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A nanoencapsulated hypocrellin A prepared by an improved microemulsion method for photodynamic treatment

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Abstract A new hypocrellin A (HA) encapsulated silica nanoparticles was prepared by an improved microemulsion method based on the unique character of cetyl trimethyl ammonium bromide (CTAB). Stable aqueous dispersions of the HA-loaded nanoparticles, with the diameter about 50 nm, owned superior photo-stability and singlet oxygen generation ability to free HA. In vitro studies demonstrated the active uptake of HA-doped nanoparticles into the cytosol of HeLa (human cervix epithelioid carcinoma) cells. Significant morphology change and phototoxicity to such impregnated tumor cells was observed upon irradiation with light. Thus, the potential of using this method to prepare silica nanoparticles as drug carriers for photodynamic therapy has been demonstrated.

1 Introduction

Photodynamic therapy (PDT) has been developed for cancer treatment over the last 40 years. It has been expanded as an emerging modality for the treatment of various cardiovascular, dermatological and ophthalmic diseases [1, 2]. This treatment involves the administration of a photosensitizer and activation by irradiation. After a time-interval to allow the photosensitizer to accumulate in the tumor tissue, the irradiation of the tumor site with light leads to the formation of an excited photosensitizer. The combined action of the excited triplet photosensitizer and molecular oxygen results in the formation of singlet oxygen ($^1O_2$), which is thought to be the main mediator of cellular death induced by PDT [3, 4].

As clearing of the photosensitizer is often a limiting factor in PDT, the studies of most photosensitizers have been receiving increasing attention. But span both fundamental and applied research illustrated that preparation of pharmaceutical formulations for parenteral administration is highly hampered because of their poor water solubility. So advances in the use of nanoparticles including inorganic oxide-, metallic-, and polymer-based nanocomposites as photosensitizer carriers are highlighted [5]. The systemic administration result showed that such drug-doped carriers are made to their aqueous dispersion and preferentially taken up by tumor tissues by virtue of the enhanced permeability and retention effect [6–8]. And among above systems, there is an increasing interest in developing silica nanoparticles as carriers for drug delivery due to their excellent properties and distinct suitability to PDT. Silica nanoparticles are highly stable and may not release any encapsulated bimolecular, but their porous matrix is permeable to molecular as well as singlet oxygen. Therefore, the desired photodestructive effect of the drug will be maintained even in the encapsulated form [9].

Microemulsion method, a surfactant based way, is an effective silica nanoparticle preparation way [10–13] and already be used in PS encapsulation. Upon systemic administration, such drug-doped carriers are preferentially taken up by tumor tissues by virtue of the "enhanced

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permeability and retention effect", which is the property of such tissues to engulf and retain circulating macromolecules and particles owing to their "leaky" vasculature [9, 14–18]. A very important step in microemulsion method is the surfactant removal procedure because the residual surfactants tend to increase the systemic toxicity of the drug formulation and surfactants always remove by dialyzing against water in a cellulose membrane for 48 h or longer [9, 14–16]. Ultrafiltration [17] is a simple and effective method to remove surfactant but this procedure increase the demanding of equipment and cost.

To solve this problem, we prepared silica nanoparticle by microemulsion method and chose cetyl trimethyl ammonium bromide (CTAB) as surfactant but not common used Aerosol-OT, tween-80 or triton X-100. CTAB can easily solve in water at room temperature (25°C) but rapidly separated and precipitated by crystallization at the temperature lower than 15°C. After silica nanoparticle was prepared at room temperature, it was putted into refrigerator at 4°C for 4 h and most of CTAB was separated and precipitated. After that, very short time dialyzing against water (about 12 h) can remove the residual small quantities of CTAB due to its electropositivity, which was same with N^+ of silica nanoparticles hydrolyzed and polymerized from amino silane [18, 19].

For demonstrating this method, we prepared and characterized of hypocrellin A encapsulated silica nano-carriers using the improved microemulsion method. Hypocrellin A is an effective PS owning the potential for photodynamic therapy (PDT) of cancer, premalignant conditions and several types of viruses, including the human immunodeficiency virus (HIV) due to their high reactive oxygen species (ROS) generation ability [20–22]. In addition, the morphology and size, optical properties, singlet oxygen production, light-stability and PDT efficacy to cancer cell in vitro was discussed.

