ACS APPLIED MATERIALS & INTERFACES

Research Article

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Multifunctional Lactobionic Acid-Modified Dendrimers for Targeted Drug Delivery to Liver Cancer Cells: Investigating the Role Played by PEG Spacer

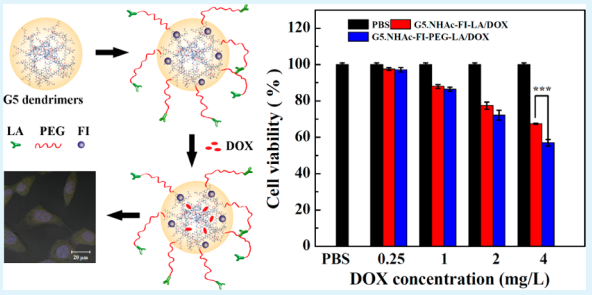
Fanfan Fu,[†] Yilun Wu,[†] Jingyi Zhu,[‡] Shihui Wen,[†] Mingwu Shen,[†] and Xiangyang Shi*[,†,‡,§]

[†]College of Chemistry, Chemical Engineering and Biotechnology, and [‡]State Key Laboratory for Modification of Chemical Fibers and Polymer Materials, College of Materials Science and Engineering, Donghua University, Shanghai 201620, People's Republic of China

[§]CQM-Centro de Química da Madeira, Universidade da Madeira, Campus da Penteada, 9000-390 Funchal, Portugal

Supporting Information

ABSTRACT: We report the development of a lactobionic acid (LA)-modified multifunctional dendrimer-based carrier system for targeted therapy of liver cancer cells overexpressing asialoglycoprotein receptors. In this study, generation 5 (G5) poly(amidoamine) (PAMAM) dendrimers were sequentially modified with fluorescein isothiocyanate (FI) and LA (or polyethylene glycol (PEG)-linked LA, PEG-LA), followed by acetylation of the remaining dendrimer terminal amines. The synthesized G5.NHAc-FI-LA or G5.NHAc-FI-PEG-LA conjugates (NHAc denotes acetamide groups) were used to encapsulate a model anticancer drug doxorubicin (DOX). We show that both conjugates are able to encapsulate approximately 5.0 DOX molecules within each dendrimer and the formed dendrimer/DOX complexes are stable under different pH conditions and different aqueous media. The G5.NHAc-FI-PEG-LA conjugate appears to have a better cytocompatibility, enables a slightly faster DOX release rate, and displays better liver cancer cell targeting ability than the G5.NHAc-FI-LA conjugate without PEG under similar experimental conditions. Importantly, the developed G5.NHAc-FI-PEG-LA/DOX complexes are able to specifically inhibit the growth of the target cells with a better efficiency than the G5.NHAc-FI-LA/DOX complexes at a relatively high DOX concentration. Our results suggest a key role played by the PEG spacer that affords the dendrimer platform with enhanced targeting and therapeutic efficacy of cancer cells. The developed LA-modified multifunctional dendrimer conjugate with a PEG spacer may be used as a delivery system for targeted liver cancer therapy and offers new opportunities in the design of multifunctional drug carriers for targeted cancer therapy applications.



KEYWORDS: *PAMAM dendrimers, lactobionic acid, PEG spacer, doxorubicin, targeted cancer therapy*

INTRODUCTION

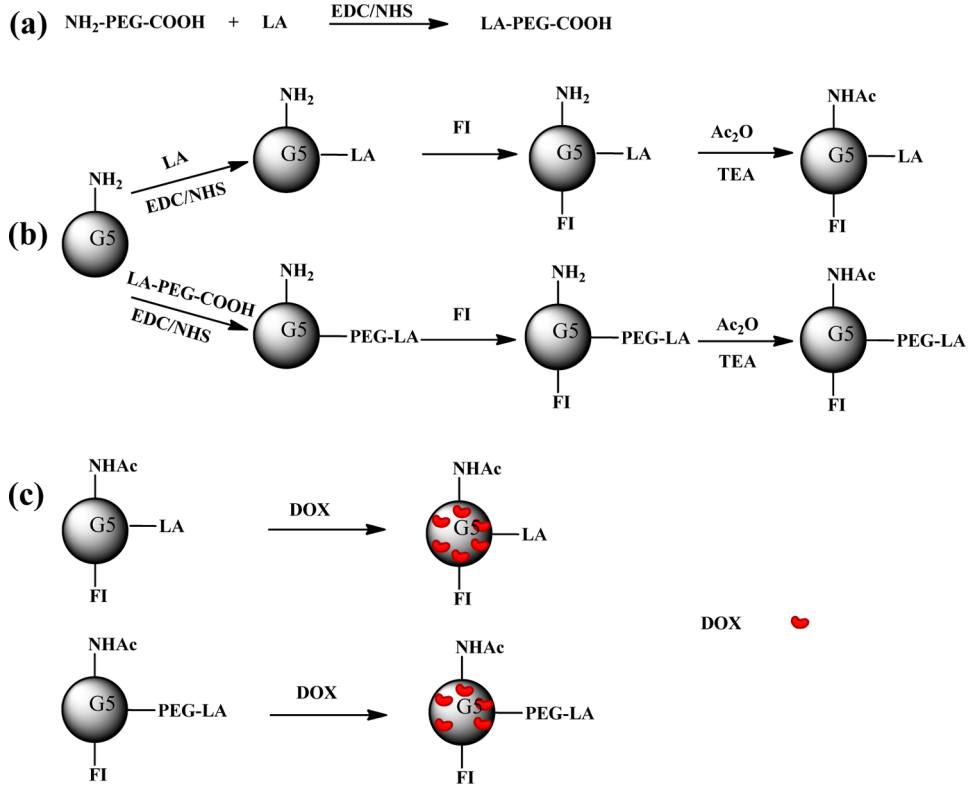
Cancer has been identified as one of the major causes of mortality in the world.[1] Chemotherapy has been frequently used to treat cancer in clinical practice. However, the problems of rapid clearance, low water solubility, nonspecific distribution, and thus normal tissue damage associated with most of the anticancer drugs limit their applications in chemotherapy. Therefore, it is prerequisite to develop a drug delivery system with targeting specificity to achieve the goal of minimizing the side effects while maximizing the therapeutic efficacy of the anticancer drugs.[2,3] Recent advances in nanotechnology show that it is possible to develop a carrier system that can deliver drug molecules directly to cancer cells either via a passive targeting pathway based on the enhanced permeability and retention (EPR) effect[4−6] or via an active targeting mediated by tumor-specific targeting ligands.[7−10] Various nanocarrier systems including but not limited to dendrimers,[3,11−19] micro- or nanoparticles,[20−24] inorganic nanoplatelets,[25] nanoclays,[26] carbon nanotubes,[27,28] micelles,[29,30] and liposomes[31] have been developed to achieve the goal of anticancer drug delivery applications.

