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Cellular internalization of doxorubicin loaded star-shaped micelles with hydrophilic zwitterionic sulfobetaine segments

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ABSTRACT

Four arm star-shaped poly(ε-caprolactone)-b-poly((N,N-diethylaminoethyl methacrylate)-r-(N-(3-sulfopropyl)-N-methacryloxyethy-N,N-diethylammoniumbetaine)) (4sPCLDEAS) micelles were loaded with anticancer drug doxorubicin to track their endocytosis in Hela cancer cell line. The effects of mean diameters and surface charges of the drug loaded micelles on the cellular uptake were studied in details. The results demonstrated that the internalization of micelles was both time and energy dependent process. Endocytic pathways including clathrin-mediated endocytosis, caveolae-mediated endocytosis and macropinocytosis were all involved in the internalization; caveolae-mediated endocytosis was the main pathway for the internalization of 4sPCLDEAS micelles. The assays for cell apoptosis and growth inhibition of tumor spheroids identified that these doxorubicin loaded micelles could induce cell apoptosis and inhibit tumor spheroids growth efficiently, which was even equal to free DOX·HCl. This study provided a rational design strategy for fabricating diverse micellar drug delivery systems with high anticancer efficiency.

1. Introduction

In recent years, polymeric micelles have attracted much interest as promising anticancer drug carriers due to their significances in reducing side effects, enhancing solubility, prolonging blood circulation time, and escaping capture from reticuloendothelial cell system [1–4]. The physicochemical properties of polymeric micelles including size, surface charge and morphology could be easily controlled via facile synthetic approaches. These characteristics of micelles directly evoked different interactions between micelles and biological environment, which affected the clearance, biodistribution, cellular uptake and metabolism of the micelles in vivo [5]. Size and surface charge are important parameters to decide the cellular internalization behaviors of nanoparticles [6,7]. Chithrani [8,9] studied the cellular uptake of gold nanoparticles with the mean diameters of 14, 50, and 74 nm in Hela cells, the kinetics of cellular uptake were different to nanoparticle sizes, the nanoparticles with 50 nm size exhibited the highest efficiency in cellular uptake. Mou examined the effect of particle size on the cellular uptake of FITC-mesoporous silica nanoparticles, the results showed that the maximum uptake of nanoparticles to Hela cells occurred at 70 nm [10]. Cho explored the role of surface charge on the internalization of gold nanoparticles [11], neutral and negatively charged nanoparticles were adsorbed less on cell membranes and consequently exhibited lower internalization extent comparing to positively charged nanoparticles. Patil investigated the cellular uptake behavior of cerium oxide nanoparticles with different zeta potentials [12], the negatively charged nanoparticles showed bovine serum albumin protein adsorption resistance and were favorable for the uptake to adenocarcinoma lung cells.

The compositions are the intrinsic nature to determine the physicochemical properties and cellular uptake behaviors of polymeric micelles. In most polymeric micelles, poly(ethylene glycol) (PEG) was used as the hydrophilic segment to avoid the aggregation and clearance from the reticular-endothelial system due to its protein adsorption resistance, which could achieve long circulation in blood vessel transportation [13,14]. However, the shielding effect of PEG shell was unfavorable for cellular uptake, therefore, other hydrophilic polymers were developed to replace PEG. Zwitterionic polymer, such as sulfobetaine, is a class of promising hydrophilic polymer to fabricating polymeric micelles. The zwitterionic

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poly(sulfobetaine) possessed anti-biofouling properties due to the tightly bound water layer in zwitterionic-based materials. The hydrated corona in polymeric micelles minimized the protein adsorption to result "stealth" in blood circulation [15–17]. The hydrophilic zwitterionic sulfobetaine in polymeric micelles was expected to overcome the drawback of PEG and enhance the internalization capability of micelles.

Endocytosis research found that there were several endocytic pathways for nanoparticles including phagocytosis, macropinocytosis, clarthrin-mediated endocytosis, caveolae-mediated endocytosis, and clarthrin- and caveolae-independent endocytosis. The options of endocytic pathways were mainly dependant on the compositions, sizes and surface charges of micelles [18]. For example, folic acid, albumin or cholesterol decorated micelles favored the caveolin-mediated endocytosis, alternatively, transferrin and CPP encouraged the uptake via clathrin-medicated endocytosis and macropinocytosis, respectively [19]. Therefore, to discover the endocytic pathway of polymeric micelles could not only track the process of internalization but also provide a rational strategy to design and develop nano-engineered drug delivery systems.

