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Doxorubicin-loaded poly(lactic-co-glycolic acid) hollow microcapsules for targeted drug delivery to cancer cells†

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We report here the fabrication, characterization and use of poly(lactic-co-glycolic acid) (PLGA) hollow microcapsules (HMs) loaded with anticancer drug doxorubicin (DOX) for targeted drug delivery to cancer cells. In this study, PLGA HMs loaded with DOX (PLGA-DOX HMs) were prepared by a double emulsion method, followed by electrostatic assembly of positively charged polyethyleneimine (PEI) pre-modified with polyethylene glycol-folic acid segments (PEI-PEG-FA). The formed multifunctional PLGA-DOX-PEI-PEG-FA HMs were characterized *via* different techniques. We show that the HMs having a size of 2.5 μm are stable, and are able to release DOX in a sustained manner with a higher release rate under an acidic pH condition than that under the physiological pH condition. Importantly, the PLGA-DOX-PEI-PEG-FA HMs displayed an effective therapeutic efficacy, comparable to that of free DOX, and were able to target cancer cells overexpressing high-affinity folic acid receptors and effectively inhibit the growth of the cancer cells. The fabricated PLGA-DOX-PEI-PEG-FA HMs may hold great promise to be used as a versatile carrier system for targeted drug delivery to different types of cancer cells.

Introduction

Current anticancer chemotherapy still deals with the fact that the administrated drug not only kills the cancer cells, but also affects the normal cells. In addition, the used anticancer drug often lacks water-solubility, hence having limited bioavailability. Therefore, it is important to develop a versatile carrier system to overcome the above problems. With the development of micro/nanotechnology, various carrier systems including but not limited to liposomes,[1-4] microparticles or nanoparticles,[5-7] nanogels,[8, 9] micelles,[10] nanoclays,[11] dendrimers,[12-17] and carbon nanotubes[18, 19] have been used for anticancer drug delivery.

Among the many different methods to generate micro/nanoparticles, double-emulsion technique has been proven to be powerful in creating polymer micro/nano particulate system that can be used to incorporate anticancer drugs and imaging agents.[20] Using the double emulsion method, anticancer drug doxorubicin (DOX)-loaded poly(lactic-co-glycolic acid) (PLGA) microspheres have been prepared for cancer therapy applications.[21-26] For targeted delivery to cancer cells, PLGA micro or nanoparticles have been modified with different targeting ligands, such as aptamers,[27, 28] folic acid (FA),[29-31] antibody[32], and RGD peptide.[33] Among the many types of targeting ligands, FA displays its inherent advantages. Besides the fact that FA can target different types of cancer cells such as ovary, kidney, uterus, testis, brain, colon, lung, and myelocytic blood that overexpress high-affinity FA receptors (FAR),[34-36] FA is cost-effective and has small molecular weight, affording the formed micro/nanoparticles with approximately similar conformation or size to the non-modified ones. However, a thorough literature investigation reveals that there are few reports related to FA-targeted PLGA micro/nanoparticles prepared using a double emulsion approach for targeted cancer therapy applications.

Inspired by our previous work associated with the electrostatic assembly of FA-functionalized dendrimers to render nanoparticles with targeting specificity,[37, 38] in this work, we fabricated FA-targeted DOX-loaded PLGA (PLGA-DOX) hollow microcapsules (HMs) for targeted drug delivery to cancer cells. First, PLGA-DOX HMs were prepared *via* a double emulsion method. The formed negatively charged PLGA-DOX HMs were then assembled with positively charged polyethyleneimine (PEI) grafted with polyethyle glycol (PEG)-FA (PEI-PEG-FA), which has been used as a highly efficient carrier in gene and drug delivery systems.[39, 40] The formed multifunctional PLGA-DOX-PEI-PEG-FA HMs were characterized *via* zeta potential measurements, scanning electron microscopy (SEM), and confocal laser scanning microscopy (CLSM). The release kinetics of DOX from the HMs was investigated under both acidic and physiological pH conditions. The *in vitro* targeting specificity and therapeutic efficacy of the multifunctional HMs were studied in detail. To our knowledge, this is the first report related to the development of FA-targeted PLGA HMs for targeted cancer therapy applications.

Experimental

Materials

PLGA (50:50, Mw = 20,000) was purchased from Jinan Daigang Biological Engineering CO., Ltd (Jinan, China). Doxorubicin hydrochloride (DOX•HCl) was from Beijing Hua Feng United Technology CO., Ltd (Beijing, China). Branched PEI (Mw = 25,000), poly(vinyl alcohol) (PVA, Mw = 25,000), FA, and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) were obtained from Aldrich (St. Louis, MO). PEG monomethyl ether with one end of carboxyl group (*m*PEG-COOH, Mw = 2000) and a dual functional PEG with one end of amine group and the other end of carboxyl group (NH_2-PEG-COOH, Mw = 2000) were from Shanghai Yanyi Biotechnology Corporation (Shanghai, China). KB cells (a human epithelial carcinoma cell line) were from the Institute of Biochemistry and Cell Biology (the Chinese Academy of Sciences, Shanghai, China). RPMI 1640 medium, fetal bovine serum (FBS), penicillin, and streptomycin were purchased from Hangzhou Jinuo Biomedical Technology (Hangzhou, China). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was acquired from Shanghai Sangon Biological Engineering Technology & Services Co., Ltd (Shanghai, China). All other chemicals were from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China). Regenerated cellulose dialysis membranes with molecular weight cutoff (MWCO) of 10,000 and 1,000 were acquired from Fisher Scientific (Hudson, NH). Unless other specified, all materials and chemicals were used as received. Water used in all experiments was purified using a Milli-Q Plus 185 water purification system (Millipore, Bedford, MA) with resistivity higher than 18 MΩ·cm.