Among many of the nanoscale drug delivery systems, dendrimers are a class of highly branched, monodispersed, and synthetic macromolecules with well-defined architecture and composition.[32,33] The unique physicochemical properties enable dendrimers to be used as a versatile nanoplatform for drug delivery applications.[34−38] The major advantage to use dendrimers as a carrier is that the size of dendrimers is quite small, affording efficient clearance via renal filtration.[2] In this context, there is no requirement for dendrimers to be biodegradable. Through the use of dendrimers as a platform, targeting ligands, imaging agents, and anticancer drugs are able to be covalently modified onto the dendrimer surfaces for targeting, imaging, and treatment of cancer cells.[13,14,35] Likewise, the hydrophobic interior of dendrimers is suitable

Received: July 23, 2014
Accepted: September 3, 2014
Published: September 3, 2014

   dx.doi.org/10.1021/am504849x | ACS Appl. Mater. Interfaces 2014, 6, 16416−16425

Scheme 1. Schematic Illustration of the Preparation of (a) LA-PEG-COOH Segments, (b) G5.NHAc-FI-LA and G5.NHAc-FI-PEG-LA Dendrimers, and (c) the Formation of the G5.NHAc-FI-LA/DOX and G5.NHAc-FI-PEG-LA/DOX Complexes[a]



[a]Ac_2O and TEA represent acetic anhydride and triethylamine, respectively.

for physical complexation or encapsulation of water-insoluble anticancer drugs, thereby significantly improving the bioavailability and water solubility of the anticancer drugs.[16,39−42] By encapsulating hydrophobic anticancer drugs within targeting ligand-modified dendrimer carriers, targeted drug delivery to cancer cells is able to be realized.[3,17,18]

To design an ideal dendrimer-based drug delivery system that is able to be accumulated in the tumor site either via EPR-based passive targeting strategy or via ligand-mediated active targeting, the dendrimer carriers should have a good antifouling property to be able to efficiently escape from the macrophage uptake. This can be actually realized by modifying dendrimers with polyethylene glycol (PEG), which is known to avoid opsonization and prolong the blood circulation time of the dendrimers.[43−46] With the advantage of PEGylation modification, Shen and co-workers discovered that the modification of generation 5 (G5) poly(amidoamine) (PAMAM) dendrimers with PEGylated folic acid (FA) enabled a better specific tumor uptake of the dendrimer devices than the direct modification of FA onto the G5 dendrimers.[47] This study highlights the importance of the PEG spacer between the dendrimer platform and the targeting ligands. It is anticipated that via a PEG spacer, the physical interaction between the targeting ligand and dendrimer surface can be readily avoided, thereby appreciably improving the accessibility of the targeting ligands to the receptors onto the cell surfaces. Unfortunately, the impact of PEG spacer onto the loading capacity, release kinetics, and targeting specificity of a dendrimer-based drug delivery system has been rarely reported in the literature.

In our previous work, we have shown that lactobionic acid (LA)-modified G5 PAMAM dendrimers with terminal acetyl groups are able to effectively target human hepatocellular carcinoma overexpressing asialoglycoprotein receptors (ASGPR).[48] In our another study,[49] we have also shown that G5 dendrimers modified with PEG-linked LA (PEG-LA) can be used as templates to form dendrimer-entrapped gold nanoparticles for targeted computed tomography imaging of hepatocellular carcinoma in vitro and in vivo. These studies stimulate us to employ LA-targeted dendrimers as a model system to investigate the impact of PEG spacer on the drug loading, release kinetics, and targeting specificity of the multifunctional dendrimer carriers.

In this present study, G5 PAMAM dendrimers were modified with LA or PEG-LA via 1-ethyl-3-(3-(dimethylamino)propyl) carbodiimide hydrochloride (EDC) chemistry, followed by modification with fluorescein isothiocyanate (FI) and acetylation of the remaining dendrimer terminal amines to form multifunctional G5.NHAc-FI-LA or G5.NHAc-FI-PEG-LA dendrimers (NHAc denotes acetamide groups, Scheme 1). The formed dendrimers were characterized via UV−vis spectroscopy and ^{1}H NMR spectrometry, and were used to physically load a model anticancer drug doxorubicin (DOX). The release kinetics of dendrimer/DOX complexes was investigated under two different pH conditions. Combined flow cytometry analysis, confocal laser scanning microscopic (CLSM) observation, and resazurin reduction assay of the viability of cancer cells were used to evaluate the targeting specificity, performance of targeted drug delivery of the dendrimer/DOX complexes, and therapeutic efficacy. To our knowledge, this is the first report related to the comparison of PEG spacer in impacting the encapsulation, release, and

targeted drug delivery of an LA-targeted dendrimer carrier system.

EXPERIMENTAL SECTION

Materials. Ethylenediamine core amine-terminated G5 PAMAM dendrimers (G5.NH_2) with a polydispersity index less than 1.08 were purchased from Dendritech (Midland, MI). FI, LA, resazurin, and Hoechst 33342 were obtained from Aldrich (St. Louis, MO). *N*-Hydroxysuccinimide (NHS) and EDC were from J&K Chemical Reagent Co., Ltd. (Shanghai, China). PEG with one end of carboxyl and the other end of amine group (NH_2—PEG-COOH, Mw = 2000) and PEG monomethyl ether with one end of carboxyl group (*m*PEG-COOH, Mw = 2000) were purchased from Shanghai Yanyi Biotechnology Corporation (Shanghai, China). Doxorubicin hydrochloride (DOX·HCl) was purchased from Beijing Huafeng Pharmaceutical Co., Ltd. (Beijing, China). HepG2 cells (a human hepatocellular carcinoma cell line) were from Institute of Biochemistry and Cell Biology, the Chinese Academy of Sciences (Shanghai, China). Dulbecco's Modified Eagle Medium (DMEM) and fetal bovine serum (FBS) were purchased from Hangzhou Jinuo Biomedical Technology (Hangzhou, China) and Shanghai Excell Biology, Inc. (Shanghai, China), respectively. Cellulose dialysis membranes (molecular weight cutoff, MWCO = 500 or 8000) were acquired from Shanghai Yuanye Biotechnology Corporation (Shanghai, China). Water used in all experiments was purified using a Milli-Q Plus 185 water purification system (Millipore, Bedford, MA) with a resistivity higher than 18 MΩ cm.

Synthesis of Multifunctional Dendrimers. The synthesis of LA-PEG-COOH was performed according to our previous work[49] with a slight modification (Scheme 1a). Briefly, LA (16.14 mg) and equal molar equivalent of EDC (8.57 mg) were dissolved in phosphate buffer (pH 6.0, 0.02 M, 10 mL) under vigorous magnetic stirring for 0.5 h, followed by dropwise addition of NHS with equal molar equivalent (5.18 mg) under stirring. After 3 h, the activated LA solution was dropwise added to a solution of NH_2-PEG-COOH (60 mg, in 10 mL of phosphate buffer, pH 6.0) under magnetic stirring for 3 days at room temperature. The reaction mixture was then dialyzed against water (9 times, 2 L) using a dialysis membrane with an MWCO of 500 for 3 days to remove the excess reactants, followed by lyophilization to obtain the PEG-linked LA (LA-PEG-COOH).

