



Targeted H⁺-Triggered Bubble-Generating Nanosystems for Effective Therapy in Cancer Cells



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ARTICLE INFO

Article history:

Received 18 May 2017

Received in revised form

10 September 2017

Accepted 12 September 2017

Available online 14 September 2017

Keywords:

Doxorubicin

Folic acid

Polydopamine

Bubble-generating nanosystem

ABSTRACT

A novel targeting drug delivery system for cancer therapy based on H⁺-triggered bubble-generating nanosystems (BGNSs) was engineered. First, hollow mesoporous silica nanoparticles (HMSNs) were used to load doxorubicin (DOX). Then, the obtained drug-loaded HMSNs were treated with NaHCO₃ to prepare the BGNSs. The BGNSs were coated with polydopamine (pDA), and finally, folic acids (FA) were anchored on the nanosystems to obtain the desired nanoscale drug delivery system (BGNSs@pDA-FA). BGNSs@pDA-FA was effectively internalized by cancer cells through folate receptor-mediated endocytosis and generated CO₂ bubbles under the acidic environment of the lysosomes, thus enhancing lysosomal membrane permeability (LMP) to release caspase-3 into the cytoplasm, resulting in cancer cell death via an apoptosis-like pathway. Notably, we demonstrated that the BGNSs@pDA-FA exhibited a significant simultaneous synergistic cytotoxicity against MCF-7 cells and remarkably overcame the multidrug resistance (MDR) of MCF-7/ADR cells. Moreover, compared to free DOX and a nanosystem without FA modification (BGNSs), the BGNSs@pDA-FA induced relatively minor side effects in the MCF-10A cells. Therefore, the results showed that BGNSs@pDA-FA, as a targeted drug delivery system, have a good probability of overcoming the MDR of tumor cells with minor side effects on normal cells.

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1. Introduction

Despite the fact many anticancer drugs have been designed and used, multidrug resistance (MDR) still remains a significant obstacle for treating cancer diseases [1–3]. A series of drug delivery systems were developed to overcome MDR [4–6]. However, normal tissue cells are simultaneously killed during the treatment of cancer, as a side effect of the anti-cancer drugs [7,8]. Hence, the design of a nano-drug delivery system not only with anti-cancer activity but also relatively low side effects on normal cells is confirmed as an extremely urgent need currently. To solve these challenging problems, at the present time, many drug delivery systems have been designed to reduce the toxicity of drugs on normal cells [9,10]. Lysosomes are defined as “suicide bags” [11]. Once lysosomal membrane permeability (LMP) occurs, lysosomal cathepsins are released into the cytoplasm and cause cell death through apo-

ptosis or apoptosis-like pathways [12,13]. Thus, using lysosomes as a potential therapeutic target has attracted increasing attention [14–16].

Among all the nanomaterials, hollow mesoporous silica nanoparticles (HMSNs), which have an excellent biocompatibility, large surface area, large pore volume and adjustable pore size, play an important role in the drug delivery system [17–21] because they can be utilized to regulate the transport of cargo molecules under external stimuli, e.g., temperature, pH and redox conditions [22]. In addition, the surface of HMSNs has the potential to be further modified with targeting agents.

Until now, the application of polydopamine (pDA) has been broadly used as a universal coating for many nanomaterials [23–25]. pDA is widely distributed throughout the human body, with excellent biocompatibility and biodegradability. pDA independently functionalizes surfaces made of virtually all materials [26]. Furthermore, substances containing amino groups or thiol groups directly achieve self-assembly on the pDA-modified surface. Folic acid modification confers the function of target recognition and has the ability to directly or indirectly modify the surface of

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many nanomaterials, such as HMSNs [27–29]. Folic acid has an amino group that can be coupled directly to the surface of the pDA-modified nanomaterials. In addition, folate receptors are expressed on the surface of cancer cells [30,31]. Therefore, folic acid, as a nonimmunogenic receptor ligand, has emerged as an extremely popular targeting agent for targeted drug delivery [32,33]. Gu et al. reported that folate ligands are readily linked to amino groups in amino-modified HMSNs [34]. Rosenholm recounted that folate ligands have the ability to graft on poly(ethylene imine) (PEI)-modified HMSNs [35]. Therefore, it is conceivable that folate-conjugated HMSNs, by integrating a receptor-mediated targeting storage capacity, can significantly enhance drug delivery efficiency and therapeutic efficiency.

We previously synthesized a H^+ -triggered bubble-generating nanosystem (BGNS), which produces CO_2 bubbles in the acidic environment of lysosomes [36,37]. The produced bubbles enhance LMP and subsequent cell death. However, the BGNSs cannot distinguish cancer cells from normal cells due to the lack of targeting agents. Herein, we modified BGNSs with targeting agents through a two-procedure method. First, we coated the BGNSs with pDA and then conjugated them with folic acid via the interaction between pDA and the amino groups of folic acids (Scheme 1). We demonstrated that BGNSs@pDA-FA possessed a high anti-cancer efficiency against MCF-7 and MCF-7/ADR cells and had relatively minor side effects on normal cells.

2. Material and methods

2.1. Materials

All chemicals were used as obtained and without further purification, and ultrapure water (18.2 M Ω), prepared with a Milli-Q system (Millipore, USA), was used in the preparation of all aqueous solutions. Doxorubicin hydrochloride was purchased from Mel-onepharma (Dalian, China). Trimethoxyoctadecylsilane ($C_{18}TMS$), tetraethylorthosilicate (TEOS), folic acid and dopamine hydrochloride was purchased from Aladdin (Shanghai, China). The Cell Counting Kit-8 (CCK-8) was purchased from Dojindo Laboratories (Kumamoto, Japan). Alexa Fluor 488-dextran (10,000 MW) was purchased from Thermo Fisher Scientific Inc. (MA, USA). The Caspase-3 Activity Assay Kit was purchased from Beyotime Institute of Biotechnology (Haimen, China).

