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A Novel Poly(L-glutamic acid) Dendrimer Based Drug Delivery System with Both pH-Sensitive and Targeting Functions

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Abstract: The functionalization of pH-sensitiveness and cellular targeting is a promising strategy to fabricate drug delivery systems with high efficiency, high selectivity and low toxicity. In this paper, a poly(L-glutamic acid) dendrimer based drug delivery system with both pH-sensitive and targeting functions is reported. Poly(L-glutamic acid) dendrimers with a polyhedral oligomeric silsesquioxane (POSS) nanocubic core were synthesized. Its globular morphology and compact structure with multiple peripheral functional groups made it suitable for drug delivery. The OAS-G_3-Glu dendrimer was conjugated with doxorubicin via pH-sensitive hydrazine bonds and targeting moiety (biotin). The cellular internalization and antitumor effects of the conjugates was evaluated in vitro. Both DLS and TEM results indicated that the conjugates aggregated into nanoparticles with diameters around 50 nm. The release rates of doxorubicin at pH 5.0 were much faster than those at pH 7.0 due to the acid cleavage of the hydrazine bonds. The internalization study revealed that the cellular uptake of the biotin modified conjugates was mainly through receptor-mediated endocytosis. These results indicate that our poly(L-glutamic acid) dendrimers with OAS core are promising vectors for fabricating smart and targeting drug delivery systems.

Keywords: Poly(L-glutamic acid) dendrimer; targeting; drug delivery; doxorubicin; pH-sensitive

Introduction

Dendrimers offer many advantages as drug carriers for their unique properties such as monodisperse, nanoscale size, semiglobular or globular structures and multiple peripheral functional groups. Extensive studies have already demonstrated that dendrimers are good carriers for drugs in transdermal, oral, ocular and pulmonary delivery systems.[1−4] Peptide dendrimers originate from amino acids, and they resemble protein-like globular structures. These characteristics provide peptide dendrimers with excellent biodegradability and biocompatibility.[5] These features have recently been explored in the development of drug carriers. Most of the drugs are immobilized on the peripheral functional groups of peptide dendrimers to form dendrimer−drug conjugates.[7]

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The drugs are then released by the chemical or enzymatic cleavage of the biodegradable labile bonds.[6]

Polyhedral oligomeric silsesquioxane (POSS) units are three-dimensional, cubic shaped building blocks that contain an inorganic inner siloxane nanocore with the possibility of chemical modification at each of the eight corners of the POSS unit.[7] The introduction of POSS core in peptide dendrimers would largely amplify the amount of peripheral groups and thus simplify the difficult synthetic process. Lu's group prepared poly(L-lysine) dendrimer with octa(3-aminopropyl) silsesquioxane (OAS) as the cubic core and used it as gene delivery vector.[8] The dendrimers possessed a globular morphology, a relatively rigid structure and a highly functionalizable surface,[9,10] which made OAS-cored dendrimers ideal carriers for drug delivery systems.[11]

Nanodrug carriers could be concentrated in tumor cells through the effect of enhanced permeability and retention (EPR) or the mechanism of receptor-mediated endocytosis (RME).[12] The targeting delivery of high dose chemotherapeutics using cancer cell-specific ligands is an attractive alternative for the successful treatment of tumors.[13,14] The specific targeting moieties included sugar,[15] folic acid,[16] antibody,[17] peptide[18] and epidermal growth factor.[19] Biotin is an essential micronutrient for cellular functions, growth and development. It must be obtained from exogenous sources via intestinal absorption.[20] The rapid proliferation of cancer cells needs extra biotin, and the cancer cells often overexpress biotin-specific receptors on the surface. Accordingly, several research groups developed different biotinylated chemotherapeutic agents for cancer cell-specific drug delivery.[21] The results have shown that biotin-conjugated macromolecular carriers can enhance the uptake of anticancer drugs to tumor cells.[22]

As the environment around tumor tissues is acidic, drug carriers with pH-sensitive and targeting functions are desired for antitumor drug delivery systems. There are many advantages for peptide dendrimers as antitumor drug carriers. The peripheral functional groups of peptide dendrimer can not only immobilize targeting moieties but also link antitumor drugs with pH-sensitive bonds. The peptide dendrimers thus provide a useful platform for easy and convenient fabrication of smart and targeting drug delivery systems. In this paper, we report the synthesis and characterization of poly(L-glutamic acid) dendrimers with OAS cores. Doxorubicin (DOX), a widely used antitumor drug, was conjugated to the peripheral groups of the dendrimers through pH-sensitive hydrazone bonds. In addition, biotin was immobilized on the dendrimers as a specific tumor cell targeting moiety. Thus the DOX−poly(L-glutamic acid) dendrimer conjugates were fabricated with dual targeting and pH-sensitive functions. We also report the subsequent studies of drug release, antitumor effects and the cellular internalization of the conjugates.

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Materials and Methods

Materials. Sulfo-NHS-LC-biotin and avidin/hydroxyazobenzene-2-carboxylic acid (HABA) reagents were obtained from Sigma-Aldrich (Shanghai, China). (3-Aminopropyl)triethoxysilane, succinic anhydride, *N*,*N*-diisopropylethylamine (DIPEA), trifluoroacetic acid (TFA), 4-(dimethylamino)pyridine (DMAP), 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide (EDC) and N_α-di-*t*-OBut-L-glutamic acid (H-Glu(OBut)-OBut·HCl) were purchased from Asta Tech Pharmaceutical (Chengdu, China). DOX hydrochloride (DOX-HCl) was purchased from Zhejiang Hisun Pharmaceutical. 2-(1*H*-Benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) and 1-hydroxybenzotriazole hydrate (HOBt) were obtained from Highfine Biotech Co. Ltd. (Jiangsu, China). All the organic solvents used in this study were purified using the standard method and distilled before use.

Methods. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker Avance II NMR spectrometer at 400 MHz using $CDCl_3$ and DMSO-d_6 as solvents with 0.5% tetramethylsilane as the internal standard. The molecular weights of the dendrimers were tested on Autoflex MALDI TOF MS (Bruker, USA) in the linear mode with α-cyano-4-hydroxycinnamic acid as a matrix. The absorbance of DOX with different concentration was recorded on a Lambda 650S UV−vis spectrometer (Perkin-Elmer) at 485 nm. The size of particles was measured by dynamic light scattering (DLS) (NANO ZSPO, Malvern) in aqueous solution. The morphology of the particles was performed on a transmission electron microscope (TEM) (H-600 electron microscope) with the sample stained by phosphotungstic acid on a carbon-coated copper grid. The laser confocal images were captured on a LEICA TCS SP5 using a 40× water-immersion objective lens and processed by Leica Confocal Software. DOX was excited at 488 nm with emissions at 595 nm.

