescent vesicles, precision medicine

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INTRODUCTION

Currently, one important objective within the field of precision medicine is to develop diagnostics with targeting functions.\textsuperscript{1-6} Owing to their versatility, mainly supramolecular self-assemblies are employed to fabricate various diagnostics. Supramolecular assemblies are often made with synthetic polymers with desired functional blocks, such as the fluorescent block, responsive block and targeting block. Drugs are usually loaded in the core of supramolecular self-assemblies (micelles or vesicles), and are delivered to the diseased sites for a therapy.\textsuperscript{7-14} Recently, self-assemblies based on drug/biomolecule multiple components are also invented,\textsuperscript{15-16} which allow combining the chemotherapy and imaging components into one nanoplatform.\textsuperscript{17-18} Although diagnostics made with molecular assemblies are facile to prepare, they often suffer from problems of stability and biocompatibility in the human physiological environment.

On the other hand, diagnostics based on silica nanoparticles are also employed for storage of active molecules owing to their excellent biocompatibility and stability.\textsuperscript{19-22} However, the fabrication of silica based diagnostics is very tedious, which usually involves multi-steps like fabrication of template, template synthesis of silica nanoparticles, removal of the template, and functionalizing the silica nanoparticles.\textsuperscript{23-26} Especially, the template is often sacrificial, which doesn’t offer any function to the obtained silica nanoparticles. As a result, various functional groups, such as imaging,\textsuperscript{27} targeting,\textsuperscript{28} and controlled releasing,\textsuperscript{29} have to be attached to the silica nanoparticles step by step. All these efforts add up to tremendous work load, which not only reduces the yield of production, increases the risk of cytotoxicity, but also decreases the number density of each functional group due to limited available surface areas.\textsuperscript{30}

To avoid these problems, herein we report an unprecedented functional template directed synthesis of hybrid siliceous fluorescent vesicles (HSFVs) with integrated ability of fluorescent imaging, drug-loading, cancer-cell targeting, and controlled rate of drug releasing. Fluorescent supramolecular vesicles displaying dual functions of
imaging and drug loading are used as templates to induce the hydrolysis of folic acid modified silica source. Since the template is not removed, the obtained vesicles immediately become targeting fluorescent diagnostics (nanodrugs if drugs are loaded) toward cancer cells. Moreover, the thickness of the silica shell can be fine-tuned by controlling the concentration of the silica source, thus allowing desired rate of drug releasing. Since the imaging and targeting groups are located at the supramolecular layer and the silicate layer of the HSFVs, respectively, it enables dual high efficiency of imaging and targeting toward cancer cells. Furthermore, dialysis separation of the unloaded drug is not needed since centrifugation can result in sedimentation of the siliceous vesicles loaded with drug, which can be readily dispersed again under gentle vortex or sonication for a few seconds. This work shows that upon integrating supramolecular vesicles and silica nanocarriers into one platform, we are able to develop advanced hybrid diagnostics facilely, which can even combine therapy ability by loading drugs. We expect that combination of supramolecular self-assembly of aggregation induced emission molecule with silica particles will provide a promising strategy for the new generation of nanodrug.

**Scheme 1.** Illustration of self-assembly of TPE-BPA@8CTAB vesicles, preparation of folic acid modified silicated fluorescent supramolecular vesicles (FA-HSFVs), and their targeted
combination with cancer cells. FA-HSFVs are conveyed to the cancer cell due to the specific interaction between folate receptor and folic acid.

RESULTS AND DISCUSSION

The fluorescent supramolecular vesicles are prepared through ionic self-assembling a propeller-shaped molecule TPE-BPA (Scheme 1) which displays aggregation induced emission (AIE). Different from conventional planar fluorescent dyes exhibiting notorious aggregation-caused quenching effect, AIE dyes are not emissive in free motion state but strongly emissive when aggregating as a result of restricted vibration. The unique light-up behavior has enabled them to be promising biolabels and visualizing agents. TPE-BPA was synthesized in our lab. This molecule contains the tetrphenylethene (TPE) group, which is the typical AIE moiety. The design of eight negative charges allows formation of ionic supramolecular vesicles TPE-BPA with positively charged amphiphiles in water. As cetyltrimethyl ammonium bromide (CTAB) was added to the aqueous solution of TPE-BPA, optimal fluorescent vesicles were formed at the charge neutral molar ratio of TPE-BPA: CTAB=1:8, as reported in our previous work. These vesicles were formed as a result of hydrophobic interaction between the supramolecular block of TPE-BPA@8CTAB, which was discussed in detail in our previous work. Figure 1A and 1B show the cryo-transmission electron microscopy (cryo-TEM) and confocal laser scanning microscopy (CLSM) image of the supramolecular TPE-BPA@8CTAB vesicles, respectively.