2.2. Synthesis of HMSNs

The HMSNs were synthesized according to a previous report with some minor modifications [36,37]. Briefly, the ethanol (71.4 mL), H_2O (10 mL), and ammonium solution (3.14 mL) were mixed and stirred at 30 °C. Then, TEOS (6 mL) was added into the mixture, and the reaction continued for 1 h. TEOS (5 mL) and $C_{18}TMS$ (3 mL) were premixed and added into the above mixture rapidly afterward, and the reaction continued for another 1 h. The obtained product was collected by centrifugation and washed with water until there was no smell of ammonia. The above collected product was dispersed into 100 mL Na_2CO_3 (0.6 mol/L) aqueous solution and was treated by sonication for several minutes. Then, it was etched at 80 °C for 4 h. After collection by centrifugation and washing with water thoroughly, the nanoparticles were calcined at 600 °C for 6 h.

2.3. Preparation of BGNSs

The BGNSs were prepared as described in our previous report work with some minor modifications [36,37]. Briefly, 60 mg of HMSNs was dispersed into a DOX aqueous solution (1 mg/mL, 7.5 mL) and was stirred in the dark for 24 h. After harvesting by

centrifugation, the precipitate was washed with ultrapure water several times, and then, it was dispersed into a $NaHCO_3$ aqueous solution (0.2 mol/L, 10 mL) and was incubated for 12 h. The BGNSs were obtained by centrifugation and were washed with ultrapure water. The above process was repeated 2 times.

To evaluate the DOX loading efficiency, the supernatant was collected, and the residual DOX content was determined using the calibration curve of the DOX standard solutions by the absorbance measurement at 480 nm, and the loading efficiency of DOX was calculated as follows:

$$\frac{\text{Initial amount of DOX} - \text{Residual DOX}}{\text{Mass of nanoparticles}} \times 100\%$$

2.4. Preparation of BGNSs@pDA and BGNSs@pDA-FA

A total of 65 mg of BGNSs was dispersed into PBS (25 mL, 50 mmol), and the pH was adjusted to 8.5. Then, 12.5 mg of dopamine was added into the mixture, and it was incubated in the dark with gentle shaking for various amounts of time (6 h, 12 h, and 24 h). Finally, BGNSs@pDA was obtained by centrifugation and was washed with ultrapure water several times.

The above synthetic BGNSs@pDA was added into PBS (25 mL, 50 mmol), and the pH was adjusted to 8.5. Then, 50 mg of FA were added into the mixture by shaking for 6 h in the dark. BGNSs@pDA-FA was collected by centrifugation and was washed with ultrapure water several times.

2.5. Characterization of nanoparticles

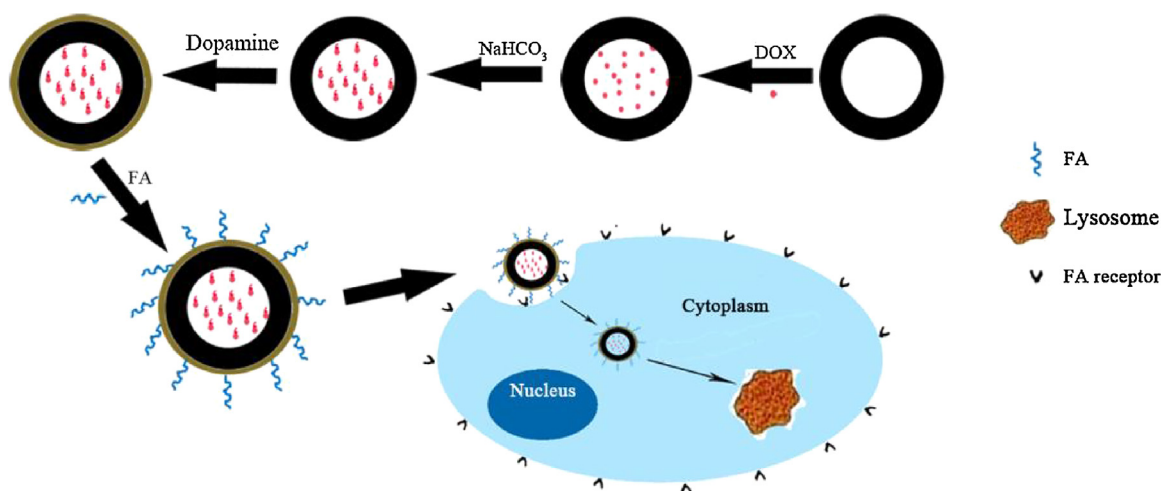
The freshly purified HMSNs, BGNSs, BGNSs@pDA, and BGNSs@pDA-FA were transferred onto a copper grid coated with carbon, dried at room temperature, and were then analyzed by a JEM-1200EX (JEOL Technics, Tokyo, Japan) transmission electron microscope (TEM). The nitrogen adsorption/desorption and the corresponding pore size distribution of HMSNs were measured by Micromeritics instrument corp (ASPS2460, Beijing). The zeta potential of the nanoparticles was measured by a Zetasizer Nano instrument (Malvern, UK). The DOX were quantified using a UV-2450 spectrophotometer (Shimadzu, Japan) at a wavelength of 480 nm.