Synthesis of OAS·HCl. Octa(3-aminopropyl)silsesquioxane hydrochloride (OAS·HCl) was prepared according to the previously reported method.[23] Briefly, (3-aminopropyl)-triethoxysilane (15 mL, 60 mmol) and 30%−36% concentrated HCl (30 mL) were dissolved in MeOH (350 mL) with stirring for 3 days at room temperature. The crude product was filtrated, washed with cold MeOH, and dried. The product was recrystallized in hot MeOH to give white solid (yield: 30%). ^{1}H NMR ($(CD_3)_2SO$, δ): 8.23 (s, 24H), 2.76 (t, 16H, $-Si(CH_2)_2CH_2$), 1.71 (m, 16H, $-SiCH_2CH_2$), 0.72 (t, 16H, $-SiCH_2$). ^{13}C NMR ($(CD_3)_2SO$) δ: 40.53, 20.13, 7.96. ^{29}SiNMR ($(CD3)_2SO$,δ): 66.4. MALDI-TOF (*m*/*z*, $[M + H]^+$): 881.50 (calculated), 881.27 (observed).

Synthesis of OAS-SA. Succinic acid terminated OAS (OAS-SA) was synthesized according to the published method.[24] Triethylamine (2 mL, 14 mmol) and succinic anhydride (3.0 g, 3 mmol) were added orderly into a methanol (150 mL) solution of OAS·HCl (2.0 g, 1.8 mmol) in an ice bath. The mixture was stirred at room temperature overnight. After the evaporation of the methanol in reduced pressure, sticky oil was formed. Chloroform was added and a white precipitation was collected via centrifugation and dried in vacuum to obtain a white powder (2.2 g, 80%). ^{1}H NMR (D_2O, δ): 0.59 (br, 2H, $-SiCH_2$), 1.44 (br, 2H, $-SiCH_2CH_2$), 2.30 (br, 2H, $-COCH_2CH_2-$), 2.41 (br, 2H, $-CH_2CH_2COOH$), 3.02 (br, 2H, $-Si(CH_2)_2CH_2$). ^{13}C NMR ($(CD_3)_2SO$, δ): 8.74, 22.48, 29.15, 29.98, 41.0, 170.92, 173.89. ^{29}Si NMR (D_2O, δ): 65.3. MALDI-TOF (*m*/*z*, $[M + H]^+$): 1680 (calculated), 1681.40 (observed).

Synthesis of OAS-Poly(L-glutamic acid) (OAS-Glu) Dendrimers. The reaction was carried out similarly to the synthesis of poly-L-lysine octa(3-aminopropyl)silsesquioxane dendrimers[8] with some modifications. Generations 1, 2, and 3 of the dendrimers were simplified as G_1, G_2 and G_3.

Synthesis of tert-Butyl Ester-Protected OAS-Poly(L-glutamic acid) Dendrimers. Generation 0 (single OAS core) to generation 2 (G_0-G_2) carboxyl terminated OAS-poly(L-glutamic acid) dendrimers (1−2 g), HBTU (1.5 equiv with respect to the carboxyl group), and HOBT (1.5 equiv with respect to the carboxyl group) were dissolved in 30 mL of anhydrous DMF in a three-necked flask equipped with a drop funnel. Excess H-Glu(OBut)-OBut·HCl (2−3 equiv with respect to the carboxyl group) was dissolved in 15 mL of DMF and added dropwise. The solution was stirred at 0 °C for 30 min in N_2 atmosphere. *N*,*N*-Diisopropylethylamine (2 equiv with respect to the amount of H-Glu(OBut)-OBut·HCl, usually 3 mL) was added. The mixture was stirred at room temperature for 2 or 3 days. After the removal of DMF, chloroform was added and the mixture was washed with saturated $NaHCO_3$, $NaHSO_4$, and brine solution several times. The solution was dried with $MgSO_4$ and the chloroform was removed to give yellow solid. The crude product was recrystallized in cold acetonitrile, and a colorless solid was obtained.

Deprotection of tert-Butyl Ester. A series of *tert*-butyl ester-terminated OAS-poly(L-glutamic acid) dendrimers (G_1p-G_3p) was treated with TFA (10 equiv, according to the number of *tert*-butyl ester groups) in an ice bath, and the mixture was stirred for 8−10 h to remove *tert*-butyl groups. The solution was concentrated to give viscous oil. The residue was treated with anhydrous diethyl ether, and a colorless solid product was obtained. The yields of the products were 90%, 85%, 75%, respectively.

Synthesis of *tert*-Butyl Hydrazinecarboxylate. *tert*-Butyl Hydrazinecarboxylate($BocNHNH_2$) was synthesized according to a published method.[25] Briefly, hydrazine monohyate (18 g, 80%) was mixed with isopropanol (300 mL), and a solution of Boc_2O (28 g, 128 mmol) in isopropanol (100 mL) was added dropwise at 0 °C for 2 h. Then the mixture

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was stirred at room temperature overnight. After the isopropanol was evaporated, the residue was dissolved in CH_2Cl_2 and dried over $MgSO_4$. CH_2Cl_2 was removed, the product was recrystallized in hexane, and colorless crystals were obtained. ^{1}H NMR ($CDCl_3$, δ): 6.13 (s, 1H, −CON*H*NH$_2$), 3.68 (s, 2H, −N*H*$_2$), 1.42 (s, 9H, (C*H*$_3$)$_3$C−). ^{13}C NMR ($CDCl_3$, δ): 58.3, 77.2, 28.5.