In our previous work, four arm star-shaped poly(ε-caprolactone)-b-poly(N,N-diethylaminoethylmethacrylate)-r-poly(N-(3-sulfopropyl)-N-methacryloxyethy-N,N-diethylammoniumbetaine) (4sPCLDEAS) micelles were synthesized and used as carriers to deliver anticancer drug in Hela cells efficiently [20]. However, the pathway of cellular internalization of those micelles was not clear. This paper focused on the exploration of internalization mechanism of these star-shaped micelles. The micelles with different sizes and surface charges were prepared, and anticancer drug doxorubicin (DOX) was trapped in the micelles. The drug loaded micelles were characterized by atomic force microscope (AFM) for morphology, dynamic light scattering (DLS) for size and size distribution, zeta potential for surface charge. The cellular uptake of the micelles in Hela cells were investigated by confocal laser scanning microscopy (CLSM) and flow cytometry (FCM). Tumor spheroid models were used to simulate solid tumors for anticancer activity evaluation of DOX loaded micelles.

2. Materials and methods

2.1. Materials

Low melting point agarose was purchased from Amresco (Solon, OH, USA). Cell culture dishes and plates were purchased from Corning incorporation (Corning, NY, USA). Dulbecco's modified Eagle medium (high glucose) cell culture medium (DMEM) and fetal bovine serum (FBS) were obtained from life technologies (Grand Island, NY, USA). 4′,6-diamidino-2-phenylindole (DAPI) and LysoTracker Green DND-2 were purchased from invitrogen. DOX was purchased from AK scientific Inc. Sodium azide (NaN_3), 2-deoxy-D-glucose (DOG) endocytosis inhibitors including chlorpromazine hydrochloride (CPZ), amiloride hydrochloride hydrate, methyl-β-cyclodextrin (mβCD) were purchased from sigma-Aldrich. All other reagents and solvents were analytical grade and used as received.

2.2. Preparation of DOX loaded micelles

The amphiphilic 4-arm star-shaped 4sPCLDEAS copolymer was synthesized according to our previously reported reference [20]. The blank 4sPCLDEAS micelles and DOX loaded 4sPCLDEAS micelles were prepared using dialysis approach [21]. DOX loaded micelles with different sizes or surface charges were controlled by the micellar concentrations from 0.01 mg/mL to 1 mg/mL or alternating the mass ratios of DOX to 4sPCLDEAS copolymer from 0.05 to 0.25.

2.3. Characterizations of micelles

The particle sizes and surface charges were performed on Zetasizer Nan-ZS (Malvern Instrument Ltd., Malvern, UK) equipped with a He–Ne laser beam at 633 nm (scattering angle: 90°). Each sample was filtered through a 0.45 μm syringe filter before analysis. The size and surface charge of micelles were the average values of three measurements.

Atomic force microscopy (AFM) was employed to observe the morphology of polymeric micelles. The AFM sample was prepared by casting a dilute micelle solution (0.01 mg/mL) on a mica substrate, and dried in vaccum. The AFM images were recorded with a Nanoscope III from Digital Instruments operated in the tapping mode in air using microfabricated Si (type NCH) cantilevers with a spring constant between 27 and 53 N m^{-1}, resonance frequency in the range of 300–365 kHz, and 1 and 2 Hz scanning speed.

The entrapped DOX was measured with UV-visible spectrophotometer. The UV absorbance was monitored at a wavelength of 483 nm. The drug loading content (%) (DLC) and loading efficiency (%) (DLE) were calculated by the following equations:

$$\text{DLC (wt\%)} = \frac{\text{mass of DOX in the micelles}}{\text{mass of DOX} - \text{loaded micelles}} \times 100\% \quad (1)$$

$$\text{DLE (wt\%)} = \frac{\text{the amount of DOX in micelles}}{\text{the amount of DOX in feed}} \times 100\% \quad (2)$$

2.4. Cell culture

Human epithelial carcinoma cells (Hela) were cultured in Dulbecco's Modified Eagle's Medium (DMEM) and McCoy's 5a Medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin G, and 100 μg/mL streptomycin (all ingredients obtained from Gibco, USA) at 37 °C in a humidified atmosphere with 5% CO_2. The cells were harvested with 0.25% trypsin (Invitrogen, USA) and rinsed. The obtained cell suspension was used in the following experiments.