2.6. Cell culture

The drug-sensitive human breast cancer cell line MCF-7 and its DOX-resistant counterpart MCF-7/ADR cell line were purchased from KeyGen Biotech Co. Ltd. (Nanjing, China). The normal cells, MCF-10A, were purchased from ATCC (Beijing, China). The MCF-7/ADR and MCF-10A cells were all cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) and 100 U/mL of penicillin and streptomycin, and the MCF-7 cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS) and 100 U/mL of penicillin and streptomycin. The three above-mentioned cell types were maintained at 37 °C in a humidified atmosphere with 5% CO_2 .

2.7. in vitro drug release study

A total of 100 μ L BGNSs and BGNSs@pDA-FA (500 μ g/mL) were added into 48-well plates that contained 900 μ L of PBS with different pH values (pH 7.4 and 5.0). The 48-well plates were gently shaken in a thermostatic rotary shaker at 250 rpm. At predetermined time points, the sample was taken out from the 48-well plates for measurement. The concentration of DOX released from BGNSs and BGNSs@pDA-FA was quantified using a UV-2450 spectrophotometer (Shimadzu, Japan).



Scheme 1. Graphical illustration of the preparation of BGNs@pDA-FA and the mechanism of their anti-tumor effect.

2.8. Inhibitory effects on cell proliferation

The MCF-7, MCF-7/ADR and MCF-10A cells were seeded at a density of 6000 cells/well in a 96-well plate and were allowed to attach for 24 h. Then, the growth medium was removed, and the cells were incubated with DOX, BGNs, and BGNs@pDA-FA at a series of concentrations and were incubated for 24 h or 48 h in 5% CO₂ at 37 °C. The CCK-8 assay was used to evaluate cell viability. After incubating the cells with the CCK-8 working solution at 37 °C for 2–4 h, the absorbance was measured at a wavelength of 450 nm using a microplate reader (Multiskan MK3, Thermo Fisher Scientific Instrument Co. Ltd., Shanghai, China). Each data point is represented as the mean $\pm$ standard deviation (SD) of three independent experiments. In each experiment, all the drug concentrations were tested in six replicates.

2.9. Cellular colocalization imaging

The MCF-7 cells were seeded into 35-mm glass-bottom culture dishes (NEST Science Co, Ltd, China) at a concentration of 5,000 cells/dish and were then treated with free DOX, BGNs, and BGNs@pDA-FA at a DOX concentration of 2 μ g/mL for 4 h or 24 h. After an incubation for a specified time, the cells were washed several times with pre-chilled PBS (pH=7.4) to remove the residual drug or nanoparticles. Then, the cells were stained with Hoechst 33342 (cellular nucleus dye) and lyso-tracker green (lysosome dye) and were visualized under a Confocal Laser Scanning Microscopy (CLSM) (Leica TCS SP5, Germany). The fluorescence images were taken under a 60 $\times$ oil-immersion objective (NA=1.42), and Hoechst 33342, lyso-tracker green and DOX were excited at the wavelength of UV, 488, and 503 nm to emit blue, green and red luminescence, respectively. There was no interference in the emission wavelengths that ranged from 450 to 500 nm for Hoechst 33342, 500–520 nm for lyso-tracker green and 580–610 nm for DOX.

2.10. LMP imaging

We investigated the effects of different drugs on the LMP of cancer cells. The MCF-7 cells were seeded in 35-mm glass-bottom culture dishes (NEST Science Co, Ltd, China) at a concentration of 6000 cells/dish, and then, the cells were washed three times with PBS after a 24 h incubation. Next, the cells were incubated with Alexa Fluor 488-dextran (100 μ g/mL) for 1 h, and then, the growth medium was substituted with new medium containing free DOX,

BGNs and BGNs@pDA-FA at a DOX concentration of 1 μ g/mL, after washing three times with PBS, and were incubated for another 4 h. A fluorescence microscope (IX70, Olympus) equipped with a 100 $\times$ oil immersion objective (NA=1.30) and a blue-illumination filter set (450–480/500/525/39 nm) was utilized to acquire the fluorescence images. The percentage of cells with LMP was calculated by counting arbitrarily selected areas, and at least 100 cells were observed for each experiment. Data are represented as the means $\pm$ standard deviation (SD) of three independent experiments.

2.11. Caspase-3 and lactic dehydrogenase (LDH) activity

We utilized a commercial Caspase-3 Activity Assay Kit to measure caspase-3 activity. First, the MCF-7 cells were seeded into 6-well plates at a density of 1×10^5 cells per well. After an incubation for 24 h, the cells were washed three times with PBS, and the culture medium was replaced by fresh culture medium containing free DOX, BGNs and BGNs@pDA-FA at a DOX concentration of 3 μ g/mL. After another incubation for 48 h, the cells were centrifuged, washed, and lysed with a cell lysis solution. After an incubation with the reaction buffer and the caspase-3 substrate at 37 °C for 4 h, caspase-3 activity was determined at 405 nm using a microplate reader (Multiskan Mk3, Thermo Fisher Scientific Instrument Co., Ltd., Shanghai, China). A commercial LDH Activity Assay Kit was used to determine LDH activity. First, the MCF-7 cells were seeded into 96-well plates at a density of 8000 cells per well. After an incubation for 24 h, the cells were washed one time with PBS, and the culture medium was replaced by fresh low serum culture medium containing 1% serum. The culture wells were divided into the following groups: background blank control, sample control, sample maximum enzyme activity control wells and drug treatment sample wells containing free DOX, BGNs and BGNs@pDA-FA at a DOX concentration of 5 μ g/mL. After another incubation for 48 h, 1 h before the scheduled detection time, the LDH release reagent provided in the kit was added to the “Sample maximum enzyme activity control wells” in the amount of 10% of the volume of the original culture medium. After the LDH release reagent was added, the mixture was repeatedly agitated several times and was then incubated at 37 °C under 5% CO₂. After a predetermined time was reached, the cell culture plate was centrifuged at 400g for 5 min. Then, 120 μ L of the supernatant from each well was added to a corresponding new 96-well plate, and the LDH Activity was determined at 490 nm using a microplate reader (Mul-

tiskan Mk3, Thermo Fisher Scientific Instrument Co., Ltd., Shanghai, China).