To synthesize the G5.NHAc-FI-LA or G5.NHAc-FI-PEG-LA dendrimers, G5.NH_2 was first conjugated with LA or LA-PEG-COOH to form the G5.NH_2-LA or G5.NH_2-PEG-LA product. In brief, in the presence of 15 mol equiv. of EDC (28.8 mg for LA; or 63.26 mg for LA-PEG-COOH) and NHS (17.26 mg for LA; or 38.0 mg for LA-PEG-COOH), the carboxyl groups of LA (3.58 mg, dissolved in 5 mL of water) or LA-PEG-COOH (51.1 mg, dissolved in 5 mL of water) were first activated under vigorous magnetic stirring at room temperature for 3 h. Then, the activated LA (10 mol equiv. of G5.NH_2) or LA-PEG-COOH (22 mol equiv. of G5.NH_2) was dropwise added to an aqueous solution of G5.NH_2 (25 mg, 10 mL) under vigorous magnetic stirring for 3 days, respectively (Scheme 1b). The reaction mixture was dialyzed against water (9 times, 2 L) for 3 days using a dialysis membrane with an MWCO of 8 000. By lyophilization of the dialysis liquid, the G5.NH_2-LA or G5.NH_2-PEG-LA product was obtained.

The formed G5.NH_2-LA or G5.NH_2-PEG-LA conjugate was successively modified with FI, followed by acetylation of the remaining dendrimer terminal amines according to our previous work. [17] Briefly, FI (1.4 mg, in 2 mL dimethyl sulfoxide (DMSO)) was dropwise added into a DMSO solution of G5.NH_2-LA (20 mg, 10 mL) or G5.NH_2-PEG-LA (29.5 mg, 12 mL) under vigorous magnetic stirring, respectively. After 24 h reaction, the reaction mixture was processed according to protocols described above to obtain the product of G5.NH_2-FI-LA or G5.NH_2-FI-PEG-LA dendrimers. Finally, the remaining terminal amines of the G5.NH_2-FI-LA or G5.NH_2-FI-PEG-LA dendrimers were transformed into acetyl groups to form G5.NHAc-FI-LA or G5.NHAc-FI-PEG-LA (Scheme 1b). In brief, the G5.NH_2-FI-LA (15 mg, in 5 mL water) or G5.NH_2-FI-PEG-LA (22 mg, in 6 mL water) solution was mixed with triethylamine (50 μL, 3.0 μmol) under vigorous magnetic stirring for 0.5 h. Subsequently, acetic anhydride (26 μL, 2.5 μmol) was dropwise added to the above dendrimer/triethylamine mixture solution under vigorous magnetic stirring. After 24 h, the mixture was purified and processed using the above procedures to obtain the G5.NHAc-FI-LA or G5.NHAc-FI-PEG-LA product. For comparison, G5.NHAc-FI and G5.NHAc-FI-*m*PEG without LA conjugation were also synthesized and purified under similar experimental conditions.

Loading of DOX within the G5.NHAc-FI-LA or G5.NHAc-FI-PEG-LA Dendrimers. The DOX loading within the G5.NHAc-FI-LA and G5.NHAc-FI-PEG-LA dendrimers was carried out according to our previous work[17] (Scheme 1c). Briefly, G5.NHAc-FI-LA (10 mg) or G5.NHAc-FI-PEG-LA (20 mg) dendrimers were dissolved in 1.5 mL water. DOX·HCl with 15 mol equiv. of the corresponding dendrimers was dissolved in 300 μL methanol, and neutralized by addition of 5 μL triethylamine. The DOX solution was then added into an aqueous dendrimer solution (1.5 mL) and the mixture was vigorously stirred overnight to allow the evaporation of the methanol solvent. Then, the G5.NHAc-FI-LA/DOX or G5.NHAc-FI-PEG-LA/DOX mixture solution was centrifuged (8000 rpm for 5 min) to remove the precipitate, which is associated with the noncomplexed DOX. The supernatant solution was lyophilized to obtain the G5.NHAc-FI-LA/DOX or G5.NHAc-FI-PEG-LA/DOX complexes. The loading of DOX within the dendrimers was quantified by subtracting the free DOX amount in the collected precipitate from the initial DOX amount. Free DOX was quantified via UV—vis spectroscopy measurement at a wavelength of 480 nm according to our previous work.[17]

Characterization Techniques. ^{1}H NMR spectra were recorded using Bruker AV400 nuclear magnetic resonance spectrometer. Samples were dissolved into D_2O before NMR measurements. UV—vis spectra were acquired by a Lambda 25 UV—vis spectrophotometer (PerkinElmer, USA). Samples were dissolved in water, methanol, or phosphate buffered saline (PBS) before measurements. Zeta potential measurements were carried out using a Malvern Zetasizer Nano ZS model ZEN3600 (Worcestershire, U.K.) equipped with a standard 633 nm laser.

In Vitro Release Kinetic Study. Taking the DOX release from the G5.NHAc-FI-PEG-LA/DOX complexes as an example, the complexes (2.92 mg) was dispersed in 1 mL PBS (pH 7.4) or acetate buffer (pH 5.0), placed in a dialysis bag with an MWCO of 8000, and dialyzed against 9 mL of the corresponding buffer medium. The experiment was done in triplicate. All the samples were incubated in a vapor-bathing constant temperature vibrator at 37 °C. At each specific time point, 1 mL buffer medium was taken out from the outer phase of the corresponding buffer medium and the concentration of the released DOX was quantified by UV—vis spectroscopy. The volume of the outer phase buffer medium was maintained constant by replenishing 1 mL of the corresponding buffer solution. The DOX release from the G5.NHAc-FI-LA/DOX, G5.NHAc-FI/DOX, and G5.NHAc-FI-*m*PEG/DOX complexes was performed under similar experimental conditions. Free DOX was also released similarly and used as control. In all cases, an equal amount of DOX from different complexes was used to ensure reasonable comparison among different groups.

Cytotoxicity Assay and Cell Morphology Observation. HepG2 cells was regularly cultured and passaged with DMEM supplemented with 10% FBS and 1% penicillin-streptomycin in a humidified incubator at 37 °C with 5% CO_2.

To determine the therapeutic efficacy of the G5.NHAc-FI-LA/DOX or G5.NHAc-FI-PEG-LA/DOX complexes, we performed a resazurin reduction assay of HepG2 cells treated with the complexes. The cells were plated into a 96-well plate (1×10^4 cells per well) for 24 h to bring the cells to confluence. The medium was then substituted with 200 μL of DMEM consisting of free DOX, G5.NHAc-FI-LA/DOX, and G5.NHAc-FI-PEG-LA/DOX with the same DOX concentrations (0.25, 0.5, 1, 2, and 4 mg/mL, respectively). The multifunctional dendrimers without DOX loading but with equivilent dendrimer concentration to the corresponding dendrimer/DOX complexes were also tested. After 24 h incubation, the resazurin reagent in PBS (1 μg/

