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Folate-polydiacetylene-liposome for tumor targeted drug delivery and fluorescent tracing

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ABSTRACT

A novel folate-polydiacetylene-liposome (FP-PL) with both targeted drug delivery and fluorescence tracing was prepared by thin film rehydration method. The simulated drug delivery was performed in Bcap-37 breast cancer cells and Hs578Bst normal cells *in vitro*. The internalization and distribution of FP-PLs in the cells were presented by fluorescence cell imaging. The results show that the FP-PLs possess low cytotoxicity, good biocompatibility and better targeting efficiency in comparison to polydiacetylene-liposomes (P-PLs).

1. Introduction

For the treatment of cancer, a great deal of research has attempted to find a drug delivery system that selectively destroys diseased cells but is not harmful to healthy tissues [1]. Drug carriers with targeting ligand and tracer are the most effective way to achieve enhanced curative effect and low side effect [2]. Multifunctional nanoparticles with appropriate inherent physicochemical properties and labels would allow us to diagnose disease and evaluate treatment efficiency, while tracking pharmacokinetics and drug releasing of the particles.

Liposomes are spherical vesicles consisting of phospholipid bilayers surrounding an aqueous cavity, which have been used widely in nanomedicine [3]. Liposomes can offer better biocompatibility, capacity for surface manipulations [4] and easy exhibition of the desired enhanced permeability and retention (EPR) effect [5]. However, the EPR effect is not suited for low vascular permeability cancers [6], and the accelerated blood clearance phenomenon will also inhibit the performance of liposome drugs [7]. Ligand-conjugated, active-targeted drug delivery systems partially solved this problem [8]. Among the ligands used for the active targeted nanocarriers, a readily available and biologically active small molecule, folic acid (vitamin B9), is widely employed for the targeting of cancer cells [9,10]. Folic acid binds with a high affinity to the glycosylphosphatidylinostiol-linked folate receptor, which is overexpressed at the surface of many types of cancer cells [11]. Nevertheless, folate ligand suffers from relatively nonselective interaction [12]. So, an imaging agent will be of great help to track the nanomedicine *in vivo*.

Up to now, imaging agents, such as fluorescent dye molecules, quantum dots, superparamagnetic iron oxide, semiconductor nanocrystals, fluorescent peptides/proteins, and conjugated polymers have been commonly used for biological tracing and diagnostic imaging [13]. However, several disadvantages like toxicity to living organisms and fluorescence bleaching still remain to be overcomed [14]. Polydiacetylenes (PDAs), a family of conjugated polymers, have unique optical and chromatic properties [15,16]. Moreover, fluorescent properties of PDAs were reported [17] and were applied in turn-on fluorescence detection [18]. Recently, a PDA-liposome (P-PL) with near infrared reversible fluorescence was reported [19], and it is particularly suitable for application in biological systems. The P-PL has been used for controlled drug delivery, where the cross-linking of diacetylene lipids could reduce drug leakage at room temperature [20,23]. However, the delivery systems allow only passive targeting in tumor uptaking of the agents, so their practical application was limited. Therefore, there is a more urgent requirement to develop a novel delivery system combined with fluorescence diagnostic imaging and active targeting [21].

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In this communication, multifunctional folate-polydiacetylene-liposomes (FP-PLs) were prepared by thin film rehydration method using docetaxel (DTX) as model drug and folate ligand as targeting ligand, which can be characterized with targeted drug delivery and fluorescence tracing. The simulated drug delivery was performed in Bcap-37 breast cancer cells and Hs578Bst normal cells *in vitro*. The internalization and distribution of FP-PLs in cells were presented by fluorescence cell imaging.

2. Experimental methods

2.1. Synthesis of cancerotropic ligand

In order to embed folic acid in liposomes, folic acid was modified using carbodiimide method [22] to form the cancerotropic ligand folic acid derivate (FAD) as shown in Fig. 1a. FAD was characterized by ^{1}H NMR, IR spectrum and elemental analysis, and the results were shown in Figs. S1 and S2 of ESI.

2.2. Preparation and characterization of liposomes

Liposomes were prepared using thin film rehydration method (Fig. 1c). Briefly, for the preparation of P-PLs, egg phosphatidylcholine (EPC) and PDA monomer (10,12-pentacosadiynecarboxylic acid, PCDA) (weight ratio 8:1) were dissolved in diethyl ether. The organic solvent was removed under a vacuum rotary evaporator to yield a thin uniform translucent film, in which film the PDA was inserted into the lipid bilayers. An appropriate amount of double distilled water was added to dissolve the lipid bilayers. Above processes were carried out under dark condition to avoid PDA polymerization. Then, in order to polymerize the PDA in the lipid bilayers, the liposome suspension was exposed to UV irradiation (254 nm) for few minutes, and was heated to 65 °C under strong stirring for 10 min. The resulted liposomal suspension was cooled to room temperature and further incubated for 4 h. For the preparation of plain liposomes (PLs), EPC was dissolved in diethyl ether and were evaporated to yield a thin film. Other processes are the same with the preparation of P-PLs. For the preparation of FP-PLs, EPC, PCDA, and FAD (weight ratio 8:1:0.8) were dissolved in chloroform methanol mixtures (chloroform:methanol = 9:1), and were evaporated to yield thin film. Other processes same with P-PLs preparation are used.