2.12. Statistical analysis

The results are expressed as the mean average $\pm$ SD, and the statistical analysis was carried out using a One-Way ANOVA. Statistical significance was set at $*p < 0.05$, and extreme significance was set at $**p < 0.01$ and $***p < 0.001$.

3. Results and discussion

3.1. Optimization of pDA-thickness

Dopamine can self-polymerize to surface-adherent pDA film onto a series of organic and inorganic materials [23–25]. We coated BGNSs with pDA by incubating them with dopamine in an alkaline solution for various time points (6, 12 and 24 h). According to a previous report [38], the incubation time affects the polymerization thickness of dopamine, thus affecting the drug release. To optimize the thickness of pDA, after incubating the BGNSs with dopamine for the different time periods, the *in vitro* cytotoxicities of the obtained BGNSs@pDA towards MCF-7 were tested. As shown in supplementary Fig. S1 (Supplementary materials), the cell viability was 28.2%, 23.3%, 31.4% for BGNSs@pDA after the 6, 12 or 24 h incubation at a DOX concentration of 4 μ g/mL, respectively. There was no significant difference in the toxicity of the three kinds of BGNSs@pDA ($p > 0.05$). Thus, we incubated the BGNSs with dopamine for 6 h in the next experiments in order to save experimental time.

3.2. Characterization of nano-carriers

The HMSNs and BGNSs were prepared according to our previous report with some minor modifications [36], and the coating of dopamine on the BGNSs and the subsequent bioconjugation with FA was performed according to previous reports [39]. The drug loading rate of the obtained BGNSs and BGNSs@pDA-FA was 10.5% and 9.7%, respectively. Fig. S2 (Supplementary materials) exhibits the color change of the different nanoparticles during the preparation. Different from the as-prepared HMSNs, which were white in ultrapure water, the BGNSs were orange-red after loading DOX and treating with NaHCO_3 . However, the BGNSs@pDA was dark brown, indicating the successful modification of pDA. Ultimately, the BGNSs@pDA-FA presented as a black-green color, demonstrating the anchor of FA.

TEM was used to investigate the morphology of the HMSNs, BGNSs, BGNSs@pDA and BGNSs@pDA-FA. As shown in Fig. 1a, the HMSNs, with a special hollow structure, were successfully synthesized and had an average diameter of approximately 360 ± 5 nm. The BGNSs were composed of uniform solid spheres with a similar diameter as the HMSNs, suggesting the successful loading of DOX (Fig. 1b). Polydopamine was modified on the surface of the BGNSs in an alkaline solution to facilitate an efficient conjugation of FA through the Schiff base reaction. The TEM image of the BGNSs@pDA is shown in Fig. 1c, the average diameter was approximately $417 \text{ nm} \pm 6 \text{ nm}$, and a 47 nm increase in diameter was observed after the coating, indicating the successful modification of pDA onto the surfaces of the BGNSs. The size of the BGNSs@pDA-FA was approximately $423 \text{ nm} \pm 9 \text{ nm}$ and was 6 nm larger than that of the BGNSs@pDA. These four types of nanoparticles were all well-dispersed and had spherical shapes.

Fig. S3 (Supplementary materials) shows the nitrogen adsorption-desorption isotherm of the HMSNs and its corresponding pore size distribution curve. The HMSNs exhibited a type IV isotherm characteristic of mesoporous material with H1 hysteresis loops. The corresponding pore size distribution

data, calculated by the BJH (Barrett-Joyner-Halenda) method, showed a pore size distribution peaking at 11.3 nm. The specific surface area reached $58.9 \text{ m}^2/\text{g}$, as calculated from the linear part of the BET (Brunauer-Emmett-Teller) plot. As shown in Table S1 (Supplementary materials), the zeta potential of the HMSNs was $-44.3 \pm 5.86 \text{ mV}$ ($n=3$). After the loading of DOX and the subsequent NaHCO_3 treatment, the zeta potential of the BGNSs became $26.2 \pm 5.85 \text{ mV}$ due to the presence of DOX with a positive charge. Compared with the BGNSs, the zeta potential of the BGNSs@pDA was reduced to $11.0 \pm 4.83 \text{ mV}$, indicating the formation of pDA onto their surfaces. However, once successfully conjugated with FA, the zeta potential of the BGNSs@pDA-FA decreased to $-31.3 \pm 6.04 \text{ mV}$ because of the presence of the large number of COOH groups of the FA. The changes in the zeta potential of these nanoparticles, after being loaded, coated, and conjugated, demonstrated the successful building of BGNSs, BGNSs@pDA and BGNSs@pDA-FA.

3.3. *in vitro* drug release assay

Fig. S4 shows the *in vitro* release of DOX in PBS with different pH values. The BGNSs and BGNSs@pDA-FA released DOX in a pH-responsive release manner. The cumulative release of the BGNSs and BGNSs@pDA-FA in 48 h was only 18% and 15% in PBS at 7.4, respectively, suggesting that the BGNSs and BGNSs@pDA-FA had a slight drug leakage in the normal physiological environment. However, the release of DOX increased dramatically with increasing incubation time in PBS at 5.0. The cumulative release of DOX from the BGNSs and BGNSs@pDA-FA reached as high as approximately 59.0% and 43.0% within 48 h, respectively. These results indicated that upon internalization by the cells, the BGNSs could release DOX faster than the BGNSs@pDA-FA in acidic intracellular compartments, attributing to the modified pDA, which inhibited the release of the drug.