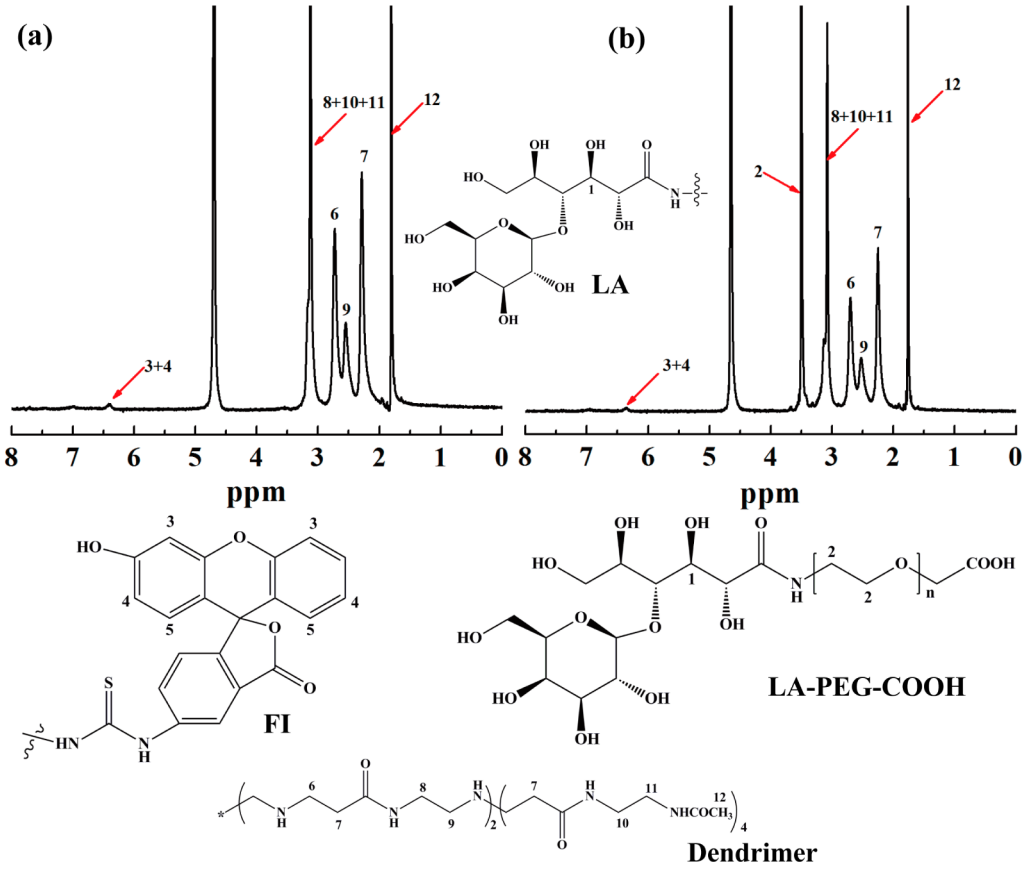


Figure 1. ^{1}H NMR spectra of the (a) G5.NHAc-FI-LA and (b) G5.NHAc-FI-PEG-LA dendrimers, respectively.

mL) was added. The assays were carried out according to the manufacturer's instructions. Mean and standard deviation of five paralleled wells of cells for each sample were reported.

After treatment with free DOX, dendrimers, or dendrimer/DOX complexes, respectively, for 24 h, the morphology of cells was observed using a Leica DM IL LED inverted phase contrast microscope with a magnification of 200× for each sample.

Flow Cytometric Analysis. To quantitatively confirm the cellular uptake of DOX within HepG2 cells, the DOX fluorescence within the cells was monitored using a FACS Calibur flow cytometer (Becton Dickinson, Mountain View, CA). HepG2 cells were seeded into a 12-well tissue culture plate (2×10^5 cells per well) for 24 h. Then, the cell medium was replaced with fresh DMEM containing the G5.NHAc-FI/DOX, G5.NHAc-FI-*m*PEG/DOX, G5.NHAc-FI-LA/DOX, and G5.NHAc-FI-PEG-LA/DOX complexes with DOX concentrations of 2 and 4 mg/L, respectively. After 4 h, the medium was discarded and the cells were washed with PBS for 3 times, trypsinized, centrifuged, and resuspended in 1 mL of PBS before flow cytometry analysis. For each sample, 1×10^4 cells were counted, and each measurement was repeated for 3 times. Cells treated with PBS were used as control.

Confocal Microscopic Observation. CLSM (Carl Zeiss LSM 700, Jena, Germany) was applied to qualitatively confirm the LA-mediated specific intracellular uptake of DOX. Coverslips with a diameter of 14 mm were pretreated with 5% HCl, 30% HNO_3, and 75% alcohol and fixed in a 12-well culture plate. After that, HepG2 cells were seeded at a density of 1×10^5 cells/well. After overnight incubation to allow the cells to attach onto the coverslips, the medium was replaced with FBS-free fresh medium containing G5.NHAc-FI-LA/DOX, G5.NHAc-FI-*m*PEG/DOX, or G5.NHAc-FI-PEG-LA/DOX complexes at the DOX concentration of 2 mg/L. About 3 h later, the cells were fixed with glutaraldehyde (2.5%) for 15 min at 4 °C. Then the cells were counterstained with Hoechst 33342 (1 μg/mL, 0.5 mL/well) for 15 min at 37 °C, followed by washing with PBS for 3 times. Finally, the cells were imaged using a 63× oil-immersion objective lens.

Targeted Antitumor Efficacy of the G5.NHAc-FI-LA/DOX or G5.NHAc-FI-PEG-LA/DOX Complexes. To evaluate the targeted cancer cell inhibition efficiency of the formed dendrimer/DOX complexes, HepG2 cells were seeded into a 96-well plate with a density of 8×10^3 cells/well in 200 μL medium at 37 °C and 5% CO_2. The next day, the medium was replaced with fresh medium containing the G5.NHAc-FI-LA/DOX, G5.NHAc-FI-*m*PEG/DOX, or G5.NHAc-FI-PEG-LA/DOX complexes with the DOX concentration of 0.25, 1, 2, and 4 mg/L, respectively. After incubation for 4 h, the cells were washed with PBS for 3 times and cultured with fresh DMEM for another 48 h at 37 °C. Finally, the cell viability was quantified by resazurin reduction assay according to the protocols described above.

Statistical Analysis. One-way ANOVA statistical method was performed to evaluate the significance of the experimental data. A value of 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 Multifunctional Dendrimers. Using a synthetic approach slightly different from our previous work,[48,49] LA-targeted G5 dendrimer devices with or without a PEG spacer were synthesized (Scheme 1). Each intermediate product was characterized using ^{1}H NMR. For the synthesis of the G5.NHAc-FI-LA dendrimers, LA was first directly conjugated onto the surface of the dendrimers via EDC chemistry. The peaks in the range of 3.8 to 4.3 ppm associated with the modified LA moieties can be clearly seen in the ^{1}H NMR spectrum (Figure S1a, Supporting Information), suggesting the successful formation of the G5.NH_2-LA dendrimers. By integration of the proton signals associated

with the LA moieties and the dendrimer methylene groups, the number of LA moieties attached onto each dendrimer was estimated to be 6.3. Further FI modification onto the G5.NH_2-LA dendrimers led to the appearance of aromatic proton signals at 6 to 8 ppm (Figure S2a, Supporting Information). By comparing the NMR integration of the FI proton peaks and the dendrimer methylene proton peaks, we were able to estimate the number of FI moieties attached onto each dendrimer to be approximately 4.8, which is close to the initial molar feeding ratio. This is in agreement with our previous work.[3] The final acetylation of the remaining terminal amines of G5.NH_2-FI-LA led to the formation of G5.NHAc-FI-LA. The emergence of a peak at 1.87 ppm (Figure 1a) that is related to the $-CH_3$ protons of the acetyl groups confirmed the success of the acetylation reaction. Furthermore, the acetylation of the remaining dendrimer terminal amines resulted in a nearly neutral surface potential of the final G5.NHAc-FI-LA product (-3.73 ± 0.7 mV, Table 1).