Synthesis of Compound 3a. The G_3·TFA (1 g, 0.12 mmol) was dissolved in DMF (30 mL). EDCI (4.2 g, 0.022 mol), HOBt (2.9 g, 0.022 mol) and DIEA (3 mL) were added. The whole solution was activated at 0 °C for a half-hour. Excess *tert*-butyl hydrazinecarboxylate (4.8 g, 0.037 mol) was added to the solution and stirred for a further 2 days in nitrogen atmosphere. After removal of the solvent, chloroform was added and the solution was washed with saturated $NaHCO_3$ and $NaHSO_4$ solutions in order. The solution was dried with $MgSO_4$. The crude product of OAS-G_3-Glu-NHNHBOC was recrystallized in acetonitrile, and a white solid was obtained (789 mg, 45%). ^{1}H NMR ((CD$_3$)$_2$SO, δ): 10.17−9.30 (m, 64H, −OCON*H*NH−), 9.06−8.44 (m, 64H, −NHN*H*CO−), 8.4−7.67 (m, 56H, −CON*H*CH− and 8H, −CH$_2$N*H*CO−), 4.26 (s, 56H, −C*H*), 3.00 (s, 16H, −Si(CH$_2$)$_2$C*H*$_2$), 2.45−2.00 (m, 32H, −COC*H*$_2$C*H*$_2$CO− and 144H, −CH$_2$C*H*$_2$CO−), 1.98−1.61 (d, 144H, −CHC*H*$_2$CH$_2$−), 1.57−1.15 (m, 592H, (C*H*$_3$)$_3$C− and 16H, −SiCH2C*H*$_2$), 0.58 (s, 16H, −SiC*H*$_2$). ^{13}C NMR ((CD$_3$)$_2$SO, δ): 179.02−168.45, 167.02−163.47, 163.93−160.90,159.01−153.12,103.61−94.92,79.53,57.19−46.84, 38.69, 36.23, 31.23, 28.50. MALDI-TOF (*m*/*z*, [M + H]$^+$): 16217.7928 (calculated for $C_{656}H_{1128}N_{192}O_{268}Si_8$), 16245.57 (observed). OAS-G_3-Glu-NHNHBOC (500 mg) was treated with TFA (2 mL) and precipitated in diethyl ether, resulting in compound **3a** as white powder (70%, 240 mg).

Synthesis of Compound (3b). OAS-G_3-Glu-NHNH$_2$ (**3a**, 500 mg) was dissolved in 4 mL of distilled water. Sulfo-NHS-LC-biotin (370 mg) was added at a molar ratio of 1:20. The mixture was reacted for 4 h and transferred to a dialysis bag (MWCO = 7000 Da) to remove the unreacted sulfo-NHS-LC-biotin. The obtained liquid was lyophilized and stored.

4-Hydroxyazobenzene-2-carboxylic Acid (HABA) Assay. 4-Hydroxyazobenzene-2-carboxylic acid (HABA) /AVIDIN REAGENT (Sigma, product No. H 2153) was used to define the content of biotin on each dendrimer. The reagent was prepared according to the manufacturer's instructions by simple reconstitution with 10 mL of deionized water. Briefly, 100 μL of biotinylated dendrimer solution was added to 900 μL of the avidin/HABA reagent solution and the absorbance was measured at 500 nm using UV−vis spectrometry.

Formation of DOX−Dendrimer Conjugates (3c). OAS-G_3-Glu-NHNH$_2$/biotin (**3b**, 12 mg) and excess DOX (300 mg) were dissolved in 50 mL of anhydrous methanol with a drop of acetic acid as a catalyst. The solution was stirred at room temperature in the dark for 40 h. The product solution was ultrafiltered and washed in Amicon Ultra tubes. The unreacted DOX was further removed with a Sephadex LH-20 column.

Drug Release Experiment. The release studies were performed at 37 °C in the phosphate buffer with different pH values (pH 5.0 and 7.4). The OAS-G_3-Glu(NHN-DOX)/biotin complex (**3c**, 4 mg) was dispersed in 2 mL of PBS medium and placed in a dialysis bag with a molecular weight cutoff of 3 kDa. The dialysis bag was then immersed in 30 mL of the release medium and kept in a horizontal shaker maintained at 37 °C for 170 rpm. 2 mL of the medium was removed periodically, and the same volume of a fresh PBS was added. The amount of released DOX was determined with UV−vis spectrophotometry at 485 nm.

Cell Cytotoxicity. HeLa cells were cocultured with dendrimers (compounds **3a** and **3b**) with different concentrations to study cell cytotoxicity. The medium was DMEM (Hyclone, Beijing) containing 10% fetal bovine serum and 1% antibiotics (antibiotic-antimycotic, Gibco BRL, USA) at 37 °C with 5% CO_2 atmosphere. Cell cytotoxicity was tested by Cell Counting Kit-8 assay.

In Vitro Antitumor Effects. The antitumor effects of OAS-G_3-Glu(NHN-DOX) and OAS-G_3-Glu(NHN-DOX)/biotin were evaluated in vitro with HeLa cells. The assay was performed in triplicate with a density of 1×10^4 cells per well in 96-well plates. After 48 h cultivation, the medium was replaced with a fresh culture solution containing free DOX, OAS-G_3-Glu(NHN-DOX), and OAS-G_3-Glu(NHN-DOX)/biotin (the DOX content ranging from 3 μg/mL to 15 μg/mL) and incubated for 48 h. Thereafter, 10 μL of CCK-8 was added to each well and incubated for another 1 h and the absorbance of each well was read on a scanning enzyme-linked immunosorbent assay (ELISA) reader at a wavelength of 490 nm.