3.4. *in vitro* cytotoxicity studies

An ideal nanocarrier for drug delivery should have a low toxicity before loading it with drugs. To investigate the possible side effects of the drug delivery vehicles on cells, the toxicities of HMSNs, HMSNs coated with pDA (HMSNs@pDA) and HMSNs@pDA conjugated with FA (HMSNs@pDA-FA) were investigated. The MCF-7 cells were incubated with these nanoparticles of different concentrations for 24 and 48 h. As shown in Fig. S5 (Supplementary materials), the cell viability remained over 80% after an incubation for 48 h, even when the concentrations of the nano-carriers were 100 μ g/mL. The maximum concentration of these nanocarriers used in drug delivery is lower than 100 μ g/mL, and thus, the nanocarriers appeared to be biocompatible.

The cytotoxicities of the free DOX, BGNSs, and BGNSs@pDA-FA against the MCF-7 cells were measured using the CCK-8 assay at different DOX concentrations after an incubation for 24 or 48 h. As shown in Fig. 2a, the free DOX, BGNSs, and BGNSs@pDA-FA all exhibited dose-dependent toxicity, and the cell viability gradually declined as the DOX concentration increased. The half-maximal inhibitory concentration (IC_{50}) of the free DOX, BGNSs and BGNSs@pDA-FA was 2.29, 3.66, and 5.31 μ g/mL, respectively, after the 24 h incubation and was 1.16, 2.15 and 3.77 μ g/mL after the 48 h incubation, respectively (Table S2). The anti-tumor efficiency of the BGNSs@pDA-FA against MCF-7 was lower than that of the BGNSs. This may be because the pDA modified onto the surfaces of the BGNSs@pDA-FA impeded the release of DOX and bubbles.

The cytotoxicities of the free DOX, BGNSs and BGNSs@pDA-FA against the MCF-7/ADR cells were also investigated. As shown in Fig. 2b, the antitumor activities of the BGNSs and BGNSs@pDA-FA against the MCF-7/ADR cells were higher than that of free DOX.

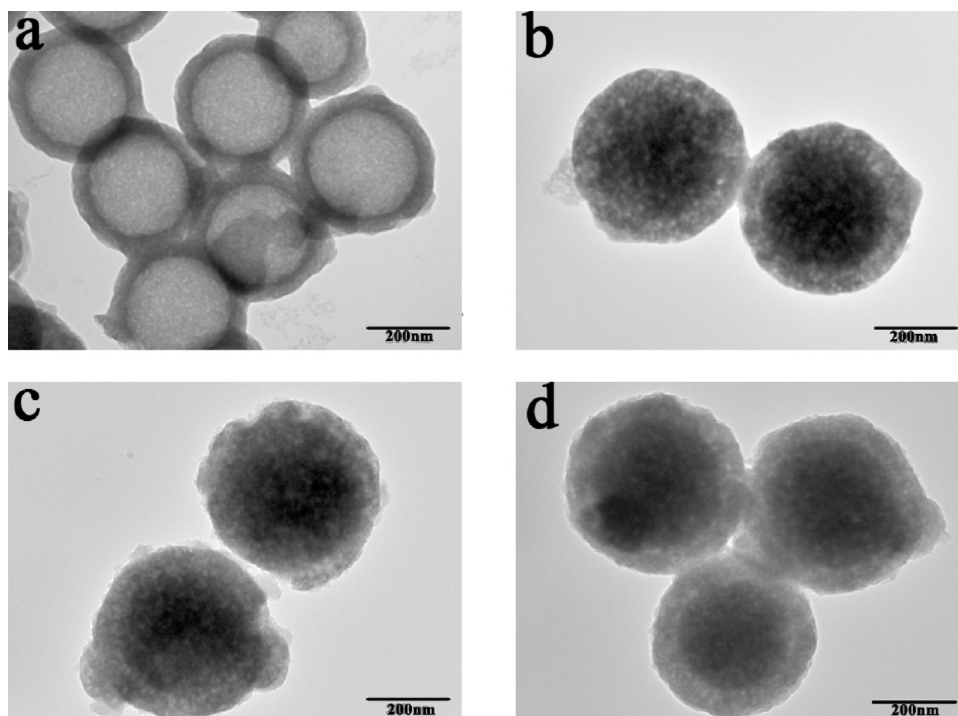


Fig. 1. TEM images of the HMSNs (a), BGNSs (b), BGNSs@pDA (c), BGNSs@pDA-FA (d); scale bar is 200 nm.

The survival rate of the MCF-7/ADR cells was approximately 60%, even after incubating the cells with 32 $\mu\text{g/mL}$ of DOX for 48 h. However, only 23% or 48% of the cells survived after incubating them with the BGNSs or BGNSs@pDA-FA at the same DOX concentration of 32 $\mu\text{g/mL}$ for 48 h. Drug-resistant cells have the ability to efflux drugs by increasing the expression of MDR proteins, such as P-glycoproteins (P-gps) of the ATP-binding cassette transporter family [3], and therefore, the higher anticancer activity of the BGNSs and BGNSs@pDA-FA might be attributed to the nanoparticle-mediated DOX delivery mechanism to bypass the P-gp-mediated efflux [2]. The anticancer activity of the BGNSs@pDA-FA was slightly lower than the BGNSs due to the fact that the modified pDA inhibited the release of both the drugs and bubbles but was still higher than that of free DOX; this result can be attributed to the generation of bubbles and the avoidance of drug efflux. The IC_{50} of the free DOX, BGNSs and BGNSs@pDA-FA against the MCF-7/ADR cells was 57.6, 23.4 and 41.3 $\mu\text{g/mL}$ after 24 h, and 43.7, 13.3 and 27.4 after 48 h, respectively. As expected, the BGNSs@pDA-FA had the ability to overcome the MCF-7/ADR cells.