Synthesis of PEI-PEG-FA

The dual functional PEG (NH_2-PEG-COOH) was used to link FA moieties on the surface of PEI. In brief, FA (0.12 mmoL) dissolved in DMSO (2.5 mL) was mixed with a DMSO solution (0.5 mL) containing EDC (0.6 mmoL) and the mixture solution was stirred for 3 h to activate the γ-carboxylic acid of FA. The activated FA (0.12 mmoL, 3 mL) was added into the NH_2-PEG-COOH solution (0.1 mmoL in 5 mL DMSO) under vigorous stirring for 3 days. Thereafter, the reaction mixture was extensively dialyzed against water (6 times, 2 L) using a dialysis membrane with MWCO of 1,000 for 3 days to remove the excess reactants, followed by lyophilization to get the product FA-PEG-COOH. Then, before reacting with PEI, FA-PEG-COOH was activated by mixing FA-PEG-COOH (0.1 mmoL, in 2.5 mL DMSO) with EDC (0.6 mmoL, in 0.5 mL DMSO) and the mixture solution was stirred for 3 h. The activated FA-PEG-COOH solution (0.1 mmoL, in 3 mL DMSO) was added to the PEI solution (0.01 mmoL, in 5 mL DMSO) under vigorous stirring for 3 days. Thereafter, the reaction mixture was extensively dialyzed against water (6 times, 2 L) using a dialysis membrane with MWCO of 10, 000 for 3 days to remove the excess reactants, followed by lyophilization to get the product PEI-PEG-FA. For comparison, PEI linked with *m*PEG without FA (PEI-*m*PEG) was also prepared under similar experimental conditions.

Fabrication of PLGA-DOX-PEI-PEG-FA HMs

PLGA-DOX HMs were fabricated by a double emulsion (water/oil/water) evaporation process. First, an oil phase and a water phase were prepared. PLGA (100 mg, dissolved in 2 mL CH_2Cl_2) was used as the oil phase. DOX•HCl (1 mg, dissolved in 200 μL water) was used as the water phase. The two phases were mixed and sonicated using an ultrasonic probe sonicator (Sonics & Materials Inc, Newtown, CT) at 130 W for 30 s to form the W/O emulsion. Then, the above emulsion was poured into an aqueous PVA solution (10 mL, 5% w/v) and homogenized with a homogenizer (FJ300-SH, Shanghai, China) at 9500 rpm for 5 min to prepare the W/O/W emulsion. The double emulsion was then poured into isopropanol alcohol solution (2% v/v, 80 mL) and mixed under mechanical stirring for 1 h to evaporate CH_2Cl_2 solvent and harden the microcapsules. The double emulsion process was carried out under an ice-water bath to maintain a low temperature in order to decrease the free movement of the droplets. Subsequently, the emulsion suspension was centrifuged at 3500 rpm for 3 min, the supernatant was discarded, and the precipitate was washed with water. The centrifugation/washing steps were repeated for 3 times. Finally, the precipitated PLGA-DOX HMs were redispersed in water (0.5 mL) before assembly with PEI-PEG-FA. For comparison, the product of PLGA HMs without DOX was also prepared under similar experimental conditions.

To assemble PEI-PEG-FA onto the surface of PLGA-DOX HMs, the suspension of PLGA-DOX HMs (100 mg, 0.5 mL in water) was mixed with PEI-PEG-FA (50 mL, 1 mg/mL in water) under mechanical stirring for 15 min. Then, the mixture was centrifuged (3500 rpm, 5 min), and the supernatant was discarded. The precipitated HMs were washed 3 times with water to remove the excessive non-adsorbed PEI-PEG-FA. Finally, the precipitate was redispersed in 10 mL water and lyophilized to obtain PLGA-DOX-PEI-PEG-FA HMs. For comparison, the products of PLGA-PEI-PEG-FA, PLGA-PEI-*m*PEG, and PLGA-DOX-PEI-*m*PEG HMs were also prepared under similar conditions.

Characterization techniques

PEI-PEG-FA and PEI-*m*PEG were characterized with ^{1}H NMR using a Bruker DRX 400 nuclear magnetic resonance spectrometer (Rheinstetten, Germany). Samples were dissolved in D_2O before measurements. Scanning electron microscopy (SEM, S-3400N, Hitachi High-Tech, Tokyo, Japan) was performed to characterize the morphology and size distribution of the PLGA-DOX-PEI-PEG-FA HMs at an accelerating voltage of 15 kV. The HMs dispersed in water (0.5 mg/mL) were dropped onto clean aluminum foil and air dried. Then, the sample was sputter coated with a 10 nm-thick gold film before SEM observation. The size distribution histogram of the PLGA particles was measured using ImageJ software (http://rsb.info.nih.gov/ij/download.html). For each sample, 300 particles from different SEM images were randomly selected to analyze the size. The morphology of the PLGA-DOX-PEI-PEG-FA HMs before lyophilization was also observed by CLSM (ZEISS LSM 700, Jena, Germany). For CLSM imaging, the PEI-PEG-FA was modified with fluorescein isothiocyanate (FI) and then assembled onto the HM surfaces. In brief, PEI was first reacted with FI at the PEI/FI molar ratio of 1:10 according to the procedure reported in the literature,[41, 42] followed by linking with PEG-FA as described above. By slight modification of the double emulsion process as described above, the microcapsules could be formulated to have an increased size

and have high visibility by CLSM under the specified experimental conditions (sonication frequency, 100 Hz; sonication time, 20 s).[43] Zeta potential and hydrodynamic size of the formed HMs were measured using a Malvern Zetasizer Nano ZS model ZEN3600 (Worcestershire, U.K.) equipped with a standard 633 nm laser.

To determine the DOX encapsulation efficiency in the PLGA-DOX-PEI-PEG-FA HMs, the concentration of DOX in the supernatants of the emulsion suspension after centrifugation and after 3 washing steps were collected and quantified by a Lamba 25 UV-vis spectrophotometer (Perkin Elmer, Boston, MA). The detection wavelength was selected at 481 nm. The loading efficiency of DOX in PLGA-DOX-PEI-PEG-FA HMs was calculated according to the following equation:

$$\text{loading efficiency (\%)} = (W_0\text{-}W_s)/W_0 \times 100\% \quad (1)$$

where W_0 and W_s represents the initial DOX mass and the DOX mass in the supernatants, respectively.

