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Hyaluronic Acid/PEGylated Amphiphilic Nanoparticulates for Pursuit of Selective Intracellular Doxorubicin Release

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Materials

Iodomethane and n-octyl acrylate were purchased from Energy Chemical (Shanghai, China). Doxorubicin hydrochloride (DOX) was purchased from Beijing Huafeng United Technology Corporation. 4′,6-Diamidino-2-phenyl-indole dihydrochloride (DAPI) and 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were purchased from Sigma and used as received. During the course of the experiment, milipore water was chosen to complete the related procedures. All other reagents were provided by Tianjin Chem. Co. Ltd. (Tianjin, China).

Preparation of polyglycidyl methacrylate (PGMA) and ethylenediamine-modified polyglycerol methacrylate (PGEDA)

The polyglycidyl methacrylate (PGMA) was synthesized by atom transfer radical polymerization (ATRP).[1] Briefly, glycidyl methacrylate (105 equivalent), 2,2'-bipyridine (1.5 equivalent) as ligand, ethyl 2-bromoisobutyrate (1 equivalent) as ATRP initiator and CuBr (1 equivalent) as catalyst were successively added into distilled tetrahydrofuran (THF) in a three-necked flask. Then the reaction solution was stirred at 90 ^{o}C overnight under a nitrogen atmosphere. After cooling down to room temperature, the reaction solution was passed through a silica gel column using THF as an eluent to exclude copper, followed by vacuum rotary evaporation of THF. The crude PGMA was dissolved in a small amount of THF and then the solution was dropped into diethyl ether to obtain pure PGMA as a white powder (yields: 80%). ^{1}H-NMR (400 MHz, DMSO): δ 4.30 (s, 216H), δ 4.02 (s, 7H), δ 3.74 (s, 222H), δ 3.33 (s, 392H), δ 3.20 (s, 276H), δ 2.80 (s, 216H), δ 2.66 (s, 227H), δ 2.50 (s, 505H), δ 1.86 (d, 11H), δ 1.23 (s, 2H), δ 1.08 (s, 1H), δ 0.98 (s, 9H), δ 0.81 (s, 18H), δ 0.62-0.14 (m, 20H).

The EDA-modified poly (glycerol methacrylate) (PGEDA) was synthesized according to a previously reported procedure.[2] To obtain the PGEDA, 1 g of PGMA was dissolved in 100 mL of distilled ethylenediamine (EDA) under a nitrogen atmosphere. The mixture was allowed to heat to 65 ^{o}C and stirred for 6 h. Then the solution was cooled down to room temperature and transferred to dialysis bag (cut-off molecular weight 7 kDa). The product was dialyzed against deionized water for 72 h and then lyophilized to obtain the PGEDA (yields: 90%). ^{1}H-NMR (400 MHz, D_2O): δ 4.70 (s, 206H), δ 3.95 (s, 4H), δ 3.08 (s, 5H), δ 2.69 (d, 7H), δ 1.89-1.84 (m, 7H), δ 1.55-1.49 (m, 1H), δ 0.97 (s, 9H), δ 0.79 (s, 13H).

Preparation of methoxy poly (ethylene glycol) benzaldehyde (mPEG-CHO)

Methoxy poly(ethylene glycol) benzaldehyde (mPEG-CHO) was synthesized according to the procedure as described in the previous report.[3] Firstly, $mPEG_{2000}$ (2.5 g, 1.2 mmol) and 4-dimethylaminopyridine (DMAP) (0.16 g, 1.3 mmol) were dissolved in 75 mL anhydrous dichloromethane (DCM). P-formylbenzoic acid (1.5 g, 10.0 mmol) and dicyclohexylcarbodiimide (DCC) (2.1 g, 10.2 mmol) were then added and stirred at room temperature for 24 h. The filtrate coming from filtration was condensed with the aid of vacuum rotary evaporation. The obtained crude product was suspended in 25 mL of deionized water, followed by stirring for 30 min. Filtration was carried out again to collect the filtrate. Subsequently, the solution was extracted with DCM (100 mL × 6). The organic layer was separated and dried over anhydrous Na_2SO_4. Then anhydrous Na_2SO_4 was removed via filtration and the clear solution was dropped into cold diethyl ether to yield the ultimate product of mPEG-CHO (yields: 46.0%). ^{1}H-NMR (400 MHz, DMSO): δ 10.10 (s, 1H), δ 8.17 (d, 2H), δ 8.07 (d, 2H), δ 4.45 (m, 2H), δ 3.78 (s, 2H), δ 3.51 (m, PEG peaks), δ 3.26 (s, 3H).