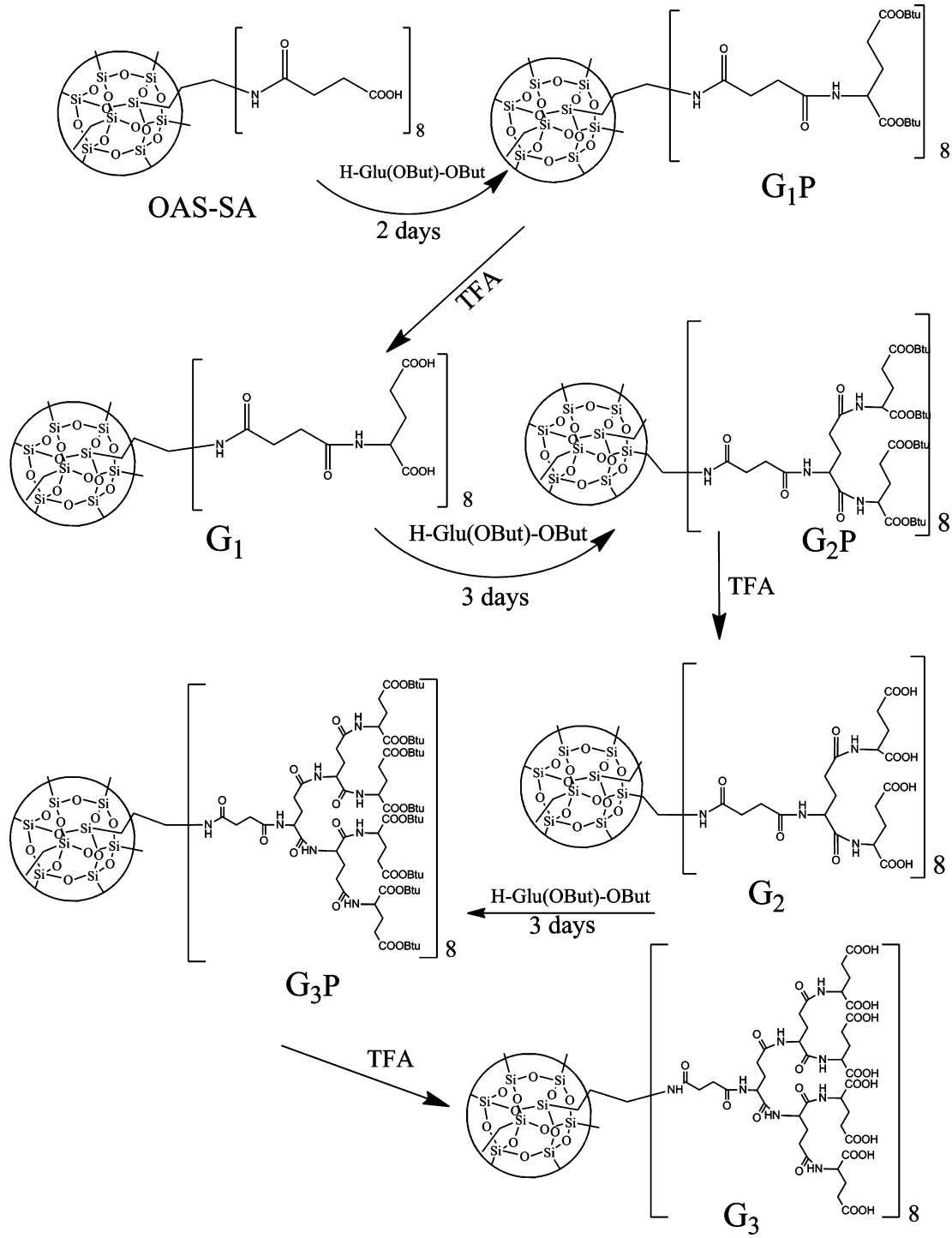
Cellular Internalization Study. The cellular uptake was examined on flow cytometry (Cytomics FC500, Beckman Coulter) and confocal laser scanning microscopy. In flow cytometry analysis, HeLa cells (about 2×10^5) were incubated in six-well plates overnight. Three samples OAS-G_3-Glu(NHN-DOX), OAS-G_3-Glu(NHN-DOX)/biotin (DOX concentration was 10 μg/mL), and OAS-G_3-Glu(NHN-DOX)/biotin with free biotin (0.5 mg/mL) were added. After 2 h treatment, the cells were rinsed with PBS buffer, trypsinized and resuspended in 500 μL of PBS buffer. The FACS Calibur flow cytometer was used and analyzed approximately 10^4 cells from each sample. The mean fluorescence of the gated viable cells was quantified and shown on a four-decade log scale.

For confocal microscopy studies (CLSM), HeLa cells were seeded on 22 mm glass coverslips in a six-well plate on the day before the experiment. The cells were treated with free DOX, OAS-G_3-Glu(NHN-DOX), and OAS-G_3-Glu(NHN-DOX)/biotin (those three with the same DOX concentration: 10 μg/mL) for 0.5 and 2 h, washed and fixed with 1.5% formaldehyde and watched under confocal laser scanning microscopy.

Results and Discussion

The synthetic process of OAS-poly(L-glutamic acid) dendrimers is shown in Scheme 1. With the consideration of the instability of the silsesquioxane units in aqueous

Scheme 1. The Formation of OAS-Poly(L-glutamic acid) Dendrimers



alkaline solutions[26] and the incomplete hydrolysis of methyl or ethyl ester under acidic catalysts,[27] *tert*-butyl ester-protected glutamic acid was used as the reagent. An excess of N_α-di-*t*-OBut-L-glutamic acid·HCl (H-Glu(OBut)-OBut·HCl) was used in each step to ensure the complete reaction of the surface carboxyl groups with *tert*-butyl ester-protected L-glutamic acid.

The formation OAS-poly(L-glutamic acid) dendrimers with *tert*-butyl ester groups was confirmed by ^{1}H NMR as shown in Figure 1. The peaks at 0.54 ppm, 1.47 ppm and 3.02 ppm were the characteristic α-, *β*- and *γ*-protons of the methylene groups adjacent to the silicon atoms of the OAS cores, consistent with the results previously reported.[8] The signal

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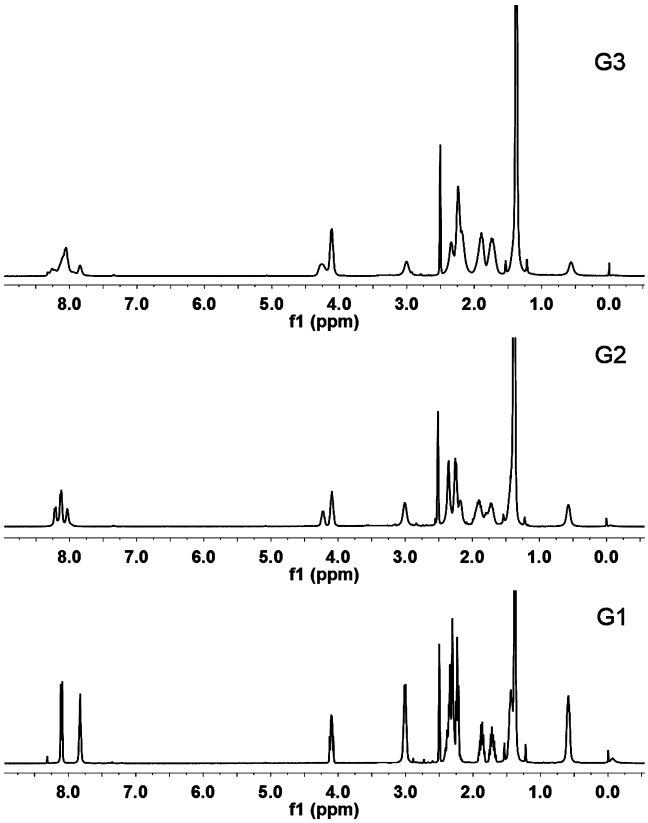

Figure 1. The ^{1}H NMR spectra of *tert*-butyl ester-protected OAS-poly(L-glutamic acid) dendrimers.

at 1.38 ppm was attributed to the protons of *tert*-butyl group in the glutamic acid skeleton. The peaks at 2.05−2.42 ppm were ascribed to the γ-protons of the L-glutamic acid side chains and the methylene groups in the succinic anhydride chains. The presence of two multiples at 1.70 and 1.86 ppm was assigned to the two methylene protons next to the α-proton of the peptide bond, and their integrity ratio was 1:1. The peak at 4.10 ppm in the ^{1}H NMR spectrum of G_1 was assigned to the α-proton of glutamic acid. When a second generation of L-glutamic acid was conjugated to the dendrimer surface, this peak split into two peaks between 4.09 and 4.23 ppm because of the different chemical environment after the conjugation to the α- and γ-carboxyl groups in the L-glutamic residue of G_1 dendrimers. The peaks at 7.71−8.24 ppm were observed due to the active proton in the formed amide bond.