Release of DOX from PLGA-DOX-PEI-PEG-FA HMs

PLGA-DOX-PEI-PEG-FA HMs (10 mg, dispersed in 3 mL phosphate buffered saline (PBS) or acetate buffer solution) was placed into a dialysis bag with an MWCO of 10,000, and suspended in 7 mL corresponding buffer medium (40 mM, pH 7.4 PBS solution or pH 5.6 acetate buffer solution). All the samples for release experiments were incubated in a vapor-bathing constant temperature vibrator at 37 ^{o}C for a period of 72 h. At the given time interval, the buffer medium (3 mL) was taken out and the concentration of the released DOX was measured by UV-vis spectroscopy. The outer phase buffer medium was maintained constant by replenishing 3 mL of the corresponding buffer solution.

MTT cell viability assay and cell morphology observation

KB cells were continuously grown in RPMI 1640 cell culture medium supplemented with penicillin (100 U/mL), streptomycin (100 μg/mL), and 10% heat-inactivated FBS. The cells grown in FA-free medium express high-level FAR (denoted as KB-HFAR), while the cells grown in FA-containing media (2.5 μM) for 24 h or longer express low-level FAR (denoted as KB-LFAR). Unless otherwise stated, the term of "KB cells" denotes KB-HFAR cells.

An MTT assay was used to quantify the viability of the cells after treatment with the HMs. Briefly, approximately 5×10^3 KB cells per well were seeded into a 96-well plate. After culturing overnight to bring the cells to confluence, the medium was replaced with fresh medium containing PLGA-DOX-PEI-PEG-FA HMs or free DOX with different DOX concentrations. After 48 h incubation, the metabolically active cells were detected by adding MTT with a final concentration of 0.5 mg/mL for 4 h at 37 °C. Then, 200 μL DMSO was added into each well to replace the culture medium and dissolve the insoluble formazan crystals. The absorbance of each well was measured using a Thermo Scientific Multiskan MK3 ELISA reader (Thermo Scientific, USA) at 490 nm and 570 nm was selected as the reference wavelength. Mean and standard deviation of 5 parallel wells for each sample were reported. For comparison, the DOX-free PLGA-PEI-PEG-FA and PLGA-PEI-*m*PEG HMs and PLGA-DOX-PEI-*m*PEG HMs were also tested under similar experimental conditions. The HM concentrations of PLGA-PEI-PEG-FA, PLGA-PEI-*m*PEG, and PLGA-DOX-PEI-*m*PEG HMs tested were similar to those of PLGA-DOX-PEI-PEG-FA HMs used for the MTT assay.

After treatment with free DOX and PLGA-DOX-PEI-PEG-FA HMs ([DOX] = 0.1 mg/L) for 24 h, the cell morphology were observed by phase contrast microscopy (Leica DM IL LED inverted phase contrast microscope). The magnification was set at 100× for all samples.

Flow cytometry

Flow cytometry was used to detect the targeting efficacy of the PLGA-DOX-PEI-PEG-FA HMs. Approximately 1×10^5 KB-HFAR cells per well were seeded in 24-well plates the day before the experiments to bring the cells to confluence. The medium was replaced with fresh medium containing PLGA-DOX-PEI-*m*PEG or PLGA-DOX-PEI-PEG-FA HMs with the same DOX concentration (0.05, 0.1, 0.2 mg/L) and the corresponding HM concentration (6.25, 12.5, 25 mg/L), respectively. After 4 h incubation, the medium in wells was totally taken out, and the cells were washed with PBS for 3 times, trypsinized, resuspended in PBS in a 1 mL eppendorf tube. The cells were analyzed using a Becton Dickinson FACS Calibur flow cytometer equipped with an argon laser (488 nm). The FL2-fluorescence of 10,000 cells was measured, and the mean fluorescence of the gated viable cells was quantified. To further confirm the targeting efficacy of the PLGA-DOX-PEI-PEG-FA HMs, approximately 1×10^5 KB-HFAR or KB-LFAR cells per well were seeded in 24-well plates the day before the experiments. The next day, the medium was discarded and replaced with fresh medium containing PLGA-DOX-PEI-*m*PEG or PLGA-DOX-PEI-PEG-FA HMs with the same concentration (12.5 mg/L, [DOX] = 0.1 mg/L). The cells were then treated and analyzed *via* flow cytometry according to the above procedures.

Targeted cancer cell inhibition

To explore the targeted cancer cell inhibition effect, approximately 5×10^3 KB cells per well were seeded into a 96-well plate the day before the experiment. Then, the medium was discarded and replaced with fresh medium containing PLGA-DOX-PEI-*m*PEG and PLGA-DOX-PEI-PEG-FA HMs with the same DOX concentrations (0.1 and 0.2 mg/L) and the corresponding HM concentrations of 12.5 and 25 mg/L. After 4 h incubation, the medium was discarded and the cells were washed with PBS for 3 times, followed by addition of fresh medium. Then the cells were incubated at 37 ^{o}C for 48 h. Finally, the cell viability was analyzed by MTT assay according to the procedure described above.

Statistical analysis

One-way ANOVA statistical analysis was performed to evaluate the experimental data. 0.05 was selected as the significance level, and the data were indicated with (*) for $p < 0.05$, (**) for $p < 0.01$, and (***) for $p < 0.001$, respectively.

Results and discussion

Synthesis and characterization of PEI-PEG-FA

To afford the fabricated PLGA-DOX HMs with targeting specificity, PEI-PEG-FA was first synthesized (Scheme S1, Electronic Supplementary Information, ESI). *Via* EDC coupling

chemistry, PEI was linked with preformed PEGylated FA (PEG-FA). The conjugation of FA with NH_2-PEG-COOH and the formation of PEI-PEG-FA and PEI-*m*PEG were confirmed *via* ^{1}H NMR spectroscopy (Figure S1, ESI). In the ^{1}H NMR spectrum of PEG-FA and PEI-PEG-FA (Figure S1a), the chemical shifts at 6.71, 7.56, 8.76 ppm, respectively can be assigned to the characteristic proton peaks of the FA moieties. The chemical shift at 3.50 ppm is assigned to the -CH_2- proton peak of PEG, while the characteristic proton peaks between 2.25 and 3.34 ppm are assigned to -CH_2- proton peaks of PEI. Based on the NMR integration, the numbers of FA molecules attached to each PEG and PEG-FA attached to each PEI were estimated to be 0.43 and 14.55, respectively. The conjugation of *m*PEG onto PEI was also confirmed *via* ^{1}H NMR spectroscopy (Figure S1b). Similarly, based on NMR integration, the number of *m*PEG attached to each PEI was estimated to be 14.86, approximately similar to that of PEG-FA attached to each PEI (14.55).