Preparation of copolymer (Q-PGEDA-PEG) after OA-dissociation of Q-PGEDA-PEG-OA

The decomposition of OA grafted to Q-PGEDA-PEG-OA was performed by hydrolysis using sodium hydroxide (NaOH). Firstly, NaOH solution (0.015 M, 5 mL) was added into Q-PGEDA-PEG-OA (13 mg) and the reaction solution was stirred for 24 h at 50 °C. Then the obtained solution was dialyzed against PBS solution (pH = 7.4, 0.01 M) for 2 days. After lyophilizing, the final product, Q-PGEDA-PEG, was obtained (yields: 45%).

Characterization of copolymer

A NanoPhotometer-NP80 Touch spectrometer (Implen, Germany) was used for measuring the UV-Vis spectra. A 400 MHz Bruker Avance-400 spectrometer (400 MHz, Bruker, Freemont, CA) is selected to record the ^{1}H-Nuclear Magnetic Resonance (^{1}H-NMR) spectra of the synthetic products. DMSO or deuterium water (D_2O) was used as solvents.

Particle size and zeta potential measurements of polyplexes

The size and zeta potentials (ζ-potential) of polyplexes were estimated in three independent experiments by Zetasizer Nano ZS90 instrument (Malvern Instruments, Southborough, MA) at 25

°C.

Preparation of DOX-loaded nanocarriers and *In vitro* release of DOX

The DOX dissolved in distilled water was added to the Q-PGEDA-PEG-OA/HA solution dropwise. After stirring overnight in the dark, free DOX was removed by dialysis, using a dialysis bag (cut-off molecular weight 3500 Da) against deionized water for 24 h, and then freeze-dried to obtain DOX-loaded Q-PGEDA-PEG-OA/HA. The DOX loading content (LC) was calculated according to the following formulations: LC (%) = (DOX loaded in nanocarriers)/(total nanocarriers weight) × 100%.

The drug release performances of the DOX-loaded Q-PGEDA-PEG-OA/HA hybrid nanocarriers were investigated in phosphate buffer solution (PBS; pH 7.4 or 5.3). The mixture was composed of Q-PGEDA-PEG-OA and HA, and their molar fraction were 2 and 1, respectively. Then the DOX (at a final DOX concentration of 5 μg mL^{-1}) was added into the aforementioned mixture. The DOX-loaded NPs was transferred into a dialysis bag (cut-off molecular weight 3500 Da), which was immersed in 15 mL of phosphate buffer solution (PBS; pH 7.4 or 5.3) and shaken at 100 rpm under 37 °C. At predetermined time intervals, 3 mL of release media was taken out and replenished with an equal volume of fresh media. The absorbency of released DOX was determined using the UV-vis spectrometer at 488 nm.

Cell culture

Roswell Park Memorial Institute Medium (RPMI) 1640 medium, supplemented with 10% fetal bovine serum (FBS) and 1% antibiotics, was used as the culture medium of human cervical carcinoma (Hela cell line), human lung carcinoma (A549), breast cancer (MCF-7) and fibroblasts cell line (L929 cell line), which were incubated in a humidified atmosphere containing 5% CO_2 at 37 °C.

Cytotoxicity assay

The cell toxicity of free DOX, Q-PGEDA-PEG-OA, Q-PGEDA-PEG-OA/HA and Q-PGEDA-PEG-OA/HA/DOX against L929, A549, MCF-7 or HeLa cells was evaluated by methyl thiazolyl tetrazolium (MTT) assay. The cells were seeded in 96-well plates (5×10^3 cells per well) in

100 μL of culture medium and incubated at 37 ºC in a 5% CO_2 atmosphere for 24 h. Then the culture medium was replaced with a volume of 100 μL of fresh medium containing 10 μL polyplex solution, followed by incubation for another 48 h. After co-incubation, the culture medium was removed and then washed with PBS twice in each well, 10 μL of filtered MTT stock solution (5 mg/mL) with 90 μL of fresh culture medium was added to each well. The MTT medium solution was carefully removed after 4 h incubation and 100 μL of DMSO was added into each well to dissolve the formed crystals. After that, the absorbance rate was measured at 570 nm using a microplate reader (Epoch, BioTek, Genecompany Limited). The cell viability (%) was calculated from $(Abs_{(test\ cell)})/(Abs_{(controlled\ cell)}) \times 100\%$. Data are presented as means ± SD (n = 6).