In order to fabricate pH sensitive drug delivery system, the *tert*-butyl ester-protected OAS-G_3-Glu dendrimer was deprotected and substituted with *tert*-butyl hydrazinecarboxylate to immobilize DOX with hydrazide bond. If hydrazine hydrate was used in usual conditions,[28,29] the silsesquioxane units would break down in the strong basic environment and an intermolecular reaction among dendrimers would occur. So, in this paper, *tert*-butyl hydrazinecarboxylate was used to avoid this problem. The obtained OAS-G_3-Glu-NHNHBOC dendrimer was confirmed by ^{1}H NMR spectra as shown in Figure 2a. With the comparison of the ^{1}H NMR spectra of OAS-G_3-Glu dendrimer, the characteristic peaks of the OAS core were both observed in the two

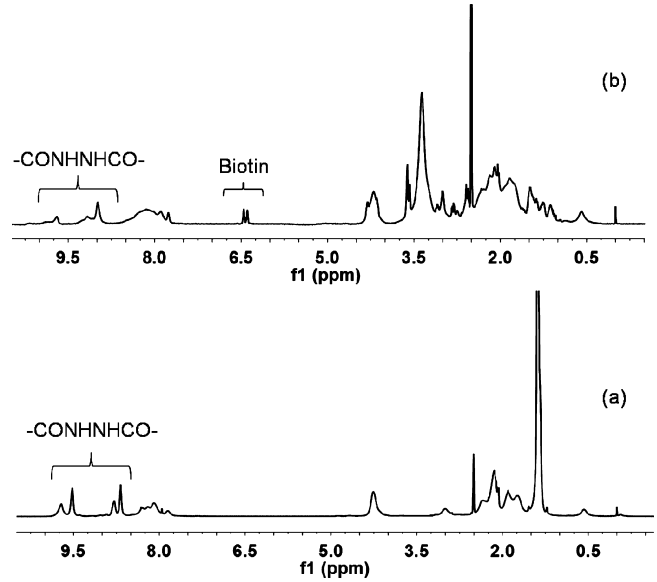

Figure 2. The ^{1}H NMR spectra of (a) OAS-G_3-Glu-NHNHBoc and (b) OAS-G_3-Glu-$NHNH_2$/biotin.

spectra. The double peaks at 8.6−9.7 ppm were attributed to the protons of newly formed bond (−NHNH−).

Biotin is a member of vitamin family (vitamin B) and is an essential micronutrient whose level is high in rapidly proliferating cells such as cancer cells.[30] Several research groups have demonstrated that dendrimers with biotin molecules contribute to the enhancement of specific cancer cell uptake.[22,31−33] Sulfo-NHS-LC-biotin, a derivative of D-biotin, acted as a biotinylation agent. The most important advantage of this reagent was that the 6-aminocaproic acid spacer prolonged the length between the covalently modified molecule and the bicyclic biotin rings,[20] which sharply reduced steric hindrance and improved drug loading efficiency.

After the removal of Boc groups, the resultant OAS-G_3-Glu-$NHNH_2$ dendrimers were reacted with sulfo-NHS-LC-

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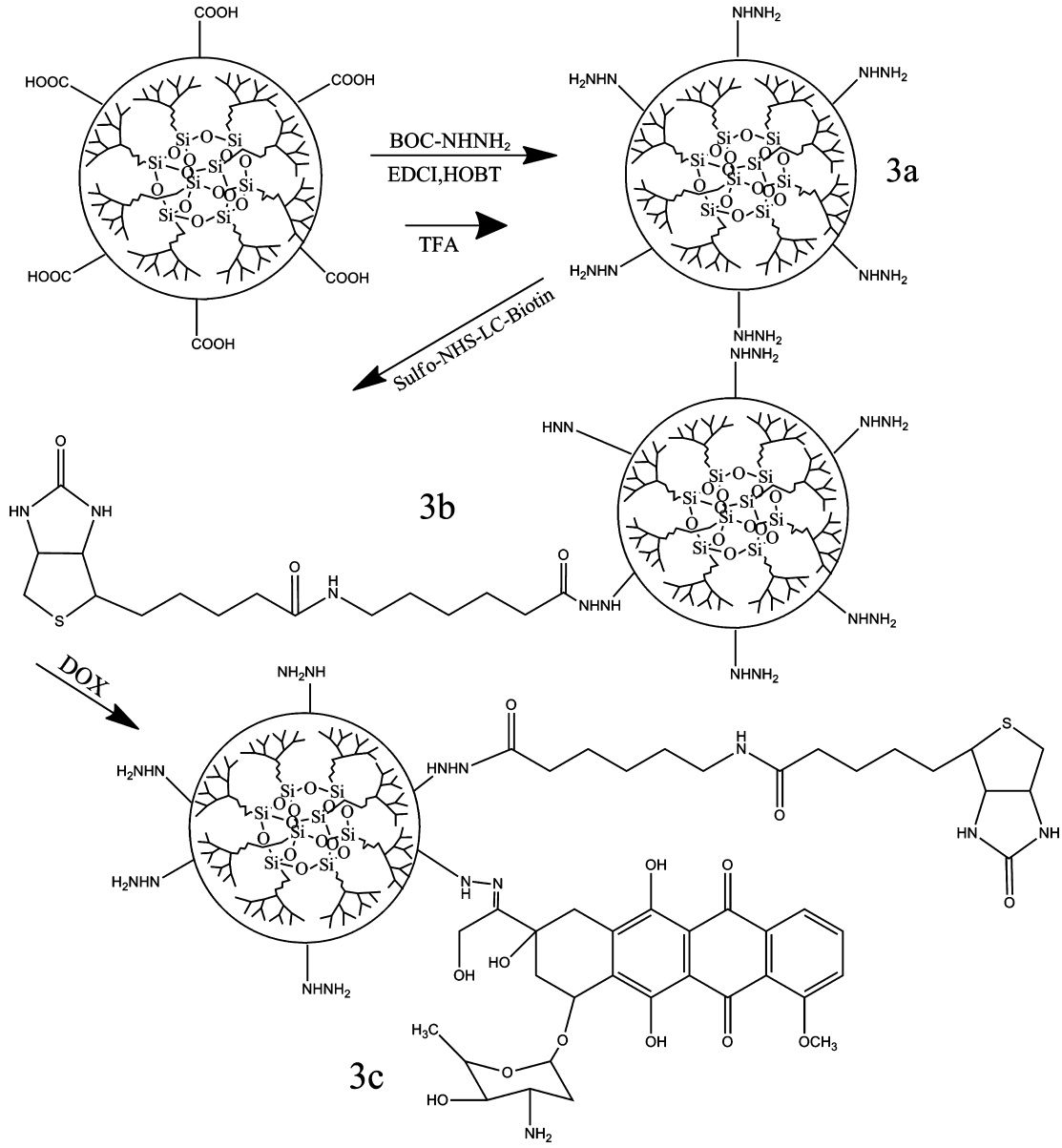
Scheme 2. The Formation of OAS-G_3-Glu(NHN-DOX)/Biotin Complex