![Figure 1](image.png)

**Figure 1** A) Cryo-TEM images of TPE-BPA@8CTAB vesicles. B) CLSM image of TPE-BPA@8CTAB vesicles. [TPE-BPA]=100 μM, [CTAB]= 800 μM.
Hybrid siliceous fluorescent vesicles (HSFVs) were generated upon introducing tetraethoxysilane (TEOS) and folic acid modified (3-aminopropyl) triethoxysilane (FA-APTS), into the aqueous system of TPE-BPA@8CTAB supramolecular vesicle under vigorous stirring. Fluorescent suspension was obtained after 24 h at pH = 5.6. Fluorescent sediments can be obtained upon centrifugation this suspension at 10000 rpm for 15 min. The sediments were readily dispersed into water upon 30 s sonication. SEM (Figure S1) observation reveals the formation of spherical particles with diameters about 80 -100 nm. These spherical particles, without staining on the sample, are found multilamellar vesicles under TEM (Figure 2A-B), featuring the presence of inorganic components in the vesicular shell. We speculate that a silica shell has been deposited in both the inner and outer surface of each single TPE-BPA@8CTAB membrane, since the silica source may interact with the polar head of this membrane via hydrogen or electrostatic interaction. This conclusion can be verified by the equally high contrast of the vesicle wall at both the inner and outer surfaces. The diameter of these silicate vesicles is about 80 nm, which is the same as the size of the multilamellar supramolecular vesicles of TPE-BPA@8CTAB in Figure 1A. It is noticed that some of the vesicles can be broken under sonication, indicating the silicate layers are fragile. In latter on text, we may find the fragile feature of the vesicles facilitates releasing of entrapped drugs.

The morphologies of the vesicles are not noticeably different as the silica source varied from TEOS to the mixture of TEOS/FA-APTS (1/1, molar ratio, Figure 2B, S2), except that the number of walls differs a little bit. For vesicles silicated with TEOS, the number of the vesicle wall is about 4-6, whereas that can be 2-6 when silication is carried out with TEOS/FA-APTS. It is possible that the bulky FA-APTS is against the formation of a homogenous silica layer. Indeed, when all the TEOS was replaced with FA-APTS, the silication process failed. The maximum molar fraction of FA-APTS for successful fabrication of folic acid modified siliceous vesicle was 0.5, so that the 1/1 mixture of TEOS/FA-APTS was employed throughout this study to ensure high folic acid grafting on the surface of the silica vesicles.
The preformation of TPE-BPA@8CTAB supramolecular vesicles is very crucial in leading to well-defined HSFVs (Figure S3). Uniform and regular hollow structures are only observed at the molar ratio of TPE-BPA: CTAB around 1:8 where well-defined vesicles are formed. Amorphous structures are produced when CTAB is deficient, such as at the molar ratio of TPE-BPA/CTAB=1:4; whereas numerous small nanoparticles (10 nm) are observed in excess of CTAB (TPE-BPA/CTAB=1:12). This fact, together with the multilamellar structure of the HSFVs and their similar size to the TPE-BPA@8CTAB vesicles, clearly manifests that the supramolecular vesicle of TPE-BPA@8CTAB acts as template for the growth of SiO$_2$ shells.

Furthermore, pH also has great impact on the siliceous vesicle formation (Figure S4). The optical pH range is around 5.0~6.0. High or low pH resulted in amorphous and polydispered structures, presumably due to the fast hydrolysis process prohibiting duplicating the vesicular structure very well.$^{44}$

The wall-thickness of the HSFVs can be controlled by varying the concentration of the silica source. With increasing the concentration of TEOS from 6.72 μL/mL, to 8.95 μL/mL, 22.4 μL/mL and 56.0 μL/mL, the obtained wall-thickness was 6.5±1.2 nm, 8.3±1.3 nm, 9.9±2.9 nm and 12.1±4.2 nm, respectively (Figure 2C-E and Figure S5 for analysis on the wall thickness).