2 Materials and methods

2.1 Chemicals

Triethoxyvinylsilane (TEVS), 3-aminopropyl-triethoxysilane (APTES) and 9,10-anthracenedipropionicacid were purchased from Sigma. Dulbecco's minimum essential medium (DMEM) was from Gibico. 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyl tetrazolium bromide (MTT) was from Amosco. All other chemicals were of analytical grade.

2.2 Synthesis and characterization of hypocrellin A-loaded silica nanoparticles

The nanoparticles were prepared in the nonpolar core of CTAB/1-butanol/water micelles. In a typical experiment, 0.3607 g CTAB and 800 μl 1-butanol were dissolving in 20 ml double distilled water by vigorous magnetic stirring to get a clear micelles solution and 60 μl HA in DMF (15 mM) was dissolved by magnetic stirring in the resulting clear solution. Then triethoxyvinylsilane (200 μl) was added to the above system, and the resulting solution was stirred until it became clear. After that, 3-aminopropyl-triethoxysilane (10 μl) was added and the system stirred for about 20 h. Finally, the resulted solution was putted into refrigerator and maintained at 4°C for 4 h and then supernatant fluid was dialyzed against water with a cellulose membrane (12–14 kDa molecular weight cut-off) for 12 h to completely remove 1-butanol and the residual CTAB. As a contrast, HA was dissolved in water using DMF as latent solvent. Transmission electron microscopy (TEM) images were obtained on a FEI-Tevnai G220 S-TWIN electron microscopy, equipped with an EDAX 32 energy disperse spectroscopy (EDS), with an acceleration voltage of 200 kV. The absorbance spectra were recorded on a VARIAN CARY 5000 spectrophotometer in a quartz cuvette with a 1 cm light path. Fluorescence spectra were measured by a Perkin-Elmer LS-50B fluorometer with an excitation wavelength of 480 nm. Fourier transforms infrared (FTIR) absorbance data were acquired with a Nicolet Nexus 670 FT-IR infrared spectrometer.

2.3 Light stability

The air-saturated solution of HANP or hypocrellin A was illuminated in a 1 cm cuvette by a high pressure mercury lamp (500 W) and its UV–Vis spectra from 350 to 800 nm were recorded every minute.

2.4 Singlet oxygen detection

Chemical oxidation of ADPA in the aqueous suspension of the nanoparticles was used as a method to characterize singlet oxygen generation efficiency [23]. In a typical experiment, aqueous solutions of ADPA (150 μl, 5.5 mM) were mixed with HA-loaded silica nanoparticle or free HA (3 ml). In this case, a decrease in the absorbance of the ADPA added to the aqueous suspensions of the nanoparticles or free HA was monitored as a function of time, following irradiation with a 500 W high-voltage mercury lamp with 470 nm cutoff filter.

2.5 In vitro studies with tumor cells: nanoparticle uptake and PDT assay

For studying nanoparticle uptake and imaging, HeLa (human cervix epithelioid carcinoma) cells were cultured in a Dulbecco's minimum essential medium (DMEM) supplemented with 10% fetal bovine serum (FBS), following

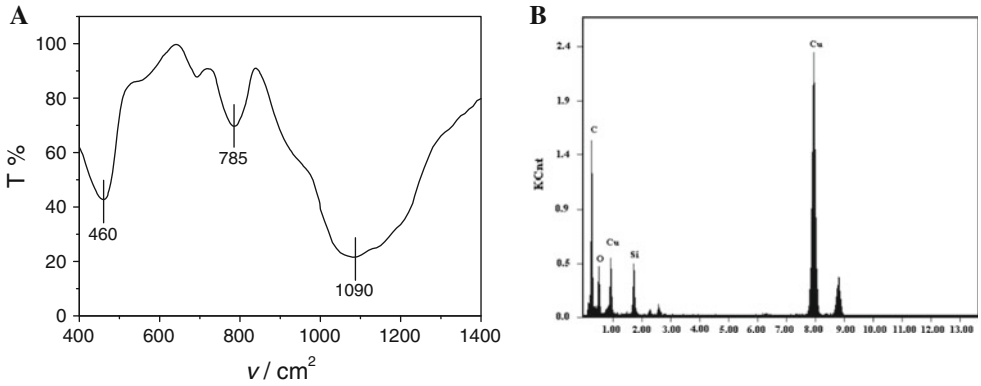

Fig. 1 **a** The FTIR spectra of HA-loaded silica nanoparticles; **b** the EDS spectra of HA-loaded silica nanoparticles

the established protocol [24, 25]. HA-loaded silica nanoparticles were added to the cell, seeded in a 6-well culture plate with 50% confluence in FBS free culture, and the treated cells were returned to the incubator (37°C, 5% CO_2) for 45 min. After incubation, the plates were rinsed with sterile phosphate-buffered saline (PBS) and directly imaged using a Zeiss fluorescence microscope.