Fabrication and characterization of PLGA-DOX-PEI-PEG-FA HMs

Through a facile double emulsion process, hydrophilic anticancer drug DOX•HCl was incorporated within PLGA microcapsules (Figure 1). By assembling positively charged PEI-PEG-FA onto the surface of PLGA-DOX HMs *via* electrostatic interaction, the final PLGA-DOX-PEI-PEG-FA HMs were formed.

Zeta potential measurements were used to confirm the formation of PLGA-DOX-PEI-PEG-FA HMs. We show that the negative surface potential of PLGA-DOX HMs (-19 mV) shifts to be close to neutral (-4 mV) after assembly with PEI-PEG-FA, suggesting the successful assembly of the PEI-PEG-FA onto the surface of PLGA-DOX HMs.

SEM was used to characterize the size and morphology of the formed PLGA-DOX-PEI-PEG-FA HMs (Figure 2a). All microcapsules displayed a round shape with an average diameter of 2.45 μm (Figure 2b). Capsular structure with holes on the wall of the HMs can be seen in the SEM images, possibly resulting from free diffusion of DOX during the formation of the microcapsules, similar to the PLGA-DOX HMs before assembly with PEI-PEG-FA (Figure S2, ESI). In contrast, the PLGA microspheres without DOX loading do not display such a holey capsular structure, confirming the role played by DOX loading (Figure S3, ESI). The hydrodynamic size of the formed HMs was measured to be 2.34 μm via dynamic light scattering (DLS), which is quite similar to that measured by SEM. This is likely due to the fact that the surface of PLGA microcapsules are sufficiently hydrophobic, quite limiting the formation of a relatively thick hydrated shell on their surfaces, in agreement with the literature.[44, 45]

To further investigate the inner and outer structure of the HMs, PLGA-DOX HMs were assembled with PEI-PEG-FA-FI and observed by CLSM (Figure 3). Both green fluorescence from FI and red fluorescence from DOX are able to track the structure of the HMs. The green fluorescent ring suggests the successful assembly of the PEI-PEG-FA-FI onto the surface of PLGA-DOX HMs, while the red fluorescence signal associated with DOX indicates the successful encapsulation of DOX within the interior of the PLGA HMs. In addition, the black holes on the surface of the HMs confirm the holey structure of the HMs, corroborating the SEM results. It should be noted that compared to other PLGA particles used for targeted drug delivery applications reported in the literature,[27-30] the developed PLGA-based HMs are of significance. Firstly, via a double emulsion approach, we were able to synthesize DOX-loaded PLGA HMs, which have never been reported before. Secondly, the multifunctional FA-targeted DOX-loaded PLGA HMs can be formed via electrostatic assembly of positively charged PEI-PEG-FA polymer onto the negatively charged PLGA HMs. As opposed to the developed PLGA-based HMs in our work, the reported PLGA particles in the literature are solid particles, and the formation of targeted PLGA particles is usually realized by first conjugating the ligands with PLGA polymer, followed by formation of the PLGA particles via a double emulsion process. Due to the fact that there is only one reactive carboxyl group per PLGA available to be modified, the efficiency of ligand modification is quite low.

The stability of the formed PLGA-DOX-PEI-PEG-FA HMs was evaluated by dispersing them in water, PBS, and cell culture medium. We show that the HMs are quite stable in different aqueous media for at least 24 h (Figure S4, ESI). Due to their larger size, the HMs tended to precipitate after 48 h. However, the precipitated particles were able to be easily redispersed in aqueous solution. Additionally, the colloidal stability of the formed HMs was occasionally checked by analyzing the change of their hydrodynamic size at different time periods (Figure S5, ESI). We show that the hydrodynamic size of the HMs does not appreciably change within one week, suggesting their good colloidal stability.

DOX loading and release

The anticancer drug DOX was encapsulated within the HMs in the double emulsion process. Under the studied conditions, 80.1% of the drug was able to be loaded within the PLGA HMs. To exert the therapeutic effect, the encapsulated DOX within PLGA-DOX-PEI-PEG-FA HMs has to be released. The release of DOX from the HMs was investigated under both acidic (pH = 5.6, representing the tumor microenvironment) and physiological pH conditions (pH = 7.4), respectively (Figure 4). It can be seen that the PLGA-DOX-PEI-PEG-FA HMs display a sustained release profile under both pH conditions with higher DOX release rate under acidic pH condition (pH = 5.6) than under physiological pH condition (pH = 7.4). Under acidic pH condition (pH = 5.6), about 73.2% of DOX was released within 24 h from the HMs, while only 41.4% of DOX was released in PBS at the same time point. At the release time of 72 h, 75.1% and 59.1% of DOX was released in the acetate buffer and PBS, respectively. The higher DOX release rate may be due to the fact that DOX has higher water solubility under acidic pH than under physiological pH condition.[11, 18, 46] Likewise, the used PLGA polymer degrades faster at pH 5 than at pH 7.4, which may also contribute to a faster DOX release rate under a slightly acidic pH condition.[47, 48] Since the micro-environment of tumors is slightly acidic (pH 5-6),[46] the higher DOX release rate from PLGA-DOX-PEI-PEG-FA HMs under acidic pH conditions could be beneficial for tumor therapy.