Morphology of polyplex and DOX-loaded nanocarriers

The morphologies of polyplex and DOX-loaded nanocarriers were investigated by scanning electron microscope (SEM) performed on a JSM-6700F instrument (JEOL Ltd., Japan) at an acceleration voltage of 10 kV. 10 μL of each sample solution was deposited onto a clean double-polished SiO_2 slice and dried at room temperature. Then the residues coated with a thin layer of gold were observed by the SEM.

Confocal laser scanning microscopy (CLSM)

The cellular uptake of free DOX and DOX-loaded nanocomposites (NPs) was determined by confocal laser scanning microscopy (CLSM) (Zeiss LSM510, Oberkochen, Germany) toward HeLa and L929 cells. The cells were seeded on a 35 mm glass bottom dish with a density of 1×10^5 cells in 1 mL of RPIM 1640 medium supplemented with 10% FBS and cultured for 24 h, and followed by the replacement of primary culture media with 1 mL of serum-free medium containing free DOX or DOX-loaded (NPs) (at a final DOX concentration of 5 μg mL^{-1}). After predetermined 1 h and 3 h incubation time, cells were washed several times by PBS and fixed by 75% ethanol for 20 minutes at room temperature. Then the culture dish was further washed twice with PBS buffer and the cells nucleus were stained with 4,6-diamidino-2-phenylindole (DAPI) for 0.5 h. Afterwards, the stained cells were washed thrice with PBS buffer and used for bioimaging subsequently. Finally, the observation was carried out by a CLSM, in which the excitation wavelength was set at 488 nm for DOX and 405 nm for DAPI.

Cellular uptake measured by flow cytometry

The cellular uptake of free DOX and Q-PGEDA-PEG-OA/HA/DOX was investigated quantitatively by flow cytometry. Briefly, HeLa and L929 cells were seeded in 24-well plates at a density of 1×10^5 cells per well in 0.5 mL culture medium and put into an incubator for 24 h. Then cells were incubated with free DOX and Q-PGEDA-PEG-OA/HA/DOX at a DOX concentration of 5 μg/mL for 3 h. After that, cells were washed three times with PBS and harvested by trypsin treatment. Then the harvested cells were suspended in PBS and spun down at 1000 rpm for 5 min. After two cycles of washing and centrifugation, cells were resuspended with 0.5 mL PBS. Finally, the cells were counted by flow cytometer (BDLSR II, BD, Franklin Lakes, NJ).

Bacterial culturing

Two kinds of sensitive single colony, Escherichia coli (*E. coli*, Gram-negative bacterium) and Staphylococcus aureus (*S. aureus*, Gram-positive bacterium) growing in a solid state Luria-Bertani (LB) agar plate, were transferred to a liquid state LB culture medium (5 mL), respectively. And both of them were cultured on a shaking incubator at 37 °C overnight. Then the bacterial concentration was quantified by measuring optical density at λ = 600 nm on a UV-vis instrument (OD600 of 2.0 corresponded to a concentration of 10^9 colony forming units per milliliter (CFU mL^{-1})). Spread plate method was selected to assess the colony forming units. The overnight cultured bacteria were diluted to glass tubes with 1 mL of PBS (pH = 7.4). And OD600 of the respective test strains was adjusted to 0.02, which corresponds to a concentration of about 10^7 CFU mL^{-1}. After that, in order to test the antimicrobial activity of the nanocomplexes, nano-carrier Q-PGEDA-PEG-OA and DOX-loaded NPs (at a final DOX concentration of 0.05 μg mL^{-1} or 5 μg mL^{-1}) were added into the prepared bacteria suspension. And then the tubes of being added NPs were kept shaking (170 rpm) at 37 °C for 2 h. Later on, the test bacterial suspensions were taken out and the concentration of the bacterial suspensions was diluted with an appropriate dilution factor. The solid nutrient agar plate was swabbed with a 100 μL portion of the dilution bacterial suspension and the colonies were counted after being kept for 16 h at 37 °C. The solution in absent of NPs was used as a control. The bacterial viability rates were determined by the ratio of colony counts to the blank groups.

