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Monodistearoylphosphatidylethanolamine-hyaluronic acid functionalization of single-walled carbon nanotubes for targeting intracellular drug delivery to overcome multidrug resistance of cancer cells

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ABSTRACT

Multidrug resistance (MDR) of cancers is a major cause for the failure of chemotherapy. Here we synthesized a multifunctional modifier of single-walled carbon nanotubes (SWCNTs), distearoylphosphatidylethanolamine-hyaluronic acid (DSPE-HA) conjugate with a single coupling point, to simultaneously disperse SWCNTs, improve the biocompatibility of SWCNTs and target SWCNTs to CD44-overexpressing MDR cancer cells for improving intracellular drug delivery and overcoming MDR. Taking epirubicin (EPI) as model drug and the DSPE-HA functionalized SWCNTs as carriers, a drug delivery system EPI-SWCNTs-DSPE-HA was constructed. The intracellular EPI delivery efficiency and MDR-reversing effects of EPI-SWCNTs-DSPE-HA were investigated in drug-resistant A549/Taxol cells and tumor spheroids. The results demonstrated that EPI-SWCNTs-DSPE-HA significantly increased the intracellular delivery and retention of EPI by circumventing the drug efflux through the mechanism of CD44 receptor-mediated endocytosis and bypassing P-glycoprotein (P-gp) pump. EPI-SWCNTs-DSPE-HA treatment effectively overcame the MDR of cancer cells and tumor spheroids and blank SWCNTs-DSPE-HA had no significant cytotoxicity, showing that SWCNTs-DSPE-HA is a promising carrier for drug delivery in MDR cancers.

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1. Introduction

The multidrug resistance (MDR) of cancer cells resulting in therapeutic failure and tumor relapses remains a major challenge faced by scientific workers in the field of cancer treatment [1]. The high expression of ATP-binding cassette (ABC) transporter family (most notably P-glycoprotein, P-gp) is responsible for the initiation of MDR [2–4]. These overexpressed transport proteins cause MDR by pumping anticancer drugs out of cancer cells. The potential key to overcoming MDR is to circumvent the action of these transporter proteins. The clinical strategy to overcome the MDR of cancer cells is to use higher doses of cytotoxic drugs or P-gp modulators (e.g., verapamil, cyclosporin A) [5]. Unfortunately, the high doses inevitably result in high toxicity and side effects and the P-gp modulators themselves also have toxicity and side effects on healthy organs and tissues. Nanoscaled drug delivery systems (NDDS) have offered an alternative strategy for reversing MDR [6–10]. NDDS reverse MDR by increasing the drug concentration in tumors and tumor cells through targeting effects of their carriers to tumor tissues and cells.

In recent years, functionalized single-walled carbon nanotubes (SWCNTs) have gained tremendous attention as a promising carrier for cancer therapies because of their distinct natures including cell membrane penetrability, high drug-loading capability, selective retention in the tumor, and so on [11]. However, some problems are required to be solved before SWCNTs become more desired drug carriers, such as its aqueous dispersibility, biocompatibility, targetability to cancer cells, easily performed modification and preparation methods. Although the available modification methods display great potential, the complicated functionalization processes involves multistep reaction and purification, thus leading to great losses in recoverable materials that attach in the precedent steps, limiting their biomedical applications. Therefore, there is a need for developing a simple and easy technique to confer SWCNTs

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multifunctionality and biocompatibility simultaneously.

The cell surface membrane-bound protein CD44 is overexpressed in many types of cancers. Thus, their MDR cancer cells also highly expressed CD44 receptors. Therefore, CD44 could be potentially used as targets for the treatment of these drug resistant tumors. A naturally occurring polysaccharide hyaluronic acid (HA) can specifically recognize its receptors CD44 and has been identified as a potential targeting ligand of tumors possessing CD44-overexpressing cells [12,13]. Besides, HA possesses unique and excellent advantages, including good biocompatibility, biodegradability, and non-immunogenicity [14]. However, HA alone was unable to render a stable suspension of SWCNTs [15]. PEG conjugates of DSPE (DSPE-PEG) can render SWCNTs surprisingly stable in various biological solutions including serum in vitro and exhibit relative long blood circulation times and low reticuloendothelial system (RES) uptake in vivo [16,17], but cannot confer SWCNTs the targetability. Phospholipids are the major component of cell membranes and tissues like the nervous system and the lung, so they have good biocompatibility and are safe to use in biological systems. In view of the prominence of DSPE and HA, we proposed here that a conjugate of DSPE and HA (DSPE-HA) would confer SWCNTs good dispersity, biocompatibility and targetability to CD44 overexpressing MDR cancer cells simultaneously. With DSPE-HA, it was possible that the functionalization of SWCNTs for dispersity, biocompatibility and targetability is completed in one step, subsequently reducing the loss of recoverable materials. Usually, phospholipids can be coupled to HA via the carboxylic groups of HA and amine groups of phospholipids by carbodiimide chemistry, resulting in multipoint attachment [18,19]. Since polysaccharide have one reductive end, the reductive end of HA could be potentially coupled to amine groups of DSPE via reductive amination, but few papers have addressed this reaction process. To retain the integrity of HA chain as possible, which was important for its affinity with CD44 receptors, we decided to use reductive amination method for attaching DSPE to the reducing end of the HA with a single coupling point which was similar to the DSPE-PEG conjugate. In the present study, using synthesized DSPE-HA to coat the epirubicin (EPI), an effective anti-cancer dug, loaded SWCNTs (EPI-SWCNTs), a targeting SWCNTs-based drug delivery system, EPI-SWCNTs-DSPE-HA, was prepared in one step for overcoming MDR of the tumor overexpressing CD44 receptors. The EPI-SWCNTs-DSPE-HA complexes were characterized and their targeting and inhibitory effects on the MDR cancer cells and tumor spheroids were evaluated.