Table 1. Zeta Potentials of Different Functionalized Dendrimers and Dendrimer/DOX Complexes[a]

materials	zeta potential (mV)
G5.NHAc-FI-LA	-3.73 ± 0.7
G5.NHAc-FI-LA/DOX	-9.2 ± 1.2
G5.NHAc-FI-PEG-LA	-10.1 ± 0.5
G5.NHAc-FI-PEG-LA/DOX	-8.6 ± 0.8

[a]Data are provided as mean ± S.D.

For the synthesis of the G5.NHAc-FI-PEG-LA dendrimers, we first synthesized and characterized LA-PEG-COOH segments. The coexistence of the proton peak appearing at 3.5 ppm (associated with PEG) and the peaks at 3.8–4.3 ppm (associated with LA) in the ^{1}H NMR spectrum (Figure S1b, Supporting Information) indicates the successful formation of LA-PEG-COOH segments, in agreement with our previous report.[49] The number of LA moieties attached to each NH_2–PEG-COOH was calculated to be 0.93 through the comparison of the NMR peak integration of PEG and LA. The formed LA-PEG-COOH segments were then conjugated onto the surface of G5.NH_2 dendrimers via EDC chemistry, which was confirmed by ^{1}H NMR spectroscopy (Figure S1c, Supporting Information). *Via* the comparison of the PEG-associated proton peak integration and the dendrimer methylene proton peak integration, the number of PEG-LA segments attached onto each G5 dendrimer was estimated to be 6.9. Similar to the synthesis of G5.NHAc-FI-LA, the formed G5.NH_2-PEG-LA was conjugated with FI, followed by acetylation of the remaining dendrimer terminal amines. On the basis of the similar NMR integration method, the number of FI moiety attached onto each G5.NH_2-PEG-LA dendrimer was estimated to be 4.9 (Figure S2b, Supporting Information). And the final acetylation of the G5.NH_2-FI-PEG-LA dendrimers led to the emergence of the $-CH_3$ proton peak, which is associated with the acetyl groups (Figure 1b). In addition, the acetylation of the G5.NH_2-FI-PEG-LA dendrimers rendered the formed G5.NHAc-FI-PEG-LA dendrimers with a slightly negative surface potential (-10.1 ± 0.5 mV, Table 1), quite similar to the acetylation of the G5.NH_2-FI-LA dendrimers to form the product of G5.NHAc-FI-LA dendrimers (-3.73 ± 0.7 mV).

The control materials of G5.NHAc-FI and G5.NHAc-FI-*m*PEG dendrimers without LA conjugation were also characterized by ^{1}H NMR spectroscopy (Figure S3, Supporting Information). It should be noted that for each dendrimer product, we attached approximately similar numbers of FI or LA moieties per dendrimer. Consequently, the comparison of the dendrimer conjugates in terms of the drug loading, release, and targeted drug delivery is solely dependent on the linked PEG spacer (for G5.NHAc-FI-PEG-LA versus G5.NHAc-FI-LA) and/or the conjugated targeting ligand LA (for G5.NHAc-FI-PEG-LA versus G5.NHAc-FI-*m*PEG).

Furthermore, UV–vis spectrometry was also used to qualitatively confirm the successful FI conjugation onto the G5 dendrimer products (Figure 2). The apparent absorption peak at 500 nm for the G5.NHAc-FI-LA and G5.NHAc-FI-PEG-LA dendrimers clearly indicates the successful conjugation of FI moieties.

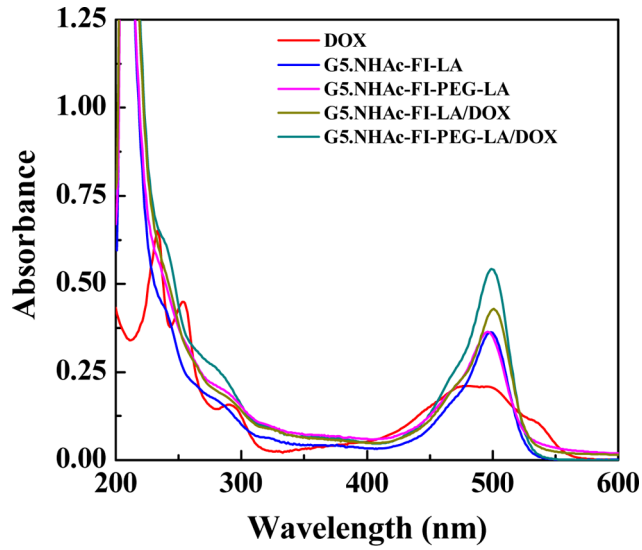


Figure 2. UV–vis spectra of free DOX dissolved in methanol, and G5.NHAc-FI-LA, G5.NHAc-FI-PEG-LA, G5.NHAc-FI-LA/DOX, and G5.NHAc-FI-PEG-LA/DOX dispersed in PBS.

Encapsulation of DOX within the G5.NHAc-FI-LA and G5.NHAc-FI-PEG-LA Dendrimers. According to protocols reported in our previous work,[17,19] the formed multifunctional G5.NHAc-FI-LA and G5.NHAc-FI-PEG-LA dendrimers were used to encapsulate anticancer drug DOX. The formed G5.NHAc-FI-LA/DOX and G5.NHAc-FI-PEG-LA/DOX complexes were characterized with UV–vis spectroscopy (Figure 2). It is clear that free DOX dissolved in methanol has a typical absorption peak at 481 nm. After DOX loading, the G5.NHAc-FI-LA/DOX and G5.NHAc-FI-PEG-LA/DOX complexes display an enhanced absorption intensity at 481 nm. In contrast, the G5.NHAc-FI-LA and G5.NHAc-FI-PEG-LA dendrimers under a similar dendrimer concentration to that of the corresponding complexes do not have the similar absorption intensity at 481 nm. By quantification via a standard DOX absorbance/concentration calibration curve, the numbers of DOX encapsulated within each G5.NHAc-FI-LA and G5.NHAc-FI-PEG-LA dendrimer were estimated to be 6.4 and 5.0, respectively. For comparison, the G5.NHAc-FI and G5.NHAc-FI-*m*PEG dendrimers were also used to load DOX, and the DOX loading was confirmed and quantified by UV–vis spectroscopy (Figure S4, Supporting Information). There were approximately 7.5 and 5.1 DOX molecules encapsulated within each G5.NHAc-FI and G5.NHAc-FI-*m*PEG dendrimer, respectively.