To understand the possible side effects of our targeted BGNSs on normal cells, we also tested the cytotoxicities of the DOX, BGNSs and BGNSs@pDA-FA against the MCF-10A cells. Free DOX had the highest toxicity against normal cells, while BGNSs@pDA-FA had the lowest toxicity (Fig. 2c). There are relatively few folate receptors on the surfaces of MCF-10A cells. BGNSs@pDA-FA are mainly taken up via folate receptor-mediated endocytosis. Thus, fewer BGNSs@pDA-FA can enter into normal cells. As expected, BGNSs@pDA-FA, as an anticancer drug delivery system, effectively targeted the cancer cells and reduced the side effects on normal cells.

3.5. CLSM imaging

After incubating the free DOX, BGNSs and BGNSs@pDA-FA with the MCF-7 cells for 4 or 12 h at 37 °C, CLSM was used to visualize the internalization and DOX release of the drug delivery systems in the

cells. As shown in Fig. 3, after a 4 h incubation with the MCF-7 cells, the red fluorescence of the free DOX was mostly distributed in the nucleus, while the red fluorescence of the BGNSs and BGNSs@pDA-FA was mostly distributed in the lysosome. Furthermore, more intense red fluorescence was found in lysosomes of the cells treated with the BGNSs@pDA-FA compared to the BGNSs-treated group, indicating a clear visual evidence of the effective internalization of the BGNSs@pDA-FA through folate ligand-mediated endocytosis. A similar result was observed for the 12 h incubation (Fig. S6, supplementary materials). The free DOX mostly existed in the nucleus and exerted toxicity by interacting with DNA. However, the BGNSs and BGNSs@pDA-FA entered the lysosomes in an acidic environment to generate lots of CO_2 bubbles [36,37], thus increasing the LMP to result in cell apoptosis. Although the BGNSs@pDA-FA were more easily taken up by the MCF-7 cells than the BGNSs, their toxicity was lower than the BGNSs, due to the surface-modified pDA of the BGNSs@pDA-FA, which prevented the generation of bubbles.

3.6. Ultrasonic imaging

Ultrasound imaging was used to investigate the pH-response bubble generation of the BGNSs and BGNSs@pDA-FA in PBS at pH 7.4 and 5.0 at 37 °C. The ultrasound imaging was obtained by a 7 MHz transducer (HD11XE, Philips, Holland). As shown in Fig. S7 (Supplementary materials), no CO_2 bubbles were generated from the BGNSs and BGNSs@pDA-FA in PBS at pH 7.4. In contrast, lots of CO_2 bubbles were formed after the BGNSs and BGNSs@pDA-FA were treated with PBS at pH 5.0. Moreover, the BGNSs@pDA-FA generated fewer bubbles than the BGNSs, possibly because pDA coating impeded the entry of protons into the BGNSs. As reported in our earlier reports [36,37], bubble generation plays an important role in killing cancer cells. As a result, the BGNSs@pDA-FA exhibited a lower anti-tumor effect than the BGNSs.