Therapeutic efficacy of PLGA-DOX-PEI-PEG-FA HMs

KB cells were selected as a model cancer cell line to study the therapeutic efficacy of PLGA-DOX-PEI-PEG-FA HMs. After incubating the cells with the PLGA-DOX-PEI-PEG-FA HMs at

the DOX concentration of 0.1 mg/L for 24 h, the morphology of cells was observed by phase contrast microscope (Figure S6, ESI). It is clear that the cells treated with DOX-free PLGA-PEI-*m*PEG and PLGA-PEI-PEG-FA HMs are quite healthy and display spindle-shaped morphology (Figures S6b and S6c), similar to the PBS control (Figure S6a). This suggests that the PLGA-PEI-*m*PEG and PLGA-PEI-PEG-FA HMs without DOX loading do not display the cytotoxic effect to KB cells. In contrast, the cells treated with PLGA-DOX-PEI-*m*PEG and PLGA-DOX-PEI-PEG-FA HMs (Figures S6e and S6f) became rounded and non-adherent, indicative of the cell death, similar to those treated with free DOX drug (Figure S6d). Our results suggest that the antitumor cytotoxicity of the PLGA-DOX-PEI-*m*PEG and PLGA-DOX-PEI-PEG-FA HMs are solely related to the loaded DOX drug.

To further quantify the therapeutic efficacy of the DOX-loaded HMs, KB cells were incubated with PLGA-DOX-PEI-*m*PEG HMs, PLGA-DOX-PEI-PEG-FA HMs, and free DOX at different DOX concentrations. DOX-free PLGA-PEI-*m*PEG and PLGA-PEI-PEG-FA HMs with the HM concentrations similar to those used to load the corresponding concentrations of DOX were also compared. After incubation of the cells for 48 h, MTT assay of cell viability was performed (Figure 5). It can be seen that when compared with the control cells treated with PBS, in the studied DOX concentration range, PLGA-DOX-PEI-*m*PEG and PLGA-DOX-PEI-PEG-FA HMs are able to cause a significant loss of cell viability ($p < 0.05$), similar to the free DOX. With the increase of the DOX concentration, the cell viability gradually decreases for each group. Moreover, the DOX-free PLGA-PEI-*m*PEG and PLGA-PEI-PEG-FA HMs do not cause any significant changes of the cell viability under different concentrations, confirming that the cytotoxicity of the DOX-loaded HMs are solely associated with the loaded DOX drug. The MTT assay data corroborated the cell morphology observation. Our results suggest that the encapsulation of DOX within the multifunctional PLGA HMs does not compromise the inherent antitumor activity of the drug. It is also noted that compared to PLGA-DOX-PEI-*m*PEG HMs, the treatment of cells using PLGA-DOX-PEI-PEG-FA HMs causes much lower cell viability under the same DOX concentrations. This is likely due to the fact that the presence of the targeting ligand FA onto the surface of PLGA-DOX-PEI-PEG-FA HMs enables enhanced binding of the HMs to the cells *via* ligand-receptor interaction, resulting in enhanced DOX delivery.

Flow cytometry

To confirm the binding affinity of the FA-modified PLGA-DOX-PEI-PEG-FA HMs to FAR-overexpressing KB cells, flow cytometry was used to quantify the cellular uptake of PLGA-DOX-PEI-PEG-FA HMs and PLGA-DOX-PEI-*m*PEG HMs *via* the inherent red fluorescence signal of DOX. As shown in Figure 6a, for KB-HFAR cells, the treatment of PLGA-DOX-PEI-PEG-FA HMs results in a significant increase in the fluorescence intensity of the cells. In contrast, the cells treated with FA-free PLGA-DOX-PEI-*m*PEG HMs display lower fluorescence intensity. These results suggest that KB-HFAR cells are able to specifically uptake PLGA-DOX-PEI-PEG-FA HMs *via* ligand-receptor interaction.

The targeting specificity of the PLGA-DOX-PEI-PEG-FA HMs was further confirmed by comparing the uptake of both KB-HFAR and KB-LFAR cells. In this case, PLGA-DOX-PEI-PEG-FA and PLGA-DOX-PEI-*m*PEG HMs at the same concentration were incubated with the cells for 4 h, followed by flow cytometry analysis. As shown in Figure 6b, for KB-HFAR cells, the treatment of PLGA-DOX-PEI-PEG-FA HMs results in a significant increase in the fluorescence signal of the cells. In contrast, the KB-HFAR cells treated with FA-free PLGA-DOX-PEI-*m*PEG HMs, and KB-LFAR cells treated with either PLGA-DOX-PEI-PEG-FA or PLGA-DOX-PEI-*m*PEG HMs do not display the same appreciably increased fluorescence signal. These results further suggest that the binding of PLGA-DOX-PEI-PEG-FA HMs preferentially occurs with KB-HFAR cells *via* FAR-mediated targeting pathway.

Targeted therapeutic efficacy of PLGA-DOX-PEI-PEG-FA HMs

We next explored the targeted therapeutic efficacy of PLGA-DOX-PEI-PEG-FA HMs in the absence of non-bound HMs by cells. KB-HFAR cells were separately treated with both PLGA-DOX-*m*PEG and PLGA-DOX-PEI-PEG-FA HMs for 4 h, followed by rinsing with PBS to remove the non-bound HMs by cells. The cells were then cultured in fresh medium for 48 h before MTT assay. As shown in Figure 7, the viability of cells treated with PLGA-DOX-PEI-PEG-FA HMs is much lower than that treated with FA-free PLGA-DOX-PEI-*m*PEG HMs at the DOX concentration of 0.1 mg/L and 0.2 mg/L, respectively ($p < 0.01$). Our results suggest that in the presence or absence of the non-bound HMs by cells, PLGA-DOX-PEI-PEG-FA HMs are able to exert specific therapeutic effect to KB-HFAR cells *via* receptor-mediated targeting (Figures 5 and 7), which is very important for targeted drug delivery to cancer cells.