EDX measurement (Figure 2F) verifies the presence of the element Si and O in the sediments, and their molar ratio is about 1:2. Furthermore, FT-IR spectrum (Figure 2G) displays the characteristic strong asymmetric vibration of Si-O-Si at 1095~1160 cm$^{-1}$, and symmetric vibration of SiO$_4$ network at 805 cm$^{-1}$, confirming the formation of SiO$_2$. The presence of a weak vibration at 950 cm$^{-1}$ indicates there are also some Si-OH groups in the silicate layer.$^{45}$ All these results support the formation of hybrid siliceous fluorescent vesicles (HSFVs). Notice that the IR spectra shows an obvious peak corresponding to carbonyl amide group (1640 cm$^{-1}$) for the HSFVs prepared with TEOS/FA-APTS (1:1) (which is denoted as FA-HSFV in Figure 2), whereas this peak is absent for those obtained from mere TEOS, indicating the successful grafting of folic acid on the hybrid FA-HSFVs.
Figure 2  A-E) TEM images of HSFVs made with [TEOS] = 22.4μL/mL (A), [TEOS] = [FA-APTS] = 11.2μL/mL (B), [TEOS] = 6.72μL/mL (C), [TEOS] = 8.95μL/mL (D), and [TEOS] = 56.0μL/mL (E), scale bar= 50 nm.  (F) EDX result of the HSFV.  (G) FT-IR spectra for the HSFVs/FA-HSFVs (red line). The blue and black lines are the FT-IR spectra for folic acid (FA) modified HSFV (FA-HSFV) and the FA, respectively.  (H) Fluorescent spectra of TPE-BPA@8CTAB solution and the suspension of HSFVs/FA-HSFVs. Inset in (H): photo of centrifuged sediments (left) and suspension in water (right) of HSFVs under 365 nm UV lamp.

The silication of the supramolecular vesicles has remarkable influence on the fluorescence (Figure 2H). The emission peak of the HSFVs (467 nm) and FA-HSFVs (458 nm) is blue shifted compared to that for the native TPE-BPA@8CTAB vesicles (479 nm), suggesting that deposition of SiO₂ on the TPE-BPA@8CTAB vesicle has strongly twisted the TPE-BPA molecules. It is possible that the polar outer layer of the TPE-BPA@8CTAB vesicles (including the CTA⁺, COOH and COO⁻ groups) has
interacted with the polar groups from the silica source generated in the process of hydrolysis of TEOS or FA-APTS. Both hydrogen bonding and electrostatic interaction may occur, which changes the configuration of the TPE-BPA molecules in the hydrophobic domain of the vesicles. Obviously, the introduction of FA-APTS to the silica shell has twisted the TBE-BPA molecules to a stronger extent, which is featured by the much bluer emission. However, FA-APTS doesn’t affect the stability of the HSFVs. The size and morphology for both the bare HSFVs and FA-HSFVs are not changed even after one year, revealing their superior stability (Figure S6). Furthermore, the macroscopic appearance of the FA-HSFVs (and also the bare HSFVs without folic acid modification) is powder after drying the sediments, which is very convenient for their storage and further application.

**Figure 3** CLSM images of cells with nanocarriers: bright field images of HeLa (A) and LO2 (B) cells with FA-HSFVs; fluorescence images of HeLa (C) and LO2 (D) cells with FA-HSFVs. The concentrations of FA-HSFVs were 10 μg/ml. Scale bar = 25 μm. (E) Cytotoxicity of FA-HSFVs with different concentrations in HeLa cells.

The imaging and targeting ability of the FA-HSFVs was investigated by incubating the HeLa cells (human cervical cancer cell line) and LO2 cells (human normal hepatic cell line) in the presence of FA-HSFVs. Blue fluorescence from cytoplasm of HeLa
cells was observed after 24 hours (Figure 3C). In clear contrast, the fluorescence is extremely weak for LO2 cells (Figure 3D), indicating the excellent targeting ability of the FA-HSFVs to cancer cells. Meanwhile, control experiment (Figure S7, the CLSM made with HSFVs) validates that the targeting ability is from folic acid groups, because HSFVs without FA shows only faint fluorescence in HeLa cells (and LO2 cells). Since folic acid receptors are highly expressed on the surface of tumor cells, this distinct targeting ability clearly manifests the successfully grafting of folic acid groups on the surface of the FA-HSFVs.

