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Pluronic mimicking fluorescent carbon nanoparticles conjugated with doxorubicin via acid-cleavable linkage for tumor-targeted drug delivery and bioimaging

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ABSTRACT

Fluorescent carbon nanoparticles based target drug delivery with bio-imaging systems offer tremendous scope for future. We described Pluronic mimicking fluorescent nanoparticles (Plu-FNPs) surface decorated doxorubicin (DOX) via acid-cleavable hydrazone linkages with tumor target folic acid (FA) [Plu-FNPs-FA-DOX]. The acid labile of hydrazine linkage to DOX can easily break off by controlled pH inducing release of conjugated DOX at site of over-expressed folate receptors (FR). This nanoparticle system can deliver DOX to the target FR and trace the delivery pathway of cancer cells. The approaching platform demonstrates the selectivity and sensitivity of molecular targets, thereby able to maximize the therapeutic efficiency.

Introduction

The nanoparticles based targeted drug delivery system (DDS) promises to expand the therapeutic regimes by selectively delivering the drug candidates [1–4]. This will effectively reduce the minimum effective dose and improve the therapeutic efficacy at equivalent plasma concentration [5–7]. At the same time, fluorescent nanoparticles (FNPs), knows as carbon dots (CDs), have appeared as an indispensable tool in fluorescence bioimaging and detection. Furthermore, it is accepted that a combination of bioimaging diagnostic and medical therapy can optimize the safety and efficacy of therapeutic regimes towards the goal of personalized medicine [8–13]. For targeted delivery to tumor, the overexpressed folate receptors (FR) for vitamin folate have been frequently identified in various types of human cancers that are generally absent in most normal tissues. Several studies have shown the FR-mediated folate-drug conjugate systems as an attractive target for tumor-selective delivery [14]. Although there are many ways to link drug to delivery vehicles, most of the delivery strategies have utilized cellular microenvironment (extra/intra cellular) for successful release of drug from vehicles. This can be accomplished by inserting an acid-sensitive linker to allow rapid release of drugs after entrapped endocytic lysosome vesicles [15].

Doxorubicin (DOX) is an anthracycline antitumor antibiotic and is ranked among the most studied drugs in oncological chemotherapy. It functions by intercalating DNA, which inhibits macromolecular biosynthesis in malignant cancer cells. However, it has poor water solubility with strong side effects containing drugs requiring effective formulation for precise delivery at selective sites [4]. In particular, pH-cleavable bonds, which dissociate under mild acidic conditions and are stable at neutral pH, are promising tools, because the pH value of extracellular spaceof solid tumor and intracellular endosomes is typically more acidic than blood plasma [16]. Several promising example of DOX delivery including polymer conjugates, polymeric nanoparticles, and self assembled micelle have been reported. To improve the therapeutic efficacy, the Pluronic block copolymers which consist of hydrophilic poly(ethylene oxide) (PEO) and hydrophobic poly (propylene oxide) (PPO) blocks conjugated DOX ensure efficient delivery to the required intracellular compartments within a specific period of time [4,15,16]. Because of biocompatibility, Pluronic have

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received significant attention for the efficient delivery of hydrophobic anticancer drugs. After injection into the bloodstream, Pluronic are not stable enough to retain their structural integrity when diluted below the critical micelle concentration. Moreover, there are no exist study about the carbonized Pluronic that resulted multicolor emission in the drug delivery vehicle system.

In our previous study, we synthesized multicolor fluorescent nanoparticles (FNPs) using Pluronic (Plu) by the carbonization method using an acidic catalyst and confirmed the presence of hydroxyl (—OH) and carboxyl (—COOH) groups on the surface of FNPs (Plu-FNPs) [10]. The current study placed —OH and —COOH groups as beneficial moieties for conjugated to FA and DOX via hydrazone linkage without another chemical modification. Based on this study, we present DOX and FA conjugated Plu-FNPs for pH-triggered drug delivery and bioimaging application with colloidal stability under biological medium. Pluronic-mimicking FNPs (Plu-FNPs) having carboxyl groups on the surface of the synthesized FNPs was chemically conjugated to DOX using an acid-labile linkage via a hydrazone linkage, and folic acid attached reasonably shuttles affinity to FR. The resulted carbonized Pluronic showed substantially high decay time after conjugated with FA and DOX which is enough time for them to tracking the cancer cells. The pH-responsive hydrazone structure was specifically released DOX form the Plu-FNPs carrier under acidic physiological conditions such as an endosomal compartment within the tumor site. In vitro targeted cellular uptake, cytotoxicity with bio-imaging behavior of the DOX/FA-conjugated Plu-FNPs was evaluated for MDCK and KB cell lines.