biotin molecules (1:20). The peaks between 6.3 and 6.5 ppm in the ^{1}H NMR spectrum were the characteristic protons of biotin as shown in Figure 2b. The content of biotinylation was calculated using the formula given by Sigma-Aldrich (the product information of HABA/AVIDIN REAGENT).

(1) μmol/mL $= 10(\Delta A_{500}/34)$

$34 =$ mM extinction coefficient at 500 nm

$10 =$ dilution factor of sample into cuvette

(2) $(\Delta A_{500}) = 0.9A_{(\text{HABA/avidin})} - A_{\text{HABA/avidin+sample}}$

$0.9 =$ dilution factor of HABA/avidin upon addition of sample

The results of both ^{1}H NMR (Figure 2b) and avidin/HABA assay revealed that four biotin molecules were in average conjugated to each dendrimer.

Table 1. The Composition of DOX−Dendrimer Conjugates

sample	terminal group	[DOX]	[biotin]	MW[a]	drug loading (%)
G_3-Glu($NHNH_2$)	64			9835	
G_3-[DOX]	56	8		14131	30
G_3-[DOX]-[biotin]	54	6	4	14026	24

[a] Molecular weights calculated from its structure.

Doxorubicin molecules were bonded to both biotinylated (OAS-G_3-Glu-$NHNH_2$/biotin) and biotin-free (OAS-G_3-Glu-$NHNH_2$) agents through Schiff bases as shown in Scheme 2. The composition of the conjugates is shown in Table 1.

Particle size and distribution are two important aspects in determining whether the conjugates are suitable for drug delivery. It was proposed that nanoparticles (NPs) with sizes less than 5 μm would be taken up via the lymphatics and sizes less than 500 nm could cross the membranes of

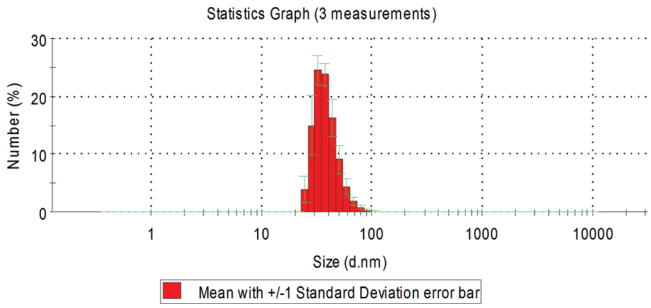


Figure 3. Dynamic laser scattering (DLS) of OAS-G_3-Glu(NHN-DOX)/biotin complexes.

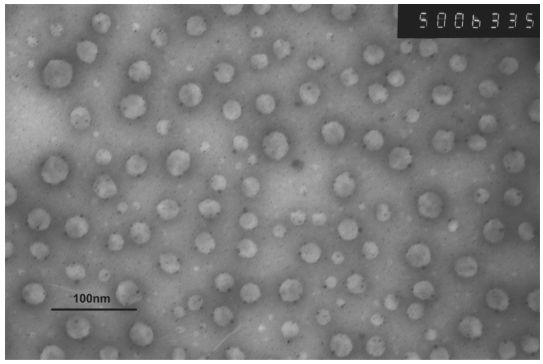


Figure 4. TEM image of OAS-G_3-Glu(NHN-DOX)/biotin complexes.

epithelial cells through endocytosis.[34,35] The size distribution of the OAS-G_3-Glu(NHN-DOX)/biotin complexes was investigated by DLS. Figure 3 shows the size distribution of the complex at a concentration of 10 μg/mL. As shown in Figure 3, the size distribution of the complex is rather narrow and the average hydrodynamic diameter is peaked at 45 nm. This size range makes these particulate conjugates ideal candidates for drug delivery as far as size is concerned.

The size and morphology of the OAS-G_3-Glu(NHN-DOX)/biotin conjugates were further evaluated by TEM. Figure 4 shows that the nanoparticles are spherical and uniform. The diameters of the complexes as observed by TEM are in the range of 30−50 nm, consistent with the size range from the DLS studies. These sizes are slightly bigger than the individual dendritic conjugate molecules themselves, and the difference is clearly due to the conjugate aggregation. The nanoparticles with diameters less than 100 nm were suitable for injectable anticancer drug carriers due to the EPR effect.[36] Hence, these nanoparticles aggregated from the conjugates are adequate for antitumor drug delivery.

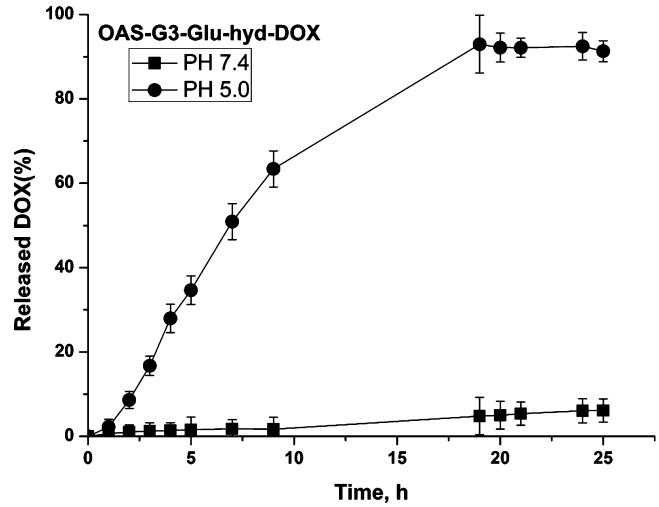


Figure 5. In vitro DOX releases from Glu(NHN-DOX)/biotin complex at pH 7.4 and 5.0.