For studying PDT efficacy through cell viability, cells were seeded in a 96-well culture plate until 80% was confluence in DMEM supplemented with 10% FBS. Then the culture was change into FBS free DMEM and HA-loaded silica nanoparticle or free HA was added. After incubation 24 h in the incubator (37°C, 5% CO_2), cells were rinsed with sterile PBS and irradiated by a 500 W high-voltage mercury lamp with 470 nm cutoff filter. Followed another 24 h incubation, cell viability was estimated by means of the colorimetric MTT assay [26–28].

3 Result and discussion

3.1 *CTAB complete removal* verification

The FTIR absorbance spectrum of the HA-loaded silica nanoparticles, presented in Fig. 1a, showed the well-established characteristic bands. The characteristic bands of Si–O–Si vibration at about 460, 785 and 1100 cm^{-1} were observed [29]. But the C–N bond of CTAB from 800 to 1,100 cm^{-1} was not observed, which illustrated that CTAB was completely removed. In order to get more evidences to support this verify, the EDS spectrum was collected and the data were analyzed. From Fig. 1b, it can be observed that no Br peak was detected from 1 to 2 keV [30], which further demonstrated that our method to remove CTAB was successful.

3.2 TEM assay

It was suggested that the size of nanoparticles was important for PS delivery system because the lifetime of $^1O_2$ in aqueous media is very short and during this interval

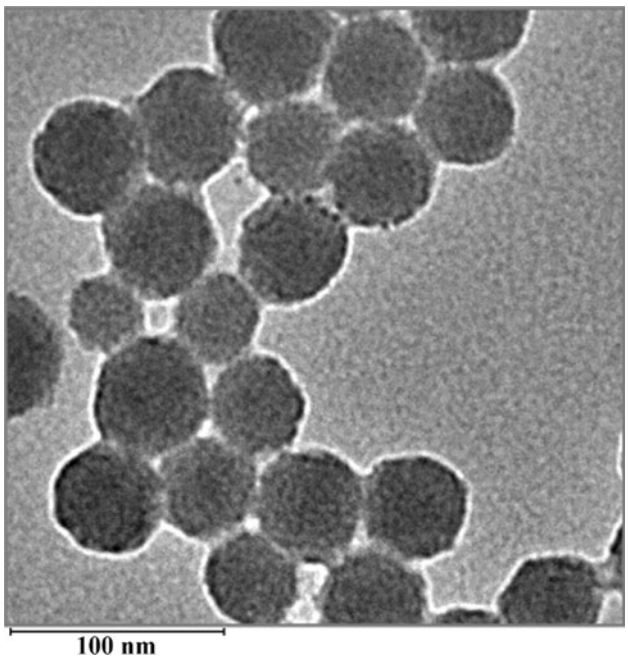

Fig. 2 TEM image of HA-loaded silica nanoparticles. *Scale bar* = 100 nm

$^1O_2$ can diffuse over a mean radical distances of about 100 nm. The size of HA-loaded silica nanoparticles was studied by TEM and the result showed that the particles were spherical, having uniform size distribution, with an average size of 50 nm (Fig. 2).