Cytotoxicity of the unloaded FA-HSFVs was evaluated using a typical (3-4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) colorimetric bioassay. Figure 3E shows that the cell viabilities within 24 h for living HeLa cells are all higher than 90% as the daily doses ranging from 0 μg/ml (control) to 12.5 μg/ml, indicative of negligible cell toxicity of the empty FA-HSFVs. This result is extremely amazing, since CTAB on its own is known to show severe instant cell toxicity. Obviously, the diffusion of CTAB molecules in the hybrid vesicles have been doubly blocked by the silica shell and the ionic self-assembly, which makes the FA-HSFVs quite promising for in vitro diagnostic applications.

To study the drug loading and releasing ability of the FA-HSFVs, Doxorubicin hydrochloride (DOX), a fluorescent dye and water soluble anticancer drug, was loaded into the TPE-BPA@8CTAB supramolecular vesicles. In a typical procedure, the hydrophilic DOX was added into water where the ionic self-assembly of TPE-BPA@8CTAB vesicles to be occur. Then there must be some DOX be entrapped into the vesicles, which would remain there during the following silication process. Then separation of the unloaded DOX from the loaded ones was made simply via centrifugation. Since this facile procedure avoids dialysis, dialysis triggered drug leaking is prohibited so that it allows high DOX loading efficiency of 21~23%. Specially, fluorescence resonance energy transfer (FRET) between DOX and the TPE-BPA molecules was observed before silication (Figure S8, the emission of TPE-BPA is 479 nm, which is in good agreement with the excitation of DOX (477 nm)). Whereas the phenomenon of FRET disappeared after depositing silica on the
TPE-BPA@8CTAB vesicles to fabricate HSFVs/FA-HSFVs (Figure S8), which can be explained by the mismatch of the dipole orientation of the TPE-BPA and DOX caused by the silication triggered twisting of the TPE-BPA molecule.

**Figure 4 A)** DOX releasing profile of HSFVs with different amounts of TEOS at pH=5.5, 37°C. The releasing efficiency can be over 80% after 10 min’s sonication. The legends represent the amount of TEOS added during the preparation of HSFVs. [TEOS]= 6.72 μL/mL, 8.95 μL/mL, 22.4 μL/mL and 56.0 μL/. **B)** Cell viability of HeLa Cells incubated with DOX loaded FA-HSFVs over time. The cell viability decreases with lowering the concentration of the silica source. The concentration of FA-HSFVs is 10 μg/ml. The legends represent the amount of TEOS added during the preparation of DOX-loaded FA-HSFVs. [TEOS] = [FA-APTS] = 28.0 μL/mL, 11.2 μL/mL and 3.36 μL/mL, T = 37°C.

Significantly, the releasing rates of DOX from the HSFVs are closely related to the wall-thickness (Figure 4A). The thicker the wall is, the slower the releasing rate becomes. For systems prepared with the concentration of TEOS being 6.72 μL/mL, 8.95 μL/mL, 22.4 μL/mL, and 56.0 μL/mL, the corresponding wall-thickness of HSFVs is 6.5±1.2 nm, 8.3±1.3 nm, 9.9±2.9 nm and 12.1±4.2 nm, respectively. In line with the increased wall-thickness, the time required to reach the releasing equilibrium also increases. Figure 4A displays that the releasing equilibrium can be reached within 2.8 h, 6.0 h, 8.8 h, and 11.8, respectively.