The stability of the formed G5.NHAc-FI-LA/DOX and G5.NHAc-FI-PEG-LA/DOX complexes under different conditions is of paramount importance for their biological applications. We show that the lyophilized powder of the G5.NHAc-FI-LA/DOX and G5.NHAc-FI-PEG-LA/DOX complexes could be completely dispersed in water, and are colloidally stable in water under different pH conditions and in cell culture medium for at least two months (Figure S5, Supporting Information). Likewise, the zeta potentials of the G5.NHAc-FI-LA/DOX (−9.2 ± 1.2 mV) and G5.NHAc-FI-PEG-LA/DOX (−8.6 ± 0.8 mV) complexes did not show obvious changes when compared to the corresponding dendrimers without DOX encapsulation (Table 1). The similarity in the stability and surface potential of the dendrimer/DOX complexes to the corresponding dendrimers without DOX loading suggests that the loaded DOX is within the relatively hydrophobic interior of the dendrimers, which is likely not to affect the cellular uptake behavior when compared to the dendrimer carriers.

In Vitro Release Kinetic Studies. PBS (pH 7.4) and acetate buffer (pH 5.0) were selected as the release media to evaluate the in vitro release kinetics of DOX from the G5.NHAc-FI-LA/DOX or G5.NHAc-FI-PEG-LA/DOX complexes at 37 °C. As shown in Figure 3, the loaded DOX can be

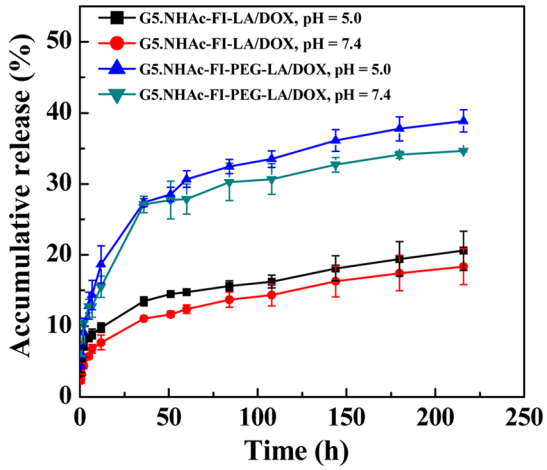


Figure 3. Cumulative release of DOX from the G5.NHAc-FI-LA/DOX or G5.NHAc-FI-PEG-LA/DOX complexes in PBS (pH 7.4) and acetate buffer (pH 5.0) at 37 °C.

released from the complexes in a sustained manner, indicating that the relatively hydrophobic dendrimer interior is able to hold the drug from burst release. Specifically, G5.NHAc-FI-LA/DOX complexes display a relatively slow DOX release rate, and 18.35 ± 2.5% and 20.59 ± 2.7% DOX are released within 216 h at pH 7.4 and pH 5.0, respectively. In contrast, at the same time point, 34.66 ± 0.13% and 38.87 ± 1.5% DOX are released from the G5.NHAc-FI-PEG-LA/DOX complexes at pH 7.4 and pH 5.0, respectively. It appears that the PEG spacer of the G5.NHAc-FI-PEG-LA dendrimers enables a faster DOX release when compared with the G5.NHAc-FI-LA dendrimers under similar conditions. This is possibly due to the fact that the hydroxyl groups of the PEG spacer may be able to interact with the encapsulated DOX drug via hydrogen bonding, promoting the DOX release, in agreement with our previous results.[50] The release rate of DOX from both dendrimer/DOX complexes is faster under acidic condition (pH 5.0) than under physiological condition (pH 7.4). This is because under an acidic condition (pH 5.0), DOX is protonated and has positive charges. The protonated dendrimer branches with positive charges under an acidic condition are likely able to repel the positively charged DOX molecules, thereby speeding up the release of DOX from the dendrimer interiors, in agreement with the literature.[17] The release kinetics of the G5.NHAc-FI-*m*PEG/DOX and G5.NHAc-FI/DOX complexes basically followed the same trend in terms of the impact of the PEG spacer and the pH-responsive DOX release behavior (Figure S6, Supporting Information). In contrast, free DOX was able to be fast released under both pH conditions. At 2 h, around 85.7% and 78.8% DOX were released under pH 5.0 and pH 7.4, respectively.

Therapeutic Efficacy of the G5.NHAc-FI-LA/DOX and G5.NHAc-FI-PEG-LA/DOX Complexes. The therapeutic efficacy of the G5.NHAc-FI-LA/DOX and G5.NHAc-FI-PEG-LA/DOX complexes was then evaluated by resazurin reduction assay of HepG2 cells treated with the dendrimer/DOX complexes (Figure 4). Similar to free DOX, both G5.NHAc-

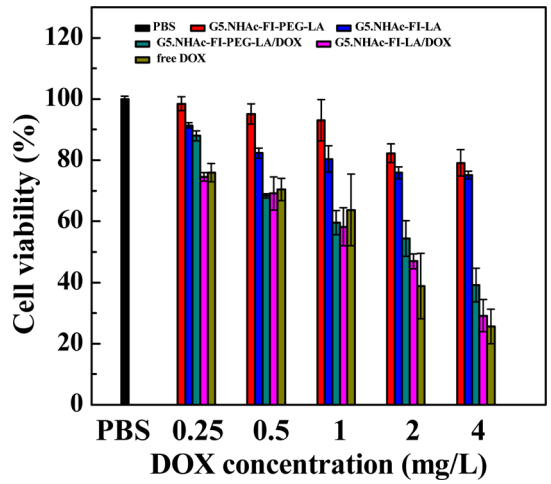


Figure 4. Resazurin reduction assay of HepG2 cells treated with PBS, G5.NHAc-FI-LA, G5.NHAc-FI-PEG-LA, free DOX, G5.NHAc-FI-LA/DOX, and G5.NHAc-FI-PEG-LA/DOX at different DOX concentrations for 24 h. The concentrations of G5.NHAc-FI-LA and G5.NHAc-FI-PEG-LA were similar to those of the corresponding dendrimer/DOX complexes.

FI-LA/DOX and G5.NHAc-FI-PEG-LA/DOX complexes are able to cause a significant loss of cell viability when compared with control cells treated with PBS. The cell viability gradually decreases with the DOX concentration. HepG2 cells incubated with free DOX, G5.NHAc-FI-LA/DOX, and G5.NHAc-FI-PEG-LA/DOX have a viability of 25.6 ± 5.5%, 29.14 ± 5.2%, and 39.12 ± 5.4% at the DOX concentration of 4 mg/L, respectively. The IC_{50} values of free DOX, G5.NHAc-FI-LA/DOX, and G5.NHAc-FI-PEG-LA/DOX were calculated to be 1.5, 1.7, and 2.5 mg/L, respectively. The higher IC_{50} values of the dendrimer/DOX complexes than that of free DOX could be due to the sustained release characteristics of the complexes, requiring more DOX to achieve the same therapeutic efficacy. Moreover, the G5.NHAc-FI-LA and G5.NHAc-FI-PEG-LA dendrimers without DOX loading at the dendrimer concentrations similar to those used to encapsulate DOX were noncytotoxic to HepG2 cells in the studied concentration range, suggesting that the therapeutic efficacy of the