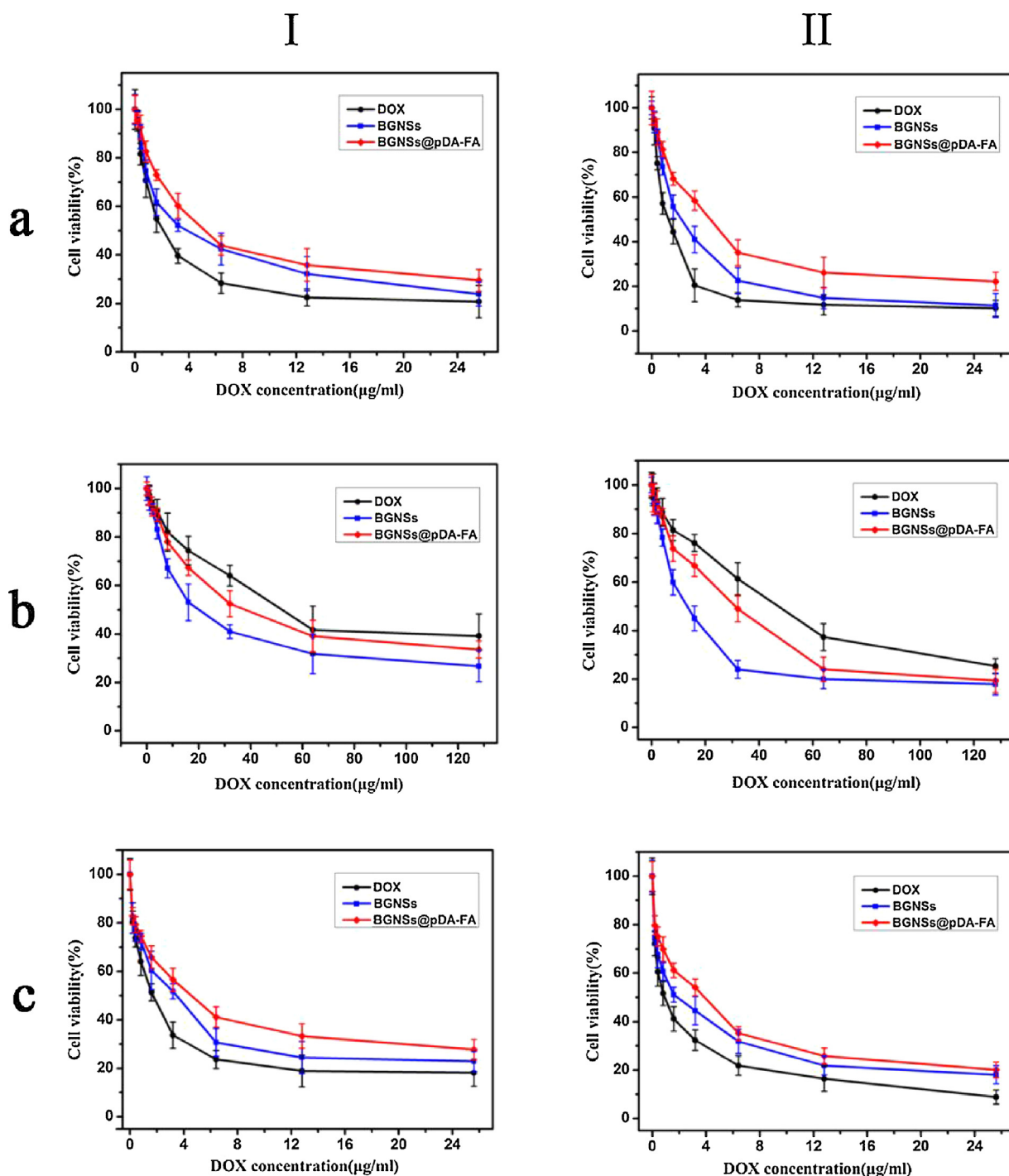


Fig. 2. *in vitro* cytotoxicity of the free DOX, BGNSs and BGNSs@pDA-FA against MCF-7 cells (a), MCF-7/ADR cells (b), and MCF-10A cells (c) incubated for 24 (I) or 48 h (II). The data are represented as the mean $\pm$ SD ($n = 6$).

3.7. LMP

The influence of the bubbles on LMP was assessed using fluorescence imaging. The fluorescent dextran developed from a punctuate lysosomal pattern to a diffuse cytosolic staining with the increase in LMP. As shown in Fig. S8 (Supplementary materials), the free DOX did not significantly enhance LMP. However, treatment with the BGNSs and BGNSs@pDA-FA caused a dramatic increase in LMP, which could be attributed to the quick generation of the CO₂ bubbles inside the lysosomes. The BGNSs@pDA-FA had

a weaker effect on LMP than the BGNSs, as they generated fewer bubbles in acidic conditions.

3.8. Assessment of the folate competition experiment

There are many folate receptors on the surface of cancer cells. FA-conjugated nanoparticles target cancer cells via folate receptor-mediated endocytosis. The folate competition experiment was employed to determine the cellular uptake of the nanomaterials modified with folic acid (BGNSs@pDA-FA). As shown in Fig. S9 (Supplementary materials), the cytotoxicity decreased in the

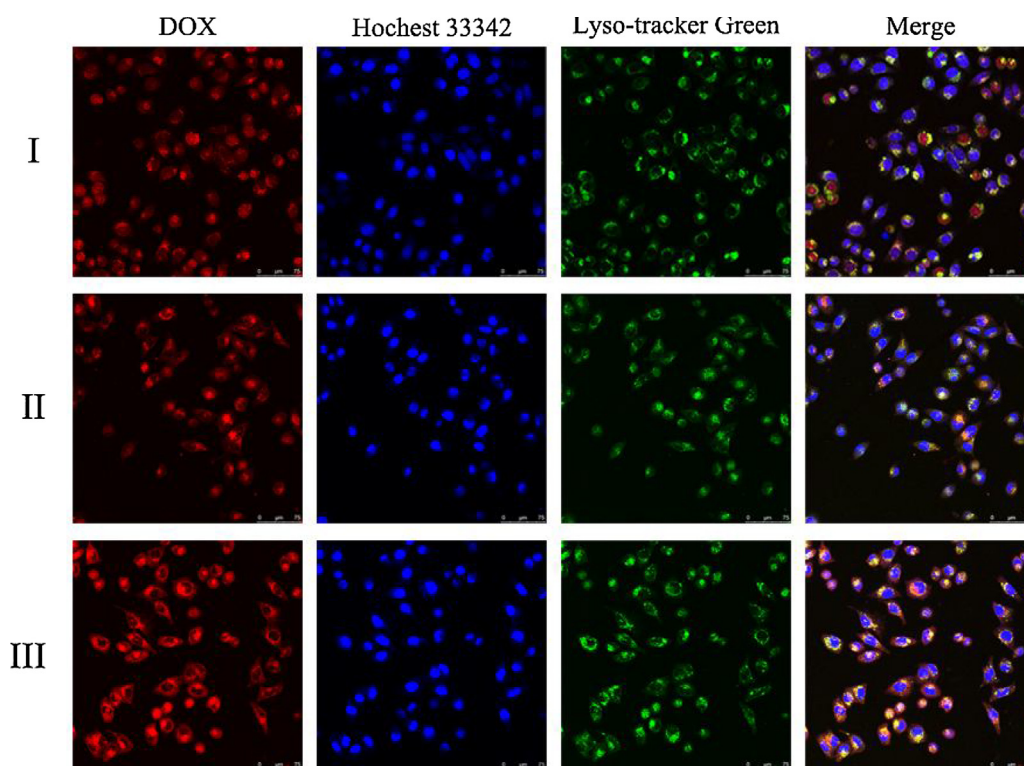


Fig. 3. CLSM images of the MCF-7 cells after a 4 h incubation with the free DOX(I), BGNSs and BGNSs@pDA-FA(III) at a DOX concentration of 1 $\mu\text{g/mL}$.