DTX methanol solution was added to the diethyl ether and chloroform/methanol solution for preparation of P-PLs with DTX and FP-PLs with DTX, respectively. The membrane preparation and hydration process are the same with the preparation for P-PLs. DTX encapsulation efficiency in the liposomes was measured by HPLC (ESI).

The morphology and size of the liposomes were obtained by transmission electron microscope (TEM) and dynamic laser light scattering (DLS) technique. The biocompatibility and cytotoxicity of the liposomes were probed with a MTT assay. The selective targeting of FP-PLs to Bcap-37 cancer cells and fluorescent tracing properties of PDA loaded liposomes were also explored by fluorescent microscope and flow cytometry.

2.3. Comparative cytotoxicity studies of DTX loaded liposomes

Cell viability was tested using MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay based on the cleavage of yellow tetrazolium salt MTT by metabolically active cells to form an orange formazan dye which was quantified using microplate reader (Biorad, USA, model 550). Cells were seeded in 96 well microliter plates at 1×10^4 cells per well, followed by overnight incubation. Supernatants from the wells were aspirated out and replaced with fresh aliquots of growth medium containing various liposomes with different DTX concentration. The solutions were further incubated for 24 h at 37 °C. Subsequently, MTT reagent (20 μl, 5 mg/ml) was added to each well, incubated for 4 h. Dimethyl sulphoxide (150 μl) was added in each well after aspirating out the supernatant and shaked for 10 min. Absorbance at 490 nm was recorded using microplate reader.

2.4. Cell uptake and intracellular distribution studies of liposomes by flow cytometry and fluorescence microscopy

Bcap-37 and Hs578Bst cells were transferred separately to 6-well tissue culture plates at 1×10^5 cells per well and incubated overnight at 37 °C. The culture medium was replaced with different liposomal formulations and then incubated for 6 h at 37 °C in medium. The culture medium was then removed and each well was washed with cold PBS solution and the fluorescent images of the cells were observed using a fluorescence microscopy (IX51, Olympus, Japan). Then 100 μl of trypsin was added to detach the cells. Detached cells were collected by centrifuging and resuspended in 500 μl of culture medium and analyzed by the flow cytometer (FACS-Calibur, BD Biosciences).

3. Results and discussion

For FP-PLs, UV–vis absorption, fluorescence excitation and emission spectra are presented in Fig. 2a. The optimal absorbance wavelength of 550 nm was observed in UV–vis spectrum as shown in dash line. At an excitation wavelength of 540 nm (black line in Fig. 2a), optimal emission wavelength was 625 nm as indicated by red line in Fig. 2a. The optical properties of FP-PLs were similar to the results obtained for P-PLs [23]. It means that the optical properties of FP-PLs are not affected by the introduction of FAD, therefore, FP-PLs are still suitable for imaging application.

The zeta potentials of the liposomes were obtained by DLS method, which were about −21.4 mV, −37.5 mV and −29.1 mV for PLs, P-PLs and FP-PLs, respectively (Table S1). The zeta potential of P-PLs is more negative than that of PLs. It is speculated that the PDA have been successfully embedded in the surface of PLs because the presence of carboxyl groups could result in a more negative zeta potential. Compared with P-PLs, the relatively positive zeta potential of FP-PLs might also prove the successful introduction of positively charged folic acid [10].

Particle sizes of the liposomes were obtained by DLS method, the results were shown in Fig. 2b and Table S1. The Z-average particle sizes of PLs, P-PLs and FP-PLs were 203.9 nm, 238.0 nm and 237.6 nm, respectively. The sizes of P-PLs and FP-PLs were dimensionally identical, and they were slightly larger than that of PLs. It could be caused by the introduction of PDA and FAD in the liposome, because the curvature of liposomes can be changed by the additives. Compared with that of PLs, P-PLs and FP-PLs both had narrower size distribution and more negative zeta potential. It suggested that insertion of PDA and FAD enhanced the stability of liposome.

TEM was used to characterize the sizes and morphologies of the PLs, P-PLs and FP-PLs, as shown in Fig. 2c–e. There were multiple compartments in the P-PLs, and there was some roughness around the edge of P-PLs. The edge of P-PLs was more rugged than that of the PLs, it further proved the introduction of PDA in the liposomes. FP-PLs were similar to P-PLs in structure. The diameters of PLs, P-PLs and FP-PLs were around 200, 230 and 230 nm, respectively, which results were consistent with that from DLS (Fig. 2b).

The amount of DTX entrapped in P-PLs and FP-PLs was measured by HPLC. The encapsulation efficiency of DTX was 91.5 ± 0.8% and 93.7 ± 1.2% for P-PLs and FP-PLs, respectively. The high encapsu-