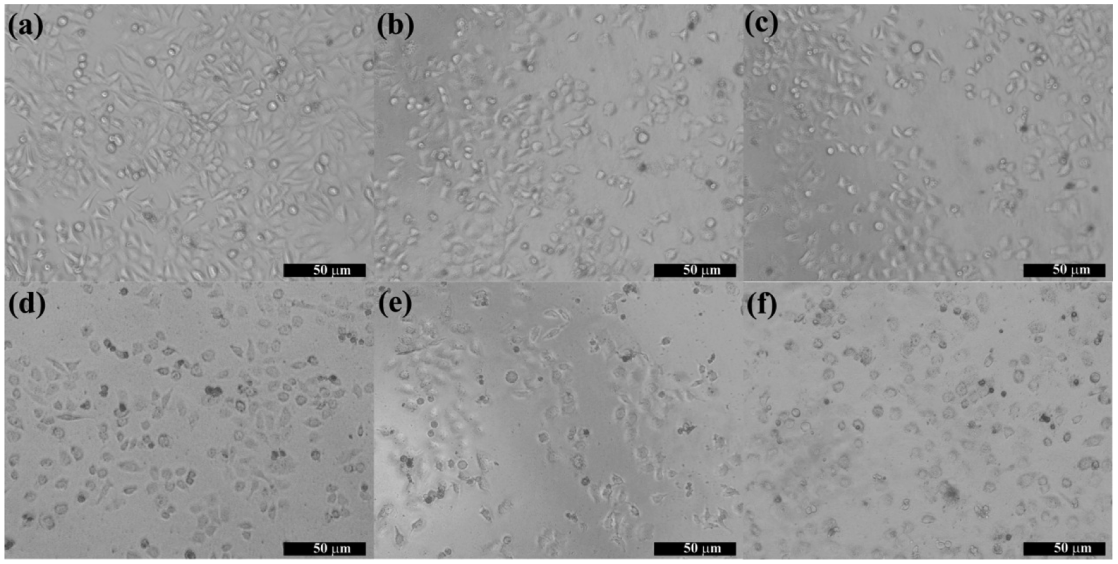


Figure 5. Phase contrast photomicrographs of HepG2 cells treated with (a) PBS, (b) G5.NHAc-FI-LA, (c) G5.NHAc-FI-PEG-LA, (d) G5.NHAc-FI-LA/DOX, (e) G5.NHAc-FI-PEG-LA/DOX, and (f) free DOX at a DOX concentration of 2 mg/L for 24 h, respectively. The concentration of the used G5.NHAc-FI-LA and G5.NHAc-FI-PEG-LA dendrimers were equivilent to the corresponding dendrimer complexes.

dendrimer/DOX complexes is solely related to the loaded DOX.

The therapeutic efficacy of the dendrimer/DOX complexes was further qualitatively confirmed by microscopic visualization of the morphology of HepG2 cells treated with the complexes at the DOX concentration of 2 mg/L (Figure 5). The morphology of HepG2 cells treated with G5.NHAc-FI-LA (Figure 5b) and G5.NHAc-FI-PEG-LA (Figure 5c) is similar to that of the control cells treated with PBS (Figure 5a), indicating that the multifunctional dendrimers without DOX loading are nontoxic to the HepG2 cells. In contrast, similar to free DOX-treated cells (Figure 5f), a significant proportion of HepG2 cells treated with the G5.NHAc-FI-LA/DOX (Figure 5d) and G5.NHAc-FI-PEG-LA/DOX (Figure 5e) complexes for 24 h became rounded and detached, indicating the cell death. The cell morphology observation results are consistent with the resazurin reduction assay data. Taken together, our results show that the encapsulation of DOX within the dendrimers does not compromise the therapeutic efficacy of the DOX, and the therapeutic effect of the complexes is solely associated with the loaded DOX.

Targeting Specificity of the G5.NHAc-FI-LA/DOX and G5.NHAc-FI-PEG-LA/DOX Complexes. To check the LA-mediated targeting specificity to liver cancer cells over-expressing ASGPR, HepG2 cells were treated with the G5.NHAc-FI-LA/DOX and G5.NHAc-FI-PEG-LA/DOX complexes for 4 h before flow cytometric analysis (Figure 6). The red fluorescence signal of DOX was utilized to quantify the targeted cellular uptake of the dendrimer/DOX complexes (Figure S7, Supporting Information). It can be seen that under similar DOX concentrations, the cells treated with G5.NHAc-FI-LA/DOX display much stronger fluorescence intensity than those treated with G5.NHAc-FI/DOX ($p < 0.01$). Similarly, the cells treated with G5.NHAc-FI-PEG-LA/DOX also show much stronger fluorescence intensity than those treated with G5.NHAc-FI-*m*PEG/DOX ($p < 0.001$). The much higher DOX uptake of the LA-targeted dendrimer/DOX complexes with or without PEG spacer under similar DOX concentrations should be due to the LA-mediated targeting of the dendrimer/

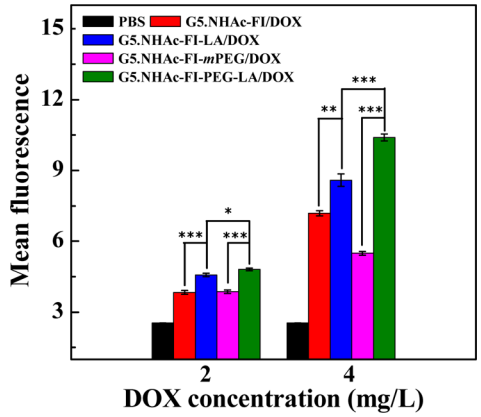


Figure 6. Flow cytometric analysis of HepG2 cells treated with the dendrimer/DOX complexes at different DOX concentrations for 4 h. HepG2 cells treated with PBS were used as control.

DOX complexes to HepG2 cells overexpressing ASGPR, in agreement with our previous reports.[48,49] Importantly, by comparison of the G5.NHAc-FI-LA/DOX complexes, the PEG spacer enables a significant higher DOX uptake of the G5.NHAc-FI-PEG-LA/DOX complexes ($p < 0.05$) under similar DOX concentrations. This is likely attributed to the fact that the PEG spacer can improve the flexibility of the targeting ligand LA, facilitating enhanced targeting to cancer cells via a receptor-mediated manner.

The LA-mediated targeting specificity of the G5.NHAc-FI-PEG-LA/DOX complexes was further confirmed by CLSM imaging of HepG2 cells (Figure S8, Supporting Information). It can be seen that the treatment of HepG2 cells with the G5.NHAc-FI-PEG-LA/DOX complexes renders the cells with strong FI-related green and DOX-related red fluorescence signals. In contrast, the cells treated with the nontargeted G5.NHAc-FI-*m*PEG/DOX complexes at the same DOX concentration display quite weak fluorescence signals. This further demonstrated the role played by the LA-mediated targeting, corroborating the flow cytometric analysis data. Furthermore, the PEG spacer-enhanced targeting specificity