Conclusions

In summary, we developed a facile method to fabricate doxorubicin-encapsulated microcapsules for targeted delivery to cancer cells. The double emulsion process enabled the generation of surface negatively charged poly(lactic-co-glycolic acid)-doxorubicin hollow microcapsules that can be assembled with positively charged polyethyleneimine-polyethylene glycol-folic acid polymer *via* electrostatic interaction to afford the hollow microcapsules with targeting specificity. The formed poly(lactic-co-glycolic acid)-doxorubicin-polyethyleneimine-polyethylene glycol-folic acid hollow microcapsules with a doxorubicin loading efficiency of 80.1% displayed a pH-responsive release behavior with higher release rate under an acidic pH condition and lower release rate under a physiological pH condition. Our results show that the encapsulation of doxorubicin within the poly(lactic-co-glycolic acid)-based hollow microcapsules does not compromise its therapeutic efficacy, importantly, the folic acid-targeted poly(lactic-co-glycolic acid)-doxorubicin polyethyleneimine-polyethylene glycol-folic acid hollow microcapsules are able to target KB cells overexpressing folic acid receptors and exert preferential therapeutic effect to the target cells. The synthesized poly(lactic-co-glycolic acid)-based hollow microcapsules may have a great potential to be used for targeted therapy of different type of cancer cells.

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Notes and references

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Figure captions

Figure 1. Schematic illustration of the preparation of PLGA-DOX-PEI-PEG-FA HMs.

Figure 2. SEM image (a) and size distribution histogram (b) of PLGA-DOX-PEI-PEG-FA HMs.

Figure 3. CLSM image of PLGA-DOX-PEI-PEG-FA-FI HMs.

Figure 4. *In vitro* release of DOX from PLGA-DOX-PEI-PEG-FA HMs as a function of time under different pH conditions.

Figure 5. MTT viability assay of KB cells treated with the formed HMs and free DOX for 48 h. KB cells treated with PBS were used as control.

Figure 6. Flow cytometry analysis of KB-HFAR cells treated with PLGA-DOX-PEI-*m*PEG HMs and PLGA-DOX-PEI-PEG-FA HMs at different HM concentrations (a) and both KB-LFAR cells and KB-HFAR cells treated with PLGA-DOX-PEI-*m*PEG and PLGA-DOX-PEI-PEG-FA HMs at an HM concentration of 12.5 mg/L (b). KB-HFAR cells treated with PBS were used as control. The fluorescence signal of DOX was monitored. Mean fluorescence was shown as mean ± S.D.

Figure 7. MTT assay of the viability of KB-HFAR cells treated with PLGA-DOX-PEI-*m*PEG and PLGA-DOX-PEI-PEG-FA HMs at the DOX concentration of 0.1 and 0.2 mg/L, respectively. The cells were treated with the HMs for 4 h, followed by rinsing with PBS, and then incubated with DOX-free fresh medium for 48 h. KB-HFAR cells treated with PBS were used as control.

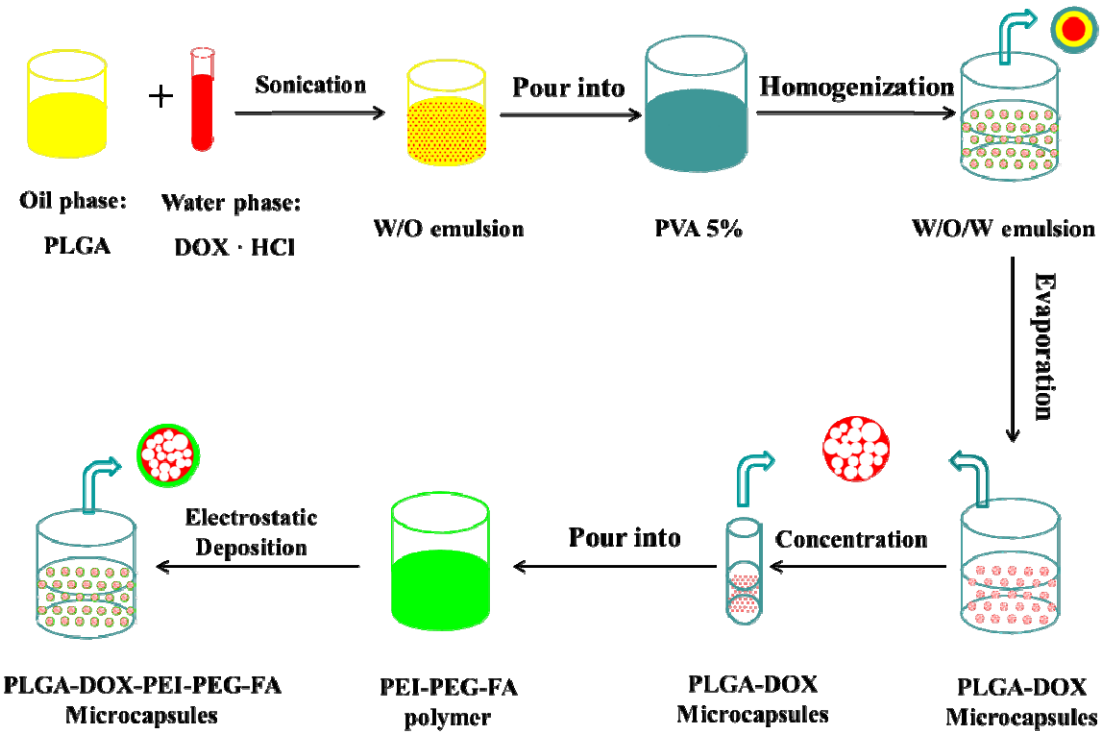

Figure 1
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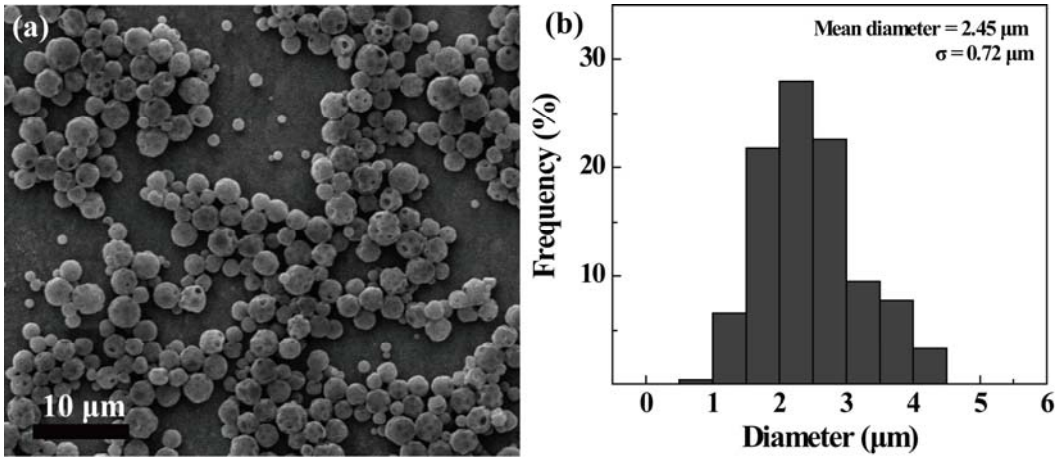