The overall fraction of released DOX from the HSFVs displays pH dependent behavior. At pH 5.5, about 40~50% DOX was released at equilibrium (Figure 4A),
whereas it lowered to 35~45% at pH 7.0 (Figure S9). This is mainly due to the increased hydrophilicity and solubility of DOX at higher pH, because the –NH$_2$ groups on DOX can be protonated at acidic pH, which increases the solubility of DOX.$^{56}$ Since pH 5.5 is often the internal environment of cancer cells,$^{57}$ the present results indicate that the HSFVs have higher releasing rate in cancer cells. Usually, the released drug from a nanocarrier is below 70% due to the interaction of drugs with nanocarriers.$^{58}$ However, in the present study, the released DOX can be further promoted to over 80% upon imposing pulse of sonication for 10 minutes. This sonication enhanced releasing rate is independent of pH (Figure 4A, blue curve), because breaking of the rigid wall of the HSFVs/FA-HSFVs may occur (Figure 2A). Since sonication has been clinically applied in therapy,$^{59}$ the FA-HSFVs represent a novel family of highly efficient releasing system.

Figure 4B shows the efficiency of DOX loaded in the FA-HSFVs in killing HeLa cell. In general, gradual decrease of the cell viability is observed for systems with different shell thickness, indicating a sustained release of DOX from all these FA-HSFVs. Significantly, the effect of wall-thickness on the drug releasing becomes evident after 8 h. Drastic difference is observed within 24 hours, by then the highest cell-killing rate can amount to 80% for FA-HSFVs with the thinnest wall-thickness of 6.5 ±1.2 nm. In contrast, the killing rates are about 60% for those FA-HSFVs with thicker walls. This result suggests that the present strategy allows design of nanocarriers with desired drug releasing rate for therapy of cancers at different developing stage, which is very useful in view of practical application.

CONCLUSION

In summary, the strategy of build-in functional template directed synthesis of hybrid siliceous vesicles is very promising in facile preparation of multifunctional diagnostics. We show in this work that using the fluorescent vesicles as build-in template, hybrid diagnostics with enhanced cyto-compatibility, sufficient fluorescence, efficient targeting, and facile drug loading can be obtained through a one-pot
approach. Since different functions are expressed at the supramolecular part and the silica part of the hybrid vesicles, respectively, it allows facilely integration of multiple desired functions at one platform without lowering their efficiency. We expect that the strategy of build-in functional template directed synthesis of siliceous nanoparticles opens a new vista in the field of precision medicine.

EXPERIMENT SECTION

1.1 Materials

TPE-BPA was synthesized according the previous report.\textsuperscript{43} Cetyltrimethyl ammonium bromide (CTAB) was used after recrystallized for five times. Tetraethoxysilane (TEOS, AR) was purchased from Xilong Chemical Co., Ltd. Folic acid (FA, >97%) was purchased from Sigma-Aldrich. 3-aminopropytriethoxysiliane (APTES, >98%) and N-hydroxysuccinimide (NHS) were purchased from Tokyo Chemical Industry Co., Ltd. N,N’-dicyclohexylcarbodiimide (DCC) was purchased from Sinopharm Chemical Reagent Co., Ltd. DMSO-d6 (99.9%) was purchased from Cambridge Isotope Laboratories, Inc. Aqueous solutions were prepared using Milli-Q water of 18 MΩ.

Synthesis of folic acid-modified (3-aminopropyl)triethoxysilane (FA-APTS)

(The $^1$H NMR experiments were performed on a Bruker ARX 400 MHz spectrometer with DMSO-d6 as solvent. Mass spectrum was performed on Bruker APEX IV Fourier Transform Ion Cyclotron Resonance Mass Spectrometer.)
**Scheme S1** Synthesis of folic acid-modified (3-aminopropyl)triethoxysilane (FA-APTS).

Folic acid (0.40 g, 0.91 mmol) was dissolved in 50 mL DMSO under 35°C. N-hydroxysuccinimide (NHS, 0.208 g, 1.81 mmol) and N,N'-dicyclohexylcarbodiimide (DCC, 0.372 g, 1.80 mmol) were added and reaction was allowed in the dark under 35°C for 48h. After floating objects (N,N'-dicyclohexylurea,DCU) being filtered from reaction system, trimethylamine(0.183 g, 1.81 mmol) and 3-aminopropyltriethoxysiliane (APTS, 0.400 g, 1.88 mmol) were added into the filtrate. The reaction was allowed for 8 h, and system was filtered to obtain clear yellow solution. Solid product was obtained by adding acetonitrile into DMSO (DMSO: acetonitrile=1:2, volume ratio). Then product was washed by acetonitrile for 2 times, and dried in vacuum to give yellowish-white powder (FA-APTS, yield 20%).