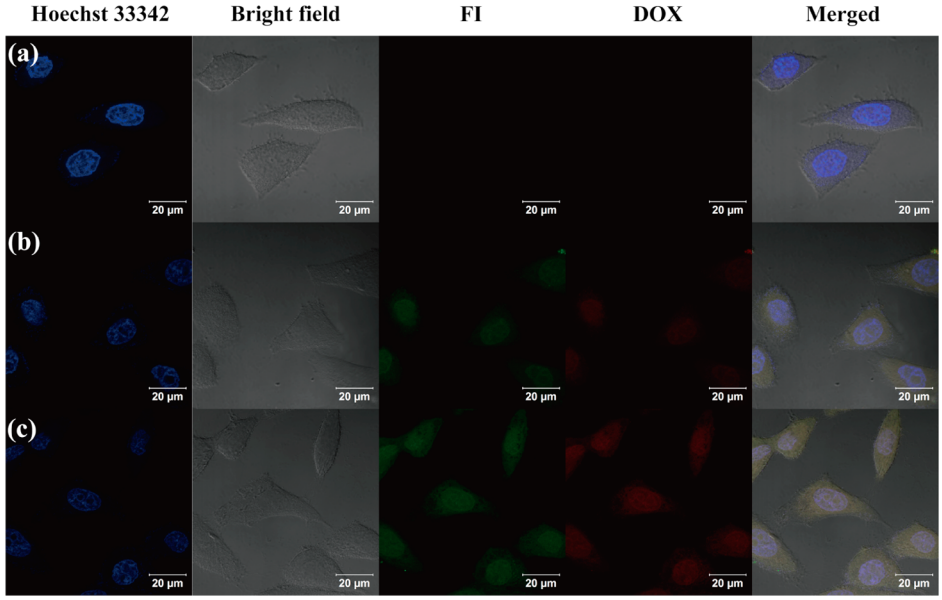


Figure 7. CLSM images of HepG2 cells treated with (a) PBS, (b) G5.NHAc-FI-LA/DOX, and (c) G5.NHAc-FI-PEG-LA/DOX at a DOX concentration of 4 mg/L for 3 h, respectively. Images were collected under similar instrumental conditions.

was also confirmed by CLSM imaging (Figure 7). After 3 h incubation with the G5.NHAc-FI-LA/DOX and G5.NHAc-FI-PEG-LA/DOX complexes, HepG2 cells display significant green and red fluorescence signals, which are associated with the specific internalization of FI-labeled dendrimer/DOX complexes into the cytoplasm of the cells. Similar to the flow cytometric analysis, the fluorescence signals of cells treated with G5.NHAc-FI-PEG-LA/DOX are much stronger than those treated with the G5.NHAc-FI-LA/DOX complexes at the same DOX concentration (4 mg/L). This further highlights the role played by the PEG spacer that can significantly improve the LA-mediated targeting specificity.

Targeted Antitumor Efficacy. With the LA-mediated targeting specificity, the G5.NHAc-FI-PEG-LA/DOX complexes are expected to have targeted antitumor efficacy. By incubating the HepG2 cells with both G5.NHAc-FI-*m*PEG/DOX or G5.NHAc-FI-PEG-LA/DOX complexes at different DOX concentrations for 4 h, the cells were rinsed and cultured with DOX-free medium for additional 48 h before resazurin reduction assay of cell viability (Figure S9, Supporting Information). It can be seen that under similar DOX concentrations, especially at high concentrations (2 or 4 mg/L), the viability of cells treated with G5.NHAc-FI-PEG-LA/DOX complexes is much lower than that treated with the nontargeted G5.NHAc-FI-*m*PEG/DOX complexes (57.0% vs 74.7% at DOX concentration of 4 mg/L, 72.2% vs 78.2% at DOX concentration of 2 mg/L). This demonstrates the role played by LA-mediated targeting specificity.

To check if the PEG spacer is able to enhance the targeted therapeutic efficacy of the G5.NHAc-FI-PEG-LA/DOX complexes, we compared the viability of HepG2 cells treated with both LA-targeted dendrimer/DOX complexes with or without PEG spacer (Figure 8). It can be observed that at the same DOX concentrations, especially at the high DOX concentration of 4 mg/L, HepG2 cells treated with G5.NHAc-FI-PEG-LA/DOX result in a significant decrease of viability (57.0%), much lower than those treated with the G5.NHAc-FI-LA/DOX complexes without PEG spacer (67.4%). This suggests that with the PEG spacer-enhanced targeting specificity as

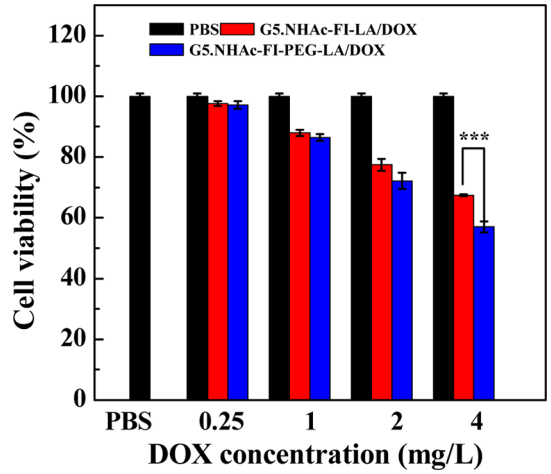


Figure 8. Resazurin reduction assay of HepG2 cells treated with the dendrimer/DOX complexes at different DOX concentrations for 4 h, followed by incubation with DOX-free fresh medium for another 48 h. HepG2 cells treated with PBS were used as control.

confirmed by flow cytometry and CLSM imaging, G5.NHAc-FI-PEG-LA/DOX complexes have enhanced targeted therapeutic efficacy in treating ASGPR-overexpressing liver cancer cells.

CONCLUSION

In summary, we synthesized multifunctional LA-targeted G5 dendrimers (G5.NHAc-FI-LA and G5.NHAc-FI-PEG-LA) with or without a PEG spacer for targeted anticancer drug delivery to ASGPR-overexpressing liver cancer cells. The formed dendrimer/DOX complexes are stable and afford sustained release of the DOX drug. The loading of DOX within both LA-targeted dendrimers does not compromise the therapeutic activity of DOX when compared with free DOX. The LA-modification onto the dendrimers enables specific targeting of both G5.NHAc-FI-LA/DOX and G5.NHAc-FI-PEG-LA/DOX complexes to ASGPR-overexpressing liver cancer cells, exerting

more toxicity to the target cells than the nontargeted dendrimer/DOX complexes. Importantly, as compared to the G5.NHAc-FI-LA dendrimers, the employed PEG spacer between the targeting ligand LA and the dendrimer platform renders the G5.NHAc-FI-PEG-LA/DOX complexes with a relatively fast DOX release rate, enhanced targeting specificity to ASGPR-overexpressing liver cancer cells, and thus enhanced antitumor therapeutic efficacy. The developed multifunctional dendrimer platform with a PEG spacer between targeting ligand and dendrimer surface may hold great promise to be used in different cancer therapy applications.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Additional materials characterization and in vivo assay data. This material is available free of charge via the Internet at http://pubs.acs.org/.

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: xshi@dhu.edu.cn.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This research is financially supported by the National Natural Science Foundation of China (21273032, 81341050), the Ph.D. Programs Foundation of Ministry of Education of China (20130075110004), the Science and Technology Collaboration Fund between China and Hungary, Ministry of Science and Technology, and the Program for Professor of Special Appointment (Eastern Scholar) at Shanghai Institutions of Higher Learning. X. S. gratefully acknowledges the Fundação para a Ciência e a Tecnologia (FCT) and Santander bank for the Invited Chair in Nanotechnology, and the FCT for funding through the project PTDC/CTM-NAN/1748/2012.

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