Figure 2
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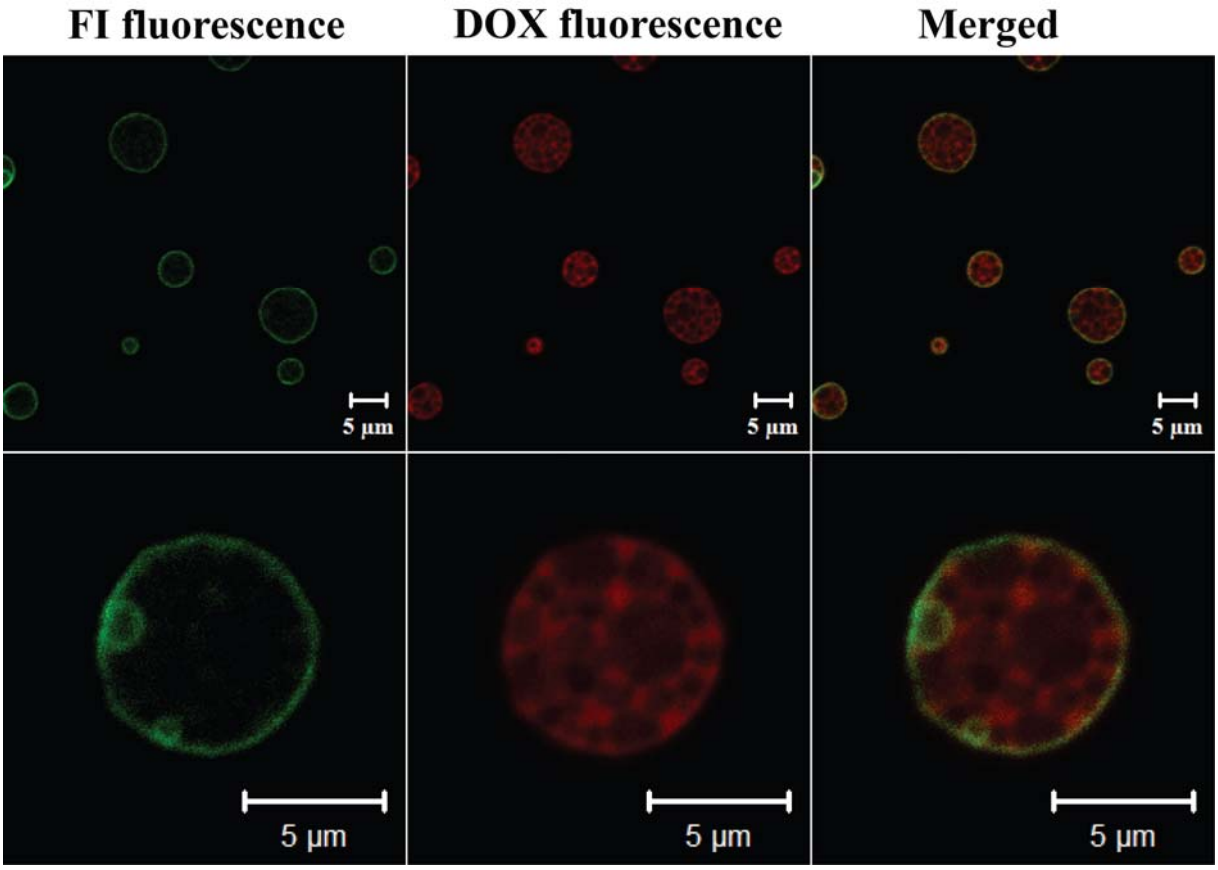

Figure 3
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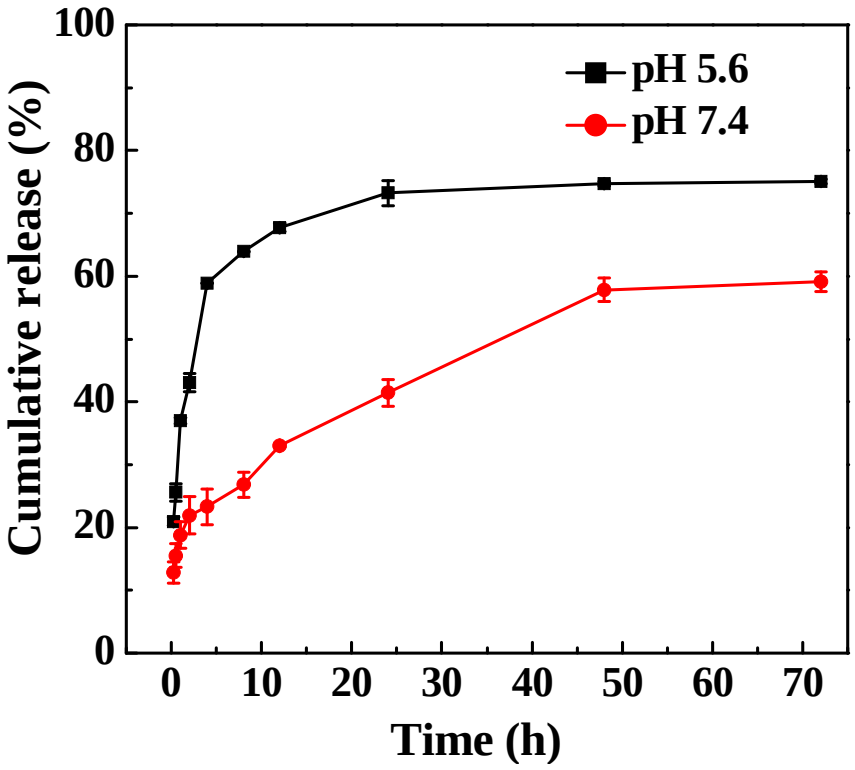