References

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2. S. Chen, Q. Chen, Q. Li, J. An, P. Sun, J. Ma and H. Gao, *Chem. Mater.*, 2018, **30**, 1782-1790.
3. Z. Jiang, Q. Chen, X. Yang, X. Chen, Z. Li, D. E. Liu, W. Li, Y. Lei and H. Gao, *Bioconjugate Chem.*, 2017, **28**, 2849-2858.

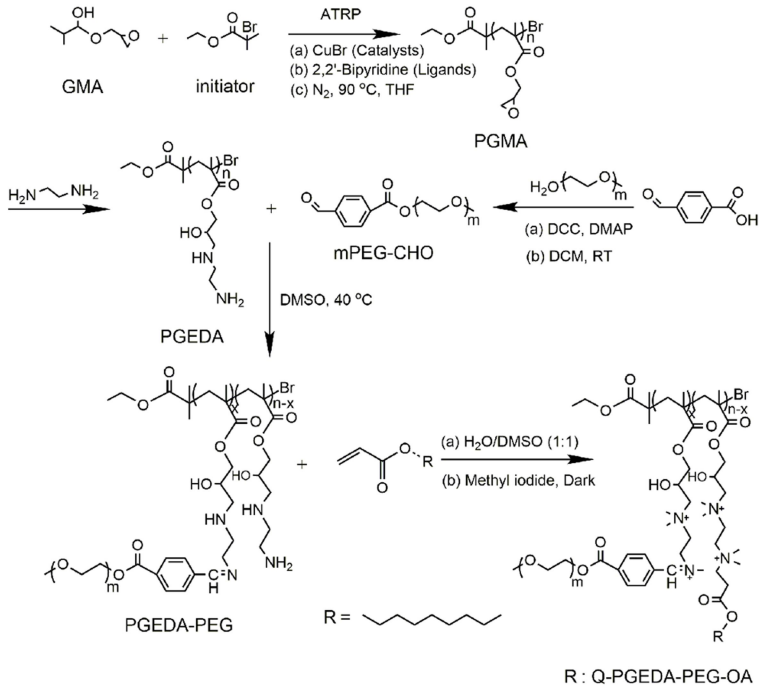

Figure S1 Synthetic route of Q-PGEDA-PEG-OA.

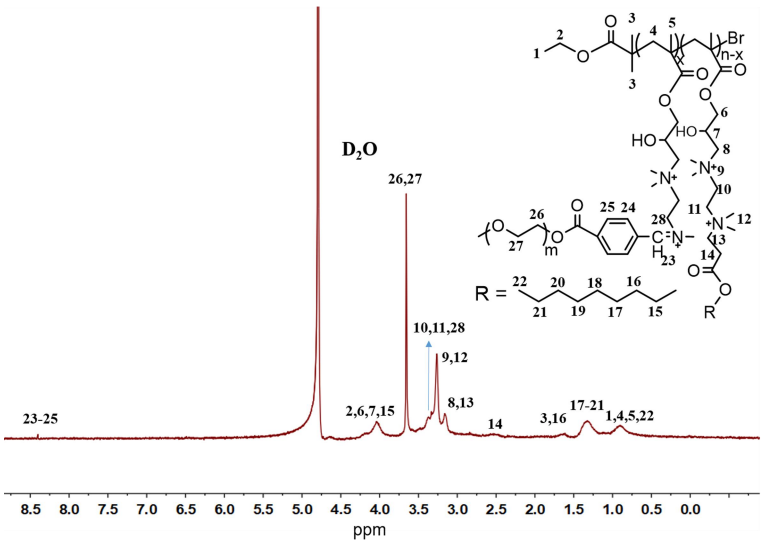

Figure S2 ^{1}H-NMR spectrum of Q-PGEDA-PEG-OA using D_2O as a solvent.