3.3 *Encapsulation efficacy* verification

HA has a characteristic emission fluorescence peak at about 600 nm; after encapsulation, the peak form and position were maintained but the peak intensity was greatly improved (Fig. 3A). The fluorescence intensity of HA (nonpolar drug) can be easily quenched in water (polar solvent) because either drug-solvent interaction promoting nonradiative decay or concentration quenching derived from self-aggregation of HA. But after encapsulation, the drug molecules can be protected from exposure to the aqueous environment

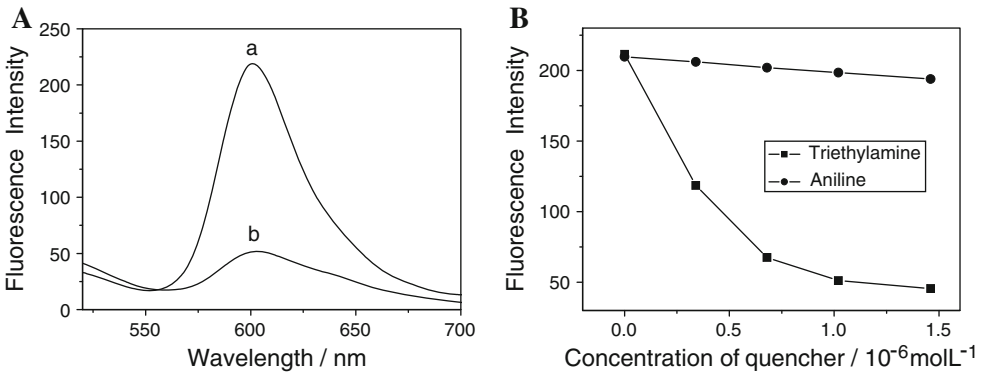

Fig. 3 **A** The emission fluorescence spectra of HA (*a*) and HA-loaded silica nanoparticles (*b*); **B** the fluorescence quenching of HA-loaded silica nanoparticles by aniline and triethylamine

by silica nanoparticles, preventing a complete loss of fluorescence. In addition, the intermolecular hydrogen bonding between –OH of HA and NH_2– of AEAPS could prevent the fluorescence quenching caused by the self-aggregation of HA molecules. This result indicated that HA was successfully encapsulated into silica nanoparticles.

In order to get more evidence to support this result, fluorescence quenching experiment was carried out. Different diameter of effective HA fluorescence quenchers, aniline and triethylamine, were used to determine whether HA was inside the silica nanoparticles. The fluorescence quenching experiment indicated that the small quencher (triethylamine) can effectively quench the fluorescence of the encapsulated HA but the big quencher (aniline) can not (Fig. 3B), which illustrated that HA was inside the particles and triethylamine can enter the particle through the micro-holes on the particle but the diameter of the hole was not big enough for aniline crossing.

3.4 Photo-stability

Direct comparisons of the light-stability of free HA and a HA-loaded silica nanoparticle was demonstrated by exposing the samples continuously for a span of time using a high-voltage mercury lamp. The insert panel of Fig. 4 showed the decrease of HA-loaded silica nanoparticles absorption intensity as a function of the irradiation time. After 5 min irradiation, 19.56% free HA was photobleached but only 8.72% was bleached after been encapsulated. The result indicated that after encapsulation, most photosensitizers could maintain their integrity under laser irradiation as compared to the case of free HA. The protection by silica nanoparticle offers better photo-stability of HA against bleaching [31].

3.5 Singlet detection by ADPA

ADPA is converted to an endoperoxide by singlet oxygen, so this reaction has been used as a test for the presence of singlet oxygen. The absorbance intensity of ADPA decreased as the irradiation time increased, which indicates that the amount of singlet oxygen production increased when HA-loaded silica nanoparticles were irradiated by light (Fig. 5 Insert panel). Figure 5 showed the decrease in absorption intensity at 378 nm of HA and HA-loaded silica nanoparticles as a function of the

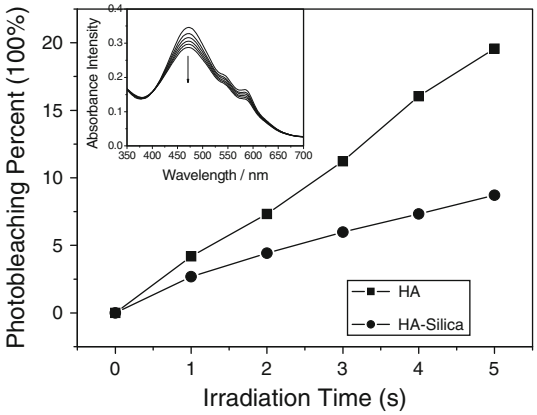

Fig. 4 Decay curves of absorption at 472 nm of free HA and HA-loaded silica nanoparticles as a function of irradiation time (*insert panel*: the photo-bleaching effect of HA-loaded silica nanoparticles by irradiation)