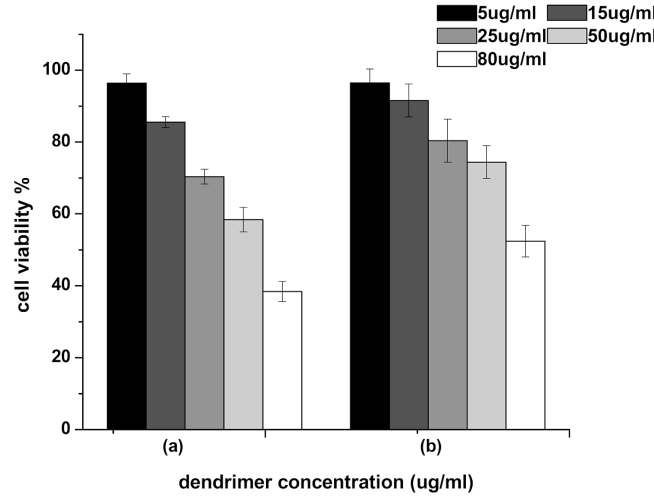


Figure 6. Cytotoxicity of drug carriers: (a) OAS-G_3-$NHNH_2$ and (b) OAS-G_3-$NHNH_2$/biotin against HeLa cell after 48 h incubation.

The release behavior of DOX from dendrimer−drug conjugates under different pH conditions was investigated to evaluate how pH variation affected the cleavage of polymer−drug linkages. Figure 5 shows the release of DOX. DOX release was found to be slow at pH 7.4, and the percentage of the released DOX was only 12% within 24 h, showing that the hydrazone bond was relatively stable at pH 7.4. On the contrary, a much faster release of DOX was observed at pH 5.0. Clearly, the cleavage of the pH sensitive hydrazone bonds became far easier in slightly acidic environment. After 20 h, the totally released DOX reached about 90% at pH 5.0.

The cytotoxicity of the dendritic carriers was evaluated. As indicated in Figure 6, the cytoxicity of the biotin free and biotin modified dendrimers is closely related to its concentrations. Both dendrimers had low cytotoxicity when the concentrations were below 15 μg/mL. The cytotoxicity then increased with the increase of dendrimer concentration. The cytotoxicity was likely to originate from the cationic

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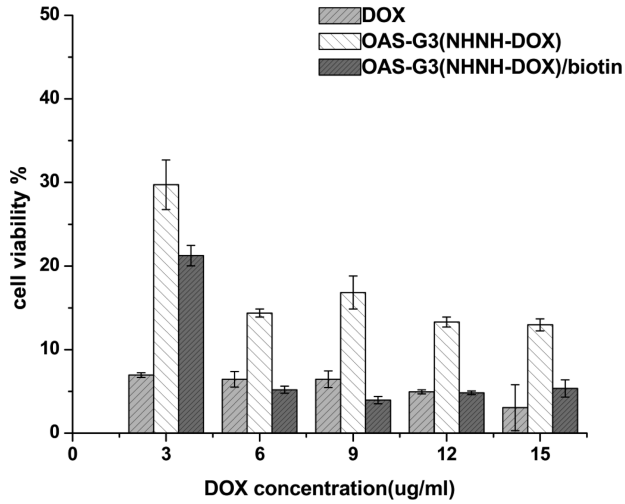

Figure 7. The cytotoxicity of free DOX, OAS-G_3-Glu(NHN-DOX), and OAS-G_3-Glu(NHN-DOX)/biotin against HeLa cells after 48 h incubation. DOX concentrations were 3, 6, 9, 12, 15 μg/mL for each group.

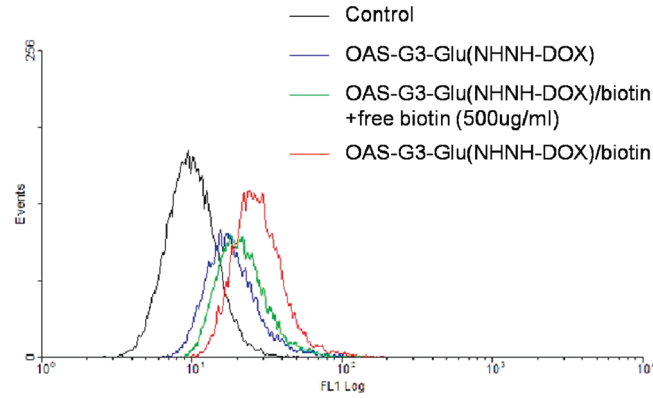

Figure 8. Flow cytometry results of HeLa cells that were incubated with OAS-G_3-Glu(NHN-DOX), OAS-G_3-Glu(NHN-DOX)/biotin (DOX concentration is 10 μg/mL) and OAS-G_3-Glu(NHN-DOX)/biotin (in the presence of 500 μg/mL free biotin) for 2 h at 37 °C.

charges[37] of the dendrimers. It was lower in biotin-immobilized dendrimers because the bond linkages reduced the level of cationic charges.

Figure 7 shows the antitumor effects of the DOX−dendrimer conjugates. The viability of HeLa cells incubated with free DOX, OAS-G_3-Glu(NHN-DOX) and OAS-G_3-Glu(NHN-DOX)/biotin (DOX concentrations ranged from 3 μg/mL to 15 μg/mL) for 48 h were tested. Cell viability dramatically decreased in the presence of free DOX, and it maintained at 4%−8% even though the concentration of DOX increased, showing that the DOX concentration of 3 μg/mL was enough to kill tumor cells in vitro. The DOX released from DOX−dendrimer conjugates exhibited lower antitumor effects. When the concentration was higher than 6 μg/mL, the cell viability of conjugates with biotin was much lower than that of conjugates without biotin. The difference demonstrated that the targeting moiety offered by biotin was efficient at enhancing the tumor cell targeting in vitro.