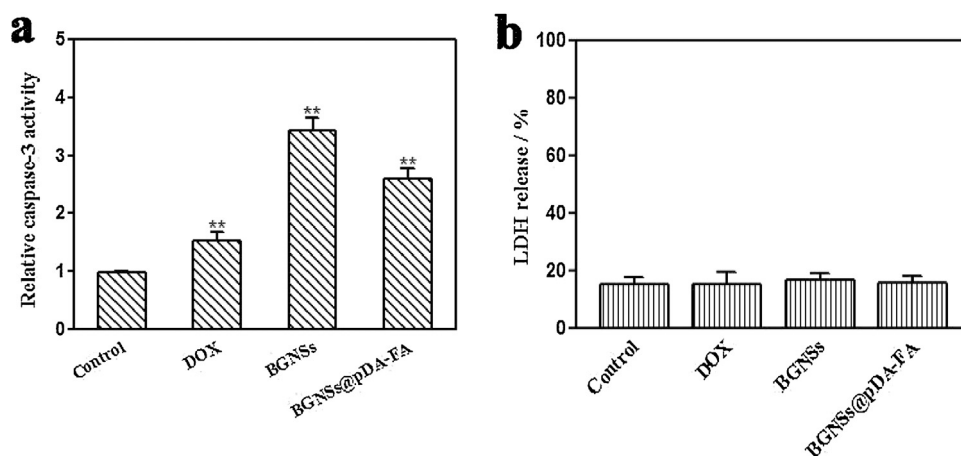


Fig. 4. (a) The caspase-3 activity of the untreated cells (control) and the cells treated with free DOX, BGNSs and BGNSs@pDA-FA at the DOX concentration of 3 $\mu\text{g/mL}$ for 48 h. (** $p < 0.001$ and ** $p < 0.01$ when compared drug treated cells with untreated cells (control)). (b) The results of the LDH assay for the cells treated with the free DOX, BGNSs and BGNSs@pDA-FA at 37 °C. ($p > 0.05$ when compared to the BGNSs and BGNSs@pDA-FA treated with untreated cells (control), respectively; There was no significant difference between the BGNSs, BGNSs@pDA-FA and control.) The data are presented as the mean $\pm$ SD ($n = 3$).

presence of 5 $\mu\text{g/mL}$ of FA, indicating the targeted binding of the BGNSs@pDA-FA with the cancer cells.

3.9. Caspase-3 and LDH activity assay

Caspase-3 is a family of proteases that plays an important role in the process of apoptosis [40,41]. Therefore, caspase-3 activity was used to determine the effect of free DOX, BGNSs and BGNSs@pDA-FA on the MCF-7 cells. As shown in Fig. 4a, the free DOX caused a slight increase in caspase-3 activity after it was incubated with the MCF-7 cells for 48 h. However, the BGNSs@pDA-FA exhibited higher caspase-3 activity than the free DOX but a lower activity than that of the BGNSs, which was in accordance with the results of the cell viability assay, the ultrasound imaging and the LMP assay.

The caspase-3 activity result implied that the BGNSs-FA triggered cell death by apoptosis or apoptosis-like pathways.

A previous report found that a dual-responsive CO_2 release system quickly releases CO_2 bubbles due to an elevated body temperature caused by an exogenous low-intensity treatment of ultrasound and tumor/internal endogenous acidic conditions. Afterwards, the resulting CO_2 bubble waves explode shortly before dissolution due to the triggering of therapeutic ultrasound waves. The explosion of the CO_2 bubbles effectively causes immediate necrosis of pancreatic-1 cells [42]. The bubble generation mechanism of our nanoparticles was different from the above report. A study found that lysosomes contain numerous hydrolases that play an important role in cell necrosis [43]. According to a previous study, the amount of LDH in the culture medium is proportional

to the degree of lysosome leakage [44]. We confirmed the presence of cavitation-induced cell necrosis by studying the release of LDH into the culture medium. Thus, the amount of LDH released in the cell supernatant was used to determine whether the cells had undergone necrosis. As shown in Fig. 4b, all the cell groups released a small amount of LDH into the medium at 37 °C. Therefore, we experimented with the LDH assay to confirm the absence of cell necrosis at 37 °C, indicating that the BGNSSs@pDA-FA do not cause cell necrosis but cause death by apoptosis or apoptosis-like pathways after entering the lysosomes, which is in accordance with the caspase-3 activity.

4. Conclusion

A targeted drug delivery system based on the coating of pDA and a subsequent FA conjugation with the H⁺-triggered bubble generating nanosystem was designed. The prepared BGNSSs@pDA-FA enhanced the permeability of lysosomes and the release of caspase-3, leading to cell apoptosis. *In vitro* assays revealed that BGNSSs@pDA-FA killed MCF-7 and MCF-7/ADR cells. Although the anti-tumor activity of the BGNSSs@pDA-FA was lower than that of the BGNSSs, for the pDA coating impeded the generation of bubbles, the severity of the side effects of the BGNSSs@pDA-FA against the MCF-10A cells was less than that of the BGNSSs. The BGNSSs@pDA-FA exhibited a higher anticancer efficiency and lower side effects than the free DOX. In view of these advantages, the BGNSSs@pDA-FA have the potential to serve as a useful drug targeting delivery system. Thus, this study provides a good direction for our next experimental research for the design of targeted drug delivery systems that generate bubbles.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (No.21175110) and the Fundamental Research Funds for the Central Universities (No.XDJK2013A022). The authors thank Prof. Xie Haiyan and Dr. Huang Lili (Beijing Institute of Technology) for their assistance with the CLSM imaging experiment.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.colsurfb.2017.09.034>.

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