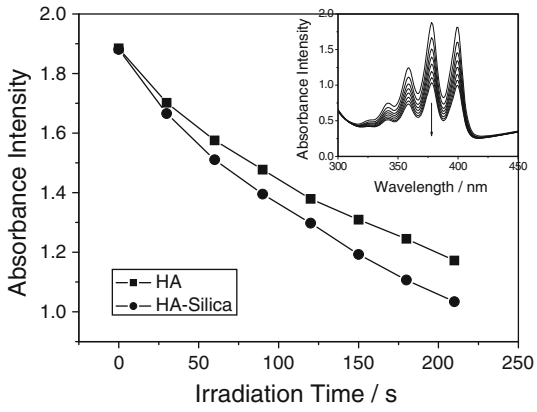

Fig. 5 Decay curves of absorption of APDA at 378 nm caused by HA and HA-loaded silica nanoparticles (*insert panel*: absorbance spectra after irradiation of HA-loaded silica nanoparticles)

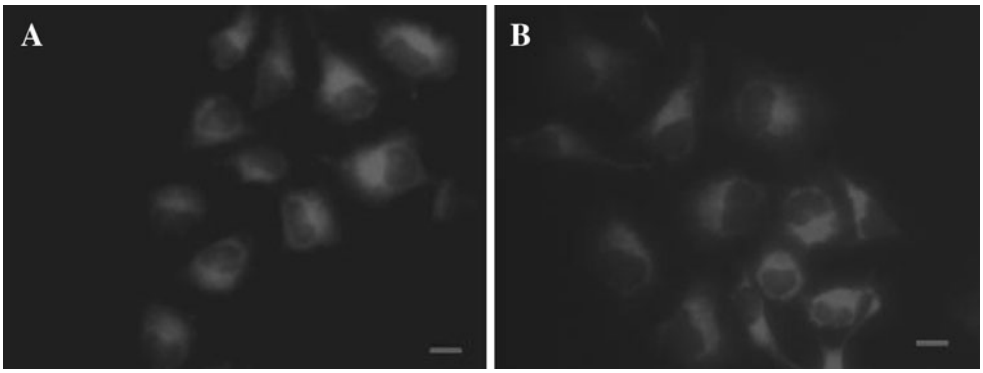

Fig. 6 Fluorescence image of HeLa cells treated with HA (**a**) and HA-loaded silica nanoparticles (**b**). ([HA] in silica nanoparticles was 5 μM). *Bar*: 20 μm

exposure time under irradiation. The slope of the curve is roughly proportional to the efficiency of generated singlet oxygen. Thus, we conclude that singlet oxygen production efficiency of HA was improved by encapsulated into silica nanoparticles. The higher $^1O_2$ generation ability of HA-loaded silica nanoparticles may find potential application in PDT.

3.6 Nanoparticle uptake and in vitro PDT

HA, using 0.05% DMSO (v/v) as latent solvent, can be taken up by cells and showed fluorescence after excitation by light (450–490 nm) (Fig. 6a). To determine the uptake of HA-loaded silica nanoparticles on intracellular drug delivery, we studied accumulation of nanoparticles. The fluorescence images of HA-loaded silica nanoparticles treated HeLa cells excitation by light (450–490 nm) show significant intracellular staining in the cytoplasm (Fig. 6b), indicating active uptake and accumulation of HA-loaded silica nanoparticles.

Light induced cytotoxic effect with nanoparticles encapsulating HA is shown in Fig. 7. HeLa cells treated overnight with nanoparticles containing HA did not cause any significant change in cell morphology, indicating low dark toxicity from the particles. On the contrary, drastic change in the morphology of HeLa cells, which was treated overnight with HA-loaded silica nanoparticles, have been observed after irradiation (Fig. 7a, b). The negative control of phototreatment of cells without HA-loaded silica nanoparticles did not cause any significant change in cell morphology (Fig. 7c, d).