The cell uptake and intracellular distribution of the conjugates were studied by flow cytometry analysis and confocal laser scanning microscopy (CLSM). Flow cytometry analysis was carried out to quantitatively evaluate the endocytosis of OAS-G_3-Glu(NHN-DOX) and OAS-G_3-Glu(NHN-DOX)/biotin (DOX concentration was kept at 10 μg/mL). Since DOX itself is a fluorophore, the fluorescence intensity is proportional to the amount of internalized DOX in Hela cells. For the results shown in Figure 8, we have used the cells without any treatment as control. These cells showed autofluorescence only. Due to the nonspecific interaction between positively charged conjugates and the negatively charged cell membranes, the OAS-G_3-Glu(NHN-DOX) showed a normal dose response after 2 h incubation. Although the DOX concentration and cell incubation time of OAS-G_3-Glu(NHN-DOX) and OAS-G_3-Glu(NHN-DOX)/biotin were the same, the fluorescence intensity of OAS-G_3-Glu(NHN-DOX)/biotin exhibited was higher than that of OAS-G_3-Glu(NHN-DOX). This difference clearly arose from the biotin facilitation of the cell uptake process. To further determine the role of biotin in cell specific uptake process, the fluorescence intensity of the OAS-G_3-Glu(NHN-DOX)/biotin conjugates (DOX concentration was kept the same as the above two samples) with additional free biotin (0.5 mg/mL) was tested. Interestingly, the presence of excess biotin effectively decreased the cellular uptake of OAS-G_3-Glu(NHN-DOX)/biotin conjugates to the HeLa cells, with a reduction of nearly 30% of the cell fluorescence intensity being observed. This funding was consistent with the previous studies,[30] though the exact mechanistic process causing the apparent "overdosing" remains unclear. Nevertheless, these results together show that the cellular uptake of DOX−dendrimer conjugates can be enhanced with the introduction of biotin and that the OAS-G_3-Glu(NHN-DOX)/biotin conjugates must be mainly internalized by the biotin-receptor mediated endocytosis.

Confocal laser scanning microscopy (CLSM) was used to visualize the internalization of the conjugates. Figure 9 shows the confocal images of HeLa cells treated with free DOX, OAS-G_3-Glu(NHN-DOX) and OAS-G_3-Glu(NHN-DOX)/biotin for 0.5 and 2 h. In each time, the cells exposed to the free DOX showed the highest fluorescence intensity in the nuclei, due to the rapid transport of DOX into the cytosol, followed by the subsequent diffusion to nuclei and aggregation there.[38] The fluorescence intensity of OAS-G_3-Glu(NHN-DOX)/biotin was much stronger than that of OAS-G_3-

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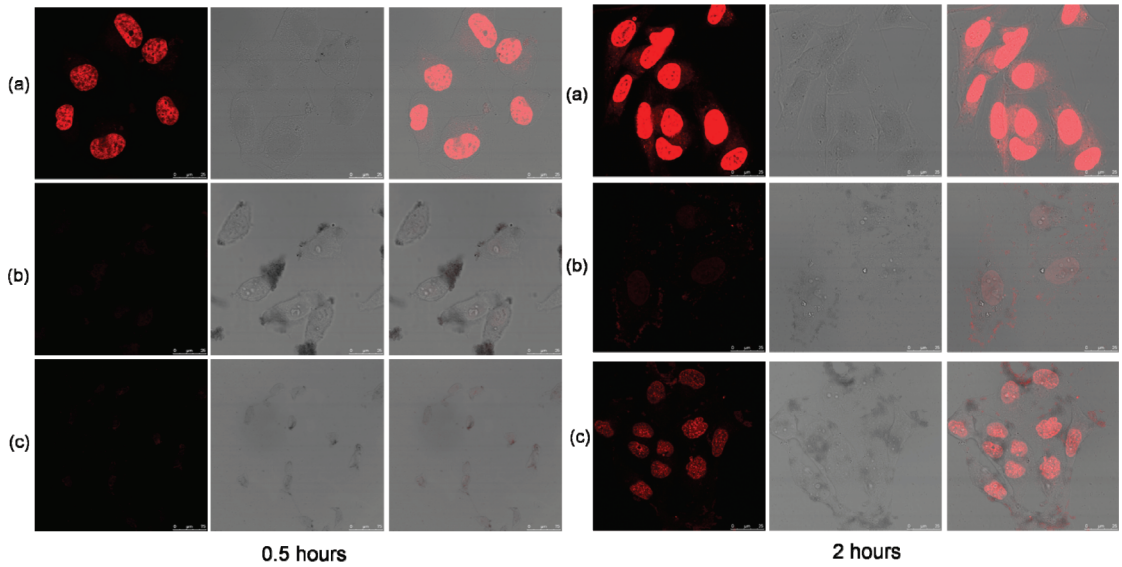

Figure 9. CLSM images of HeLa cells. The cells were incubated with (a) free DOX, (b) OAS-G_3-Glu(NHN-DOX) and (c) OAS-G_3-Glu(NHN-DOX)/biotin for 0.5 h and 2 h.

Glu(NHN-DOX), consistent with the enhancement of cellular internalization associated with the biotin's targeting effect via the receptor-mediated endocytosis. In contrast, the biotin-free conjugates were taken up by the HeLa cells through the simple endocytosis process. The amount of conjugates internalized was less, resulting in the weaker fluorescence intensity of OAS-G_3-Glu(NHN-DOX). The fluorescence intensity of both OAS-G_3-Glu(NHN-DOX) and OAS-G_3-Glu(NHN-DOX)/biotin increased with time, and this effect was attributed to the cleavage of the pH-sensitive bonds in the acidic endosome. These results demonstrated the sustained release of the DOX from the conjugates and that the general trend was consistent with the previous observations from other systems.[39] But the dendritic and polypeptide nature has made the current systems more attractive for further exploration toward the development of cancer therapy.

Conclusion

Poly(L-glutamic acid) dendrimers with silsesquioxane core were synthesized and characterized by ^{1}H NMR, ^{13}C NMR and MALDI-TOF mass spectrometry. The anticancer drug doxorubicin was conjugated to the OAS-G_3-glutamate dendrimers via an acid-labile hydrazine linkage. Biotin was also immobilized on the peripheral groups of the dendrimers as targeting moiety. The diameters of the aggregated conjugates were in the range of 30−50 nm. The conjugates with biotin showed a pH-activated drug release feature and a much higher cellular uptake compared to the conjugates without biotin. The in vitro antitumor effects of the conjugates with biotin, as studied from HeLa cell culturing, were much better than those obtained from the conjugates without biotin. These results have indicated that the introduction of both pH sensitive bonds and targeting moiety to the OAS-G_3-Glu dendrimers with nanocubic structure can improve drug delivery efficiency.

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Supporting Information Available: Detailed descriptions of synthetic procedures for the dendrimers described here and supporting mass spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

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