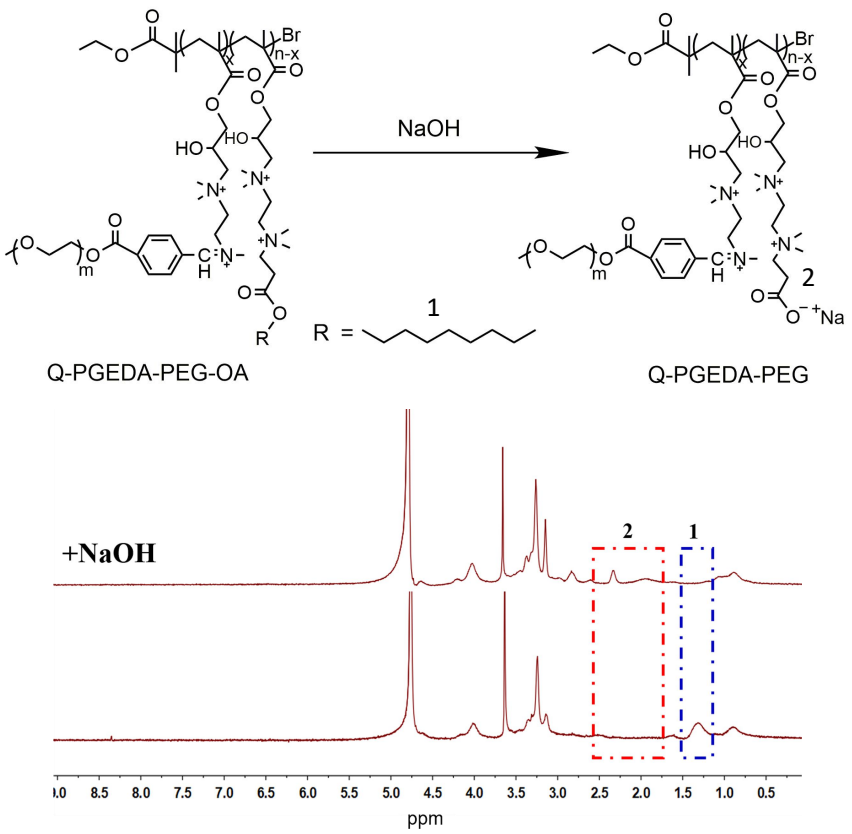

Figure S3 Structural formula and ^{1}H-NMR spectra of Q-PGEDA-PEG-OA before and after being treated with 0.015 M NaOH solution (D_2O as a solvent).

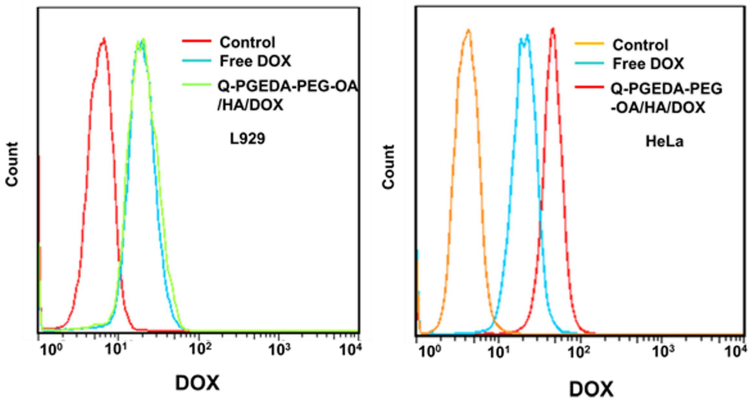

Figure S4 The flow cytometer assay of L929 and HeLa cells incubated with free DOX and Q-PGEDA-PEG-OA/HA/DOX for 2 h.

Table S1 IC_{50} values (μg/mL) of free DOX and Q-PGEDA-PEG-OA/HA/DOX against different cancer cells.

IC_{50} Values	A549	MCF-7	HeLa
Free DOX	0.552	0.559	0.707
Q-PGEDA-PEG-OA/HA/DOX	0.553	0.457	0.832

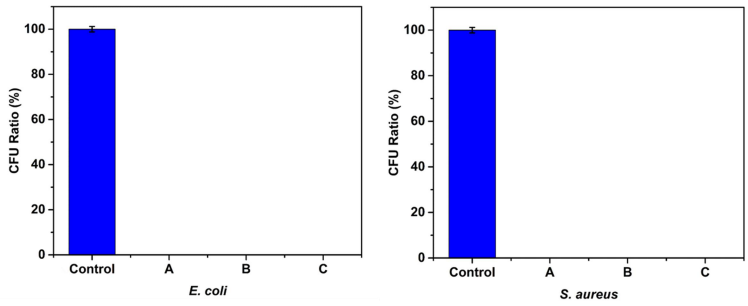

Figure S5 CFU ratio of *E. coli* (left) and *S. aureus* (right) treated with Q-PGEDA-PEG-OA (A), Q-PGEDA-PEG-OA/HA/DOX (5 μg mL^{-1}) (B) and Q-PGEDA-PEG-OA/HA/DOX (0.05 μg mL^{-1}) (C).