Furthermore, the dark-toxicity before irradiation was studied to evaluate the biocompatibility of the nanoparticles and the results showed that the IC_{50} of HA and HA-loaded silica nanoparticles were 12.08 and 12.87 μmol/l (the concentration of encapsulated HA), which improved the good biocompatibility of silca nanoparticles. In order to determine the photodynamic effect of encapsulation of HA in nanoparticles, HeLa cells were exposed to light following treatment with free HA or that encapsulated in nanoparticles. As can be seen from Fig. 8, encapsulation of HA in nanoparticles enhanced the tumor cell kill over the dose range studied.

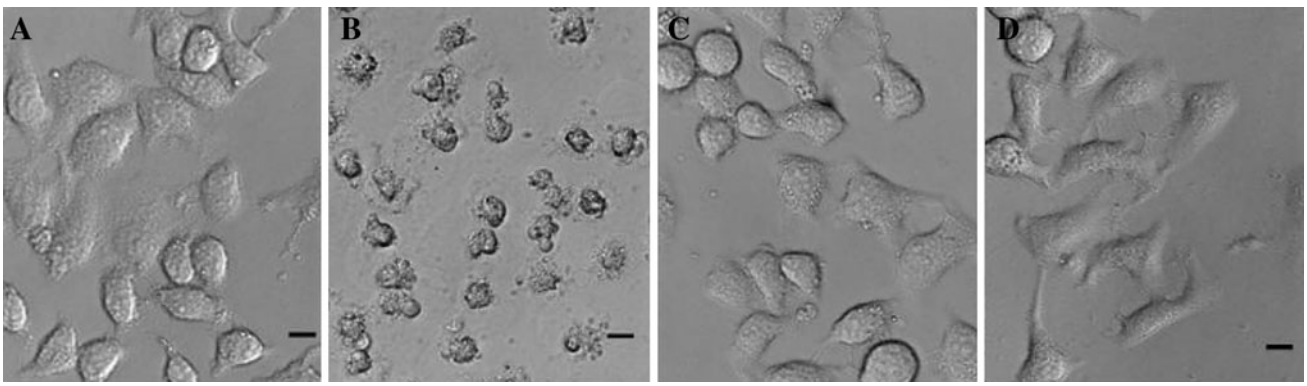

Fig. 7 Transmission images of HeLa cells treated overnight with HA-loaded silica nanoparticles (**a**), the cells after irradiation under the same conditions (**b**) and the morphology of the cells, without HA-loaded silica nanoparticles, before (**c**) and after phototreatment (**d**). *Bar*: 20 μm

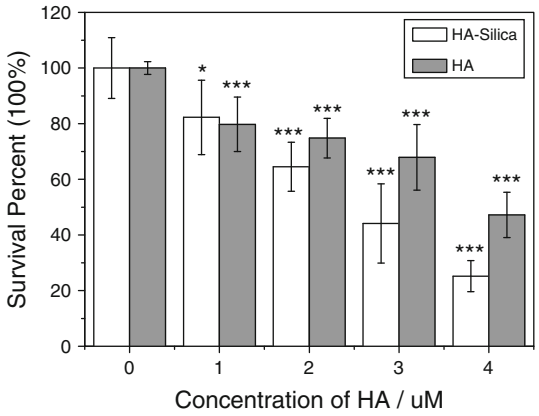

Fig. 8 Percentage of cell survival of Hela cells, after treatment with HA or HA-loaded silica nanoparticles and subsequent irradiation by light (2 J/cm^2) (* $P < 0.05$ vs. control; *** $P < 0.001$ vs. control; control: cells were irradiated but untreated cells as having 100%)

4 Conclusions

Ultrafine HA-loaded silica nanoparticles have been synthesized by microemulsion method with an improved surfactant removal procedure based on the unique character of CTAB, which is precipitation by crystallization at the low temperature and same charge with silica nanoparticles hydrolyzed and polymerized by amino silane. Using this method, the surfactant removal procedure can be effectively carried out with simple device but shorten the time comparing with traditional method, which was verified by EDS and FTIR spectra. The HA-loaded silica nanoparticles are uniform in size distribution with an average diameter of 50 nm, and can be formulated as a stable dispersion for a long time. The result HA-loaded silica nanoparticles own improved light-stability and can be actively taken up by cancer cells. The significant improved light -induced cytotoxicity were observed, which is most likely due to the superior singlet oxygen generation ability of HA-loaded silica nanoparticles to free HA. Based on these results, we concluded that the HA-loaded silica nanoparticle system is a promising modality for anticancer PDT.

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