Materials and methods

Materials

Doxorubicin hydrochloride (DOX), [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (MTT), *N*,*N*′-dicyclohexylcarbodiimide (DCC), *N*-hydroxysuccinimide (NHS), dimethyl sulfoxide (DMSO), 4-(dimethylamino)pyridine (DMAP), dichloromethane (MC), and folic acid (FA) were purchased from Sigma–Aldrich, Korea. Penicillin–streptomycin, fetal bovine serum (FBS), 0.25% (w/v) Trypsin, 0.03% (w/v) EDTA (ethylenediaminetetraacetic acid) solution, and RPMI-1640 medium were purchased from Gibco BRL (Carlsbad, CA, USA). FNP-Plu (Mn: 5400) was prepared following our previous report by the dehydration of Pluronic F-127, poly(ethylene glycol)$_{98}$-*b*-poly(propylene glycol)$_{67}$-*b*-poly(ethylene glycol)$_{98}$ (PEG-*b*-PPO-*b*-PEG) in the presence of concentrated sulfuric acid. In details, 1 gm of Pluronic F-127 was dissolved in 5 mL of de-ionized water and then mixed with 10 mL of concentrated H_2SO_4 in a vial with significant caution. The reaction vial were then preheated in a oil bath at 100 °C for 1 min and immediately cooled to room temperature in an ice bath with great caution. At the end of reaction, the sample were diluted by adding 185 mL of de-ionized water and dialyzed (3500 kDa) against de-ionized water for 3 days, and freeze-drying to get the final products (Plu-FNPs) [10].

^{1}H NMR spectra were recorded using a Bruker Advance 400 MHz spectrometer with deuterated dimethyl sulfoxide (d_6-DMSO) as the solvent. UV–vis spectra were recorded using an Optizen 2120 UV spectrophotometer (Mecasys Co.). AFM imaging was performed in the tapping mode using a MultiMode8 (Bruker) with a silicon probe. For AFM images, samples were prepared on silicon wafers. Particle size was measured using dynamic light scattering (DLS) (Zetasizer Nano, Malvern-Germany). Photoluminescence (PL) spectra were obtained using an FluoroMate fluorometer from Scinco. Zeta potential data were obtained using a particle size analyzer (ELS-Z) of Otsuka Electronics Corporation. NIR laser had a wavelength of 808 nm (PSU-III-LRD, CNI Optoelectronics Tech. Co., LTD., China). Multimode microplate reader Filter MaxF3 (Varioskan Flash, Thermo Electron Corp., Waltham, MA, USA) was used for the MTT assay. Transmission electron microscopy (TEM, JEM-2100F, JEOL) was carried out using an electron gun with potential in the range 80–200 kV. Confocal laser scanning microscope (CLSM) images of the samples were recorded using an LSM510 confocal microscope (Carl Zeiss, Oberkochen 73447, Germany). The molecular weights of Plu-FNPs, Plu-FNPs-FA, and Plu-FNPs-FA-DOX were measured using gel permeation chromatography (GPC). The GPC measurements were performed using a Waters 2410 equipped with a RI (refractive index) detector and 0.1 M NaCl in H_2O as the eluent.

Conjugation of hydrazine and FA on Plu-FNPs [Plu-FNPs-FA]

Hydrazine monohydrate (0.0574 g) dissolved in 25 mL of dimethyl sulfoxide was activated using DCC (1.22 g) and NHS (0.681 g) for 15 min and then reacted with 0.6 g of Plu-FNPs for 24 h at room temperature. The resulting Plu-FNPs-FA was purified by dialysis (MW: 3500) against water and finally freeze-dried. Subsequently, Plu-FNPs (1 g) with excess amounts of DCC and DMAP were reacted with FA (0.1324 g) in dichloromethane (30 mL) at room temperature for 24 h. After evaporating methylene chloride, the product was dissolved in deionized water and dialyzed against water and then lyophilized.

Conjugation of DOX on Plu-FNPs-FA [Plu-FNPs-FA-DOX]

Hydrazine functionalized Plu-FNPs-FA (1 g) and DOX (0.095 g) was dissolved in 10 mL anhydrous DMSO, and the resulting reaction mixture was kept for 48 h at 60 °C to complete the conjugation. Non-reacted DOX and residues were removed by dialysis for 12 h against pH 9.0. The reaction was performed in the dark by wrapping in an aluminum foil, and the product was stored after freeze-drying. The DOX conjugation was confirmed by GPC (Younglin instrument, South Korea) with YL9112 Isocratic pump and KD-804 and KD-803 columns (Shodex, Japan) with DMF (0.01 wt%, LiBr). The degree of substitution of DOX was determined using a spectrophotometer (Mecasys, South Korea) at 480 nm.

MTT assay

The cytotoxicity was measured by the [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] MTT assay method. 200 mL of KB cells (human epidermoid carcinoma cells) and MDCK cells at a density of 2×10^5 cells/mL were placed in each well of a 96-well plate. Afterwards, the cells were incubated for 24 h at 37 °C in a 5% humidified CO_2 atmosphere. To determine the cellular viability, a stock solution of Plu-FNPs-FA-DOX and DOX was dissolved in RPMI medium at a concentration of 1 mg/mL and diluted up to 0.01 mg/mL. The media was removed, and the cells were treated with different concentrations of Plu-FNPs-FA-DOX and DOX. The cells were then incubated as previously for another 24 h. The media containing Plu-FNPs-FA-DOX and DOX were then replaced with 180 μL fresh medium and 20 μL of a stock solution containing 15 mg of MTT in 3 mL PBS and incubated for another 4 h. Finally, the medium was removed and 200 μL MTT solubilizing agents were added to the cell, and the reaction mixture was shaken for 15 min. Absorbance was measured at a 570 nm wavelength using a microplate reader. The relative cell viability was measured by comparing the control 96-well containing only the cell.

Quantitative evaluation of cellular uptake

The cellular uptake of Plu-FNPs-FA-DOX against cancerous KB and normal MDCK cells were selected and seeded in a 96-well plate

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