$^1$H-NMR(400MHz, DMSO-d6): 8.64(s,1H), 7.62(s, 2H), 6.89(s,N-1H), 6.61(s,2H), 4.47(s,2H), 4.35(s,1H), 3.71(m,6H), 2.33(s,2H), 2.08(s,2H), 1.40(s,2H), 1.09(t,9H), 0.52(s,2H). 2H were missing due to overlay with DMSO residue signal around 2.5.

High resolution MS (negative mode): 643.26479. Molecular formula C$_{28}$H$_{40}$N$_8$O$_8$Si, ion formula C$_{28}$H$_{39}$N$_8$O$_8$Si [M-H], err 2.8 ppm.

**Preparation of HSFVs and FA-HSFVs**

Fluorescent vesicles were obtained by mixing TPE-BPA and CTAB in water with molar ratio of 1:8 (100 µM: 800 µM), where the pH was 5.6±0.1, and then vesicle solution was kept for 2 days at 25°C. TEOS (100 mM) was added into vesicle solution and the mixture was violently stirred for 24h at 30°C to give whitish fluorescent suspension. Upon centrifugation at a speed of 10000 rpm for 15 min, fluorescent sediments (HSFVs) were obtained, which were washed for three times before use. The preparation for FA-HSFVs is the same as HSFVs except that only TEOS (100 mM) was replaced with the mixture of TEOS (50 mM)+FA-APTS (50mM). The
silicate vesicle sediments are yellow in this case. The shell thickness of HSFVs or FA-HSFVs was related to the amount of TEOS or the mixture of TEOS and FA-APTS.

1.2 Characterization of HSFVs and FA-HSFVs

Scanning electron microscopy (SEM) was performed on a Hitachi S4800 microscope. Transmission electron microscopy (TEM) images were recorded with a JEM-2100 instrument at an acceleration voltage of 200 kV. For SEM and TEM measurements, a drop of suspension (dispersed into distilled water) was placed on clean silicon sheets and carbon-coated copper grids and dried in the air, respectively.

Cryogenic transmission electronic microscope (Cryo-TEM) observation was performed on EM922 EFTEM (Zeiss NTS GmbH, Oberkochen, Germany). A few microliters of samples were placed on a bare copper TEM grid (Plano, 600 mesh), and the excess liquid was removed with filter paper. This sample was cryo-fixed by rapidly immersing into liquid ethane cooled to -170 to -180 °C in a cryo-box (Carl Zeiss NTS GmbH). The specimen was inserted into a cryo-transfer holder (CT3500, Gatan, Munich, Germany) and transferred to a Zeiss EM922 EFTEM. Examinations were carried out at temperatures around -180 °C. The TEM was operated at an acceleration voltage of 200 kV. Zero-loss filtered images were taken under reduced dose conditions (500-2000 e/nm²). All images were recorded digitally by a bottom-mounted CCD camera system (UltraScan 1000, Gatan) and processed with a digital imaging processing system (Digital Micrograph 3.9 for GMS 1.4, Gatan).

Ultraviolet-visible (UV-vis) spectral measurements were performed on a Shimadzu UV-1800 spectrophotometer. Fluorescence (FL) measurements were carried out using a Hitachi F-4500 instrument. Fourier transform infrared (FT-IR) spectra were recorded with a Bruker Vector-22 spectrophotometer. Fluorescence images were recorded on Ti-S Nikon inverse fluorescence microscopy. Energy dispersive X-ray (EDX) measurements were performed on FEI Tecnai G2 T-20 transmission electron microscope. Quantitation method is Cliff Lorimer thin ratio section. All elements were analyzed (Normalised).
1.3 Cell experiments

Cell culture

HeLa cell lines, were generous gifts, courtesy of Yujie Sun, (Peking University, Beijing, China), routinely cultured in Dulbeccoo’s modified Eagle’s medium (Gibco, Grand Island, NY, USA) supplemented with 10% (v/v) fetal bovine serum (Hyclone, Logan City, UT, USA), 100 U/mL of penicillin, and 100 μg/mL of streptomycin (Gibco, Grand Island, NY, USA) at 37°C in a humidified incubator containing 5% CO₂. LO2 cell lines were generous gifts, courtesy of Junlong Zhang, (Peking University, Beijing, China), routinely cultured in RPMI-1640 (Gibco) supplemented with the same components as described above.