2.5. Cellular uptake of micelles

Monolayers of Hela cells were cultured with 4sPCLDEAS micelles (0.5 mg/mL) in DMEM culture medium for 24 h. After the treatment, cells were washed three times with cold PBS, and then were harvested in a 2 mL EP tube by trypsinization. The sample for TEM observation was prepared by conventional way [22].

Hela cells were seeded in 35 mm diameter glass dishes (Invitrogen, USA) at a density of 1×10^4 cells/mL. When the cells were almost 80% confluent, DOX loaded 4sPCLDEAS micelles with various surface charges or sizes were added into the serum-free medium. The glass dishes were incubated at 37 °C for 2 h in a 5% CO_2 incubator, washed three times with PBS and fixed with 4% paraformaldehyde for 10 min. The nuclei were counterstained by DAPI for 5 min. The culture media were replaced with PBS. The cells were observed by confocal laser scanning microscopy (CLSM, Leica TCP SP5) with the excitation wavelengths of 340 and 485 nm and emission wavelengths of 488 and 550 nm for DAPI and DOX, respectively [23].

The quantitative cellular uptake of micelles with different sizes or surface charges was measured by flow cytometry. The Hela cells were seeded in 6-well plates at a density of 1×10^5 cells/well in serum-free medium and incubated at 37 °C for 24 h, DOX loaded 4sPCLDEAS micelles ([DOX] = 5 μg/mL) with various surface charges and sizes were added and incubated for 2 h. The cells were washed with PBS for three times. The cells were harvested by trypsinization and centrifuged at 1000 rpm for 5 min, re-suspended in 0.5 mL PBS medium and examined by flow cytometry using FACScan instrument (Becton Dickinson, San Jose, CA, USA). 10,000 events were collected for each sample. Cell-associated DOX was detected with excitation wavelength at 485 nm and emission wavelength at 595 nm [23].

Hela cells were seeded in 35 mm diameter glass dishes (Invitrogen, USA) at a cell density of 1×10^5 cells/mL. After reaching 80% confluence, the cells were treated with DOX loaded 4sPCLDEAS micelles ([DOX] = 10 μg/mL) with various surface charges and sizes. After 2 h incubation, Lyso-Tracker Green (100 nM) was added in the medium and the cells were incubated for another 30 min. The cells were rapidly washed with ice cold PBS to prevent the removal of the attached LysoTracker Green, the live cells were observed by confocal laser scanning microscopy (CLSM, Leica TCP SP5) with the excitation wavelengths of 504 nm and 485 nm and emission wavelengths of 511 nm and 550 nm for Lyso-Tracker Green and DOX, respectively [23].

2.6. Cellular uptake pathways

The effects of temperature and NaN_3/DOG on the cellular uptake of micelles (d = 137.3 nm, ζ = 36.8 mV) were studied by pre-incubating the Hela cells at 4 °C for 3 h or pre-treated with NaN_3/DOG (0.1%, 30 mM) at 37 °C for 1 h and then incubated with DOX loaded 4sPCLDEAS micelles ([DOX] = 5 μg/mL) at 4 °C and 37 °C for 2 h, respectively. Hela cells were pre-incubated with different inhibitors of chlorpromazine (CPZ, 10 μg/mL) to inhibit the formation of clathrin vesicles, methyl-β-cyclodextrin (7 mM) to inhibit caveolae, or amiloride (50 μM) to inhibit macropinocytosis at 37 °C for 30 min. The group incubated with DOX loaded micelles without inhibitor treatment was used as the control. The inhibitor solutions were removed, and the DOX loaded 4sPCLDEAS micelles (S1′-MLs) in media containing inhibitors at the same concentrations were added and further incubated at 37 °C for 2 h. The cells were washed with ice cold PBS for three times, and fixed with fresh 4% paraformaldehyde for 15 min at room temperature. The nuclei of cells were stained with DAPI and visualized under confocal laser scanning microscopy (CLSM). The fluorescence intensities were measured by flow cytometry for quantitative analysis.

2.7. Cell apoptosis of DOX loaded micelles

After Hela cells were seeded in 6-well plates at a density of 1×10^5 cells/well and cultured for 24 h, the cells were incubated with DOX·HCl, DOX loaded micelles