2. Experimental

2.1. Materials

Pristine SWCNTs (95% in purity, 5–20 μm in length, 1–2 nm in diameter) were obtained from Shenzhen Nanotech Port Co., Ltd. (Shenzhen, China). 1,2-distearoyl-sn-glycero-3-phosphoethamolamine (DSPE), Avanti Polar Lipids (Alabaster, AL, USA). Sodium triacetoxyborohydride and tetra-n-butylammonium (TBA) hydroxide, Sigma–Aldrich (St. Louis, MO, USA). Hyaluronic acid (HA, sodium salt, Mw17500Da), Zhenjiang Dongyuan Biotech Co., Ltd. (Zhenjiang, China). Epirubicin hydrochloride (EPI), Nanjing Tianzun Zezhong Chemicals Ltd. (Jiangsu, China). Cellulose membrane (0.22 μm pore size), Shanghai ANPEL Instrument Co., Ltd. Dimethyl sulfoxide (DMSO), Amresco (Solon, OH, USA). Anti-CD44-FITC and anti-P-glycoprotein-FITC or their isotype controls, Abcam (UK). Cell counting kit-8 (CCK-8), Dojindo Laboratories (Kumamoto, Japan). Dialysis membrane (Spectra Por regenerated cellulose), Spectrum Laboratories, Inc. (Canada). Other reagents, Beijing Chemical Reagents (Beijing, China).

2.2. Synthesis and characterization of DSPE-HA conjugate

HA sodium salt (0.2 g) was dissolved in 20 mL deionized water and dialyzed against 0.01 M HCl (1 L) for 24 h, followed by dialysis against deionized water (1 L) for 24 h to obtain the acidic form of HA. Then tetra-n-butylammonium hydroxide (TBA-OH) solution was added dropwise to HA solution and pH was adjusted to 9 and the mixture was stirred for 2 h. The resulting product was purified by dialysis against deionized water overnight. Activated HA-TBA was obtained by freeze-drying.

DSPE-HA conjugate was synthesized from DSPE and HA-TBA by reductive amination reaction, in which the amino group of DSPE was attached onto the aldehydic group of HA-TBA at the reducing end. Briefly, HA-TBA (10 μmol) was dissolved in 8 mL of dry DMSO and stirred at 60 °C until all solid completely dissolved. In a separate vial, DSPE (50 μmol) and 15 μL of triethylamine (100 μmol) were dissolved in 2 mL chloroform and sonicated until all solid dissolved. This DSPE solution was added to the HA-TBA solution. Then, the mixture was stirred at 60 °C for 2 h. Subsequently, sodium triacetoxyborohydride ($NaBH(OAc)_3$, 100 μmol) dissolved in 2 ml of dry DMSO was added dropwise. The reaction proceeded for 72 h at 60 °C under magnetic stirring, then cooled to room temperature. The suspension was then subjected to an N_2 gas stream until chloroform could no longer be smelled. The remaining mixture was poured into 30 mL distilled water and centrifuged at 10 000 g for 30 min. The supernatant was collected and the precipitate was decanted off.

We converted the tetrabutylammonium salt to the acidic form by dialyzing (regenerated cellulose dialysis tubing, MWCO 3500Da) the TBA form of the DSPE-HA conjugate against a large excess amount of 0.01 M HCl solution and the deionized water. The DSPE-HA conjugate was lyophilized to get dry powder.

The obtained DSPE-HA conjugate was confirmed by nuclear magnetic resonance spectrometer (400 MHz ^1H NMR, Bruker AVANCE III 400) and Fourier-transform infrared spectrometer (FTIR, JASCO FT/IR-4200 type A, JASCO Co., Tokyo, Japan).

The standard addition method was employed to quantitate the yield of DSPE-HA conjugate [20]. Briefly, serial known aliquots of pure HA were added into the sample, yielding a linear increase in the signal from the sugar N-acetate hydrogen. As the sugar N-acetate hydrogen of HA was remained on the DSPE-HA conjugate, the signal peak was used to semi-quantify the DSPE-HA content based on calculations.

2.3. Preparation of EPI-SWCNTs-DSPE-HA

Purification, cutting and oxidation of SWCNTs were carried out according to the previous method [21]. Pristine SWCNTs were purified via refluxing in 2.6 M HNO_3 for 24 h, collected by filtration, washed with deionized water, and dried with vacuum. The purified SWCNTs were dispersed in a mixture of 98% H_2SO_4 and 65% HNO_3 (3:1, V: V) and exposed to ultrasonication at 40 °C for 8 h. After washing with deionized water to neutrality, the obtained oxidized SWCNTs (Ox-SWCNTs) were then dried under vacuum at 60 °C for further use.

EPI loading onto Ox-SWCNTs was achieved according to the previous method [22]. Briefly, Ox-SWCNTs and EPI (1:1, w/w) were dispersed in pH 9.0 PBS and stirred overnight at room temperature. Unbound excess EPI was removed by ultracentrifugation and washed thoroughly with water until the supernatant became colorless. The amount of unbound EPI was determined by UV–Vis absorbance spectra using an UV2800 spectrophotometer (Unico, China) at 490 nm (the characteristic absorbance of EPI). Finally EPI loaded Ox-SWCNTs (denoted as EPI-SWCNTs complexes) were collected by lyophilization of the formed complexes.

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