Cell imaging

HeLa cells or LO2 cells were grown overnight in 35 mm Petri dishes. The nanocarriers (HSFVs, FA-HSFVs) were respectively transferred into the medium, immediately incubated with cells at 37°C at a dose of 10 μg/ml. Include wells with cells without test compound (but with equal proportion of sterile PBS) as controls. After a 24-hour incubation, the cells were imaged under an inverted fluorescence optical microscope (Nikon, ECLIPSE Ti; λ<sub>ex</sub> = 405 nm for nanocarriers; fluorescent signals were collected at 405 nm). The images of the cells were captured using a photomultiplier.

Cytotoxicity assay of nanocarriers in vitro

MTT assay was employed to measure the cell viability. As an indicator system of cytotoxicity, MTT assay has been reported to exhibit equal or even higher precision than [³H] thymidine incorporation assay.⁶⁰⁻⁶¹ As the intensively colored formazan sediments metabolized by MTT clearly reflect cell respiration, the amount of sediments is proportional to the level of cell viability. Therefore, cytotoxicity can be measured by obvious differences in quantified cell survival. 5×10³ HeLa cells were seeded into a 96-well plate per well, which were incubated overnight at 37°C in an
5% CO₂ atmosphere. Then various doses of 0-12.5 μg/ml unloaded carriers (i.e. FA-HSFVs) were transferred into the aforementioned medium, immediately incubated at 37°C. Include wells with medium without cells or nanoparticles, cells without test compound (but with equal proportion of sterile water) were used as controls. After 24 h incubation, 10 μL of MTT solution (5 mg/mL in PBS) was added into each well. After further incubating for 4 hours, 150 μl of DMSO was added to dissolve the formazan sediments. The cell survival rate was quantified by the optical density readings at 490 nm taken with a plate reader. Every experiment was performed 6 times.

1.4 Doxorubicin loading and cell viability assay in vitro

Doxorubicin (DOX)-loaded HSFVs and FA-HSFVs was prepared by a one-pot procedure: TPE-BPA, DOX and CTAB (100 μM: 100μM: 800 μM) were mixed together in water at pH = 5.6 at 25°C and the solution was kept at 25°C in dark for 48 h. Then TEOS or the mixture of TEOS and FA-APTS were added. After violently stirred for 24 h at 30°C, red suspension were obtained. Upon centrifugation at 10000 rpm for 15 min, red solids, which are the DOX loaded HSFVs or FA-HSFVs, were obtained. Then the solids were washed with water for several times to remove the adsorbed DOX. UV-Vis spectra were used to track whether the adsorbed DOX has been removed entirely. All the experiments were carried in dark to avoid photo damage of DOX.

The encapsulation percentage of DOX was determined with UV-Vis scanning spectrophotometry at 480 nm in water according to the calibration curve of different DOX concentrations. The DOX loading efficiency = (amount of loaded DOX /amount of total DOX) ×100%. For DOX-loaded HSFVs, the loading efficiency was 19.1%, 21.3%, 19.1% and 16.0% corresponding to TEOS of 6.72 μL/mL, 8.95 μL/mL, 22.4 μL/mL and 56.0 μL/mL, respectively. For DOX-loaded FA-HSFVs, the loading efficiency was 22.7%, 21.8% and 21.2% corresponding to TEOS+ FA-APTS of 6.72 μL/mL, 22.4 μL/mL and 56.0 μL/mL, respectively (The molar ratio of TEOS: FA-APTS = 1:1).
In cell viability assay, the DOX-loaded FA-HSFVs with different thickness were transferred into the medium, immediately incubated with HeLa cells at 37°C at a dose of 10μg/ml. Include wells with medium without cells or nanoparticles, cells with equal proportion of sterile water without test compound as controls. After 4h, 8h, 24h, the cell viability was tested by MTT assay, as described above. Each assay was repeated 12 times.

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website.

SEM images of HSFVs, TEM images of FA-HSFVs, Preparation conditions of HSFVs, Stability of HSFVs and FA-HSFVs, Thickness analysis of HSFVs and so on.

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20


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