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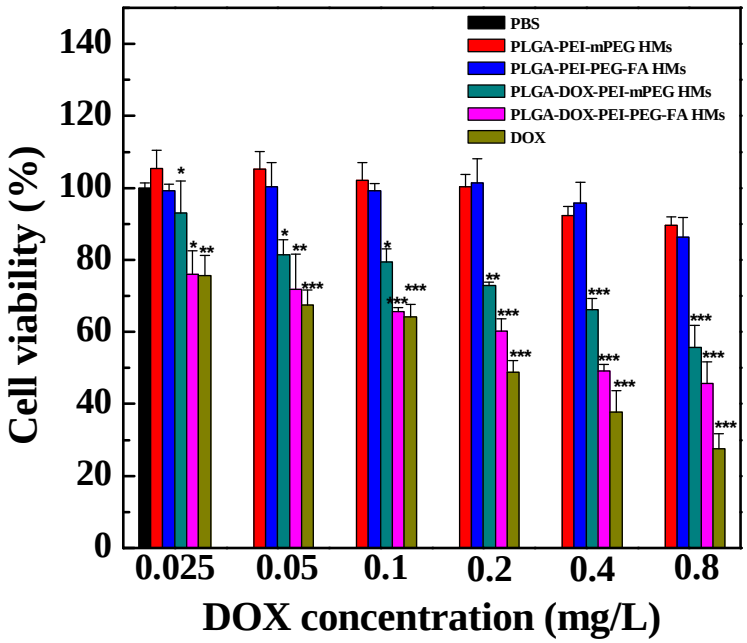

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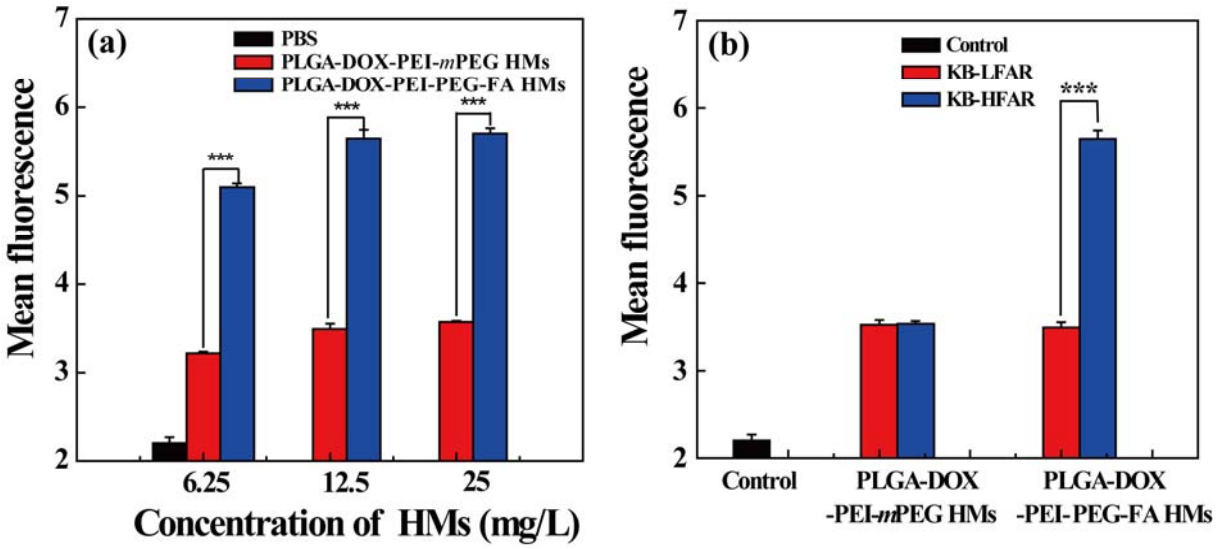

Figure 6
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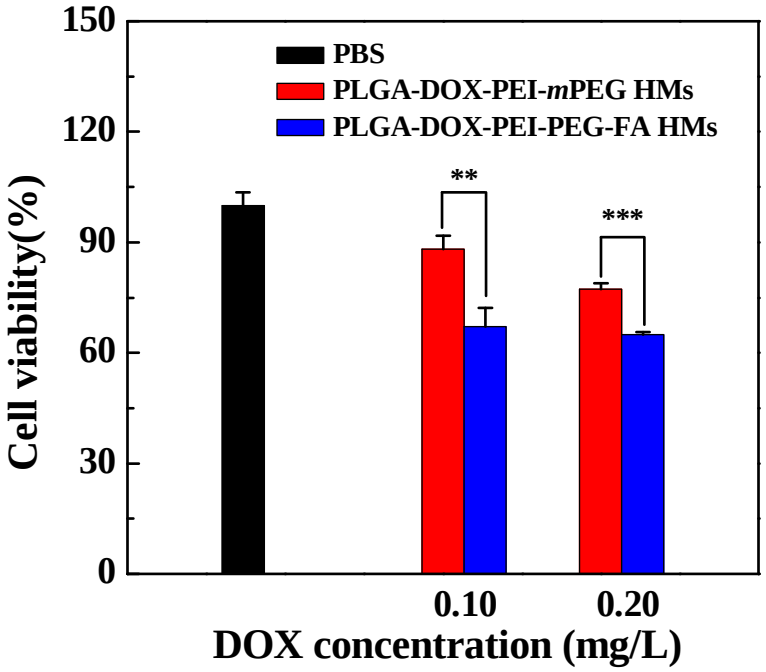

Figure 7
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