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Folate mediated self-assembled phytosterol-alginate nanoparticles for targeted intracellular anticancer drug delivery

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ABSTRACT

Self-assembled core/shell nanoparticles (NPs) were synthesized from water-soluble alginate substituted by hydrophobic phytosterols. Folate, a cancer-cell-specific ligand, was conjugated to the phytosterol-alginate (PA) NPs for targeting folate-receptor-overexpressing cancer cells. The physicochemical properties of folate-phytosterol-alginate (FPA) NPs were characterized by nuclear magnetic resonance, transmission electron microscopy, dynamic light scattering, electrophoretic light scattering, and fluorescence spectroscopy. Doxorubicin (DOX), an anticancer drug, was entrapped inside prepared NPs by dialysis method. The identification of prepared FPA NPs to folate-receptor-overexpressing cancer cells (KB cells) was confirmed by cytotoxicity and folate competition assays. Compared to the pure DOX and DOX/PA NPs, the DOX/FPA NPs had lower IC_{50} value to KB cells because of folate-receptor-mediated endocytosis process and the cytotoxicity of DOX/FPA NPs to KB cells could be competitively inhibited by free folate. The cellular uptake and internalization of pure DOX and DOX/FPA NPs was confirmed by confocal laser scanning microscopy image and the higher intracellular uptake of drug for DOX/FPA NPs over pure DOX was observed. The FPA NPs had the potential as a promising carrier to target drugs to cancer cells overexpressing folate receptors and avoid cytotoxicity to normal tissues.

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1. Introduction

The main approaches in cancer treatment are surgical excision, radiation therapy and chemotherapy. The choice of therapy depends on cancer type and its development stage. Chemotherapy, which is used alone or in combination with other forms of treatment, is the treatment of cancer with one or more cytotoxic anticancer drugs for the aim to cure cancer, prolong life or palliate symptoms. Conventional chemotherapeutic drugs are distributed nonspecifically in the body where they affect both cancerous and normal cells, resulting in dose-related side effects and inadequate drug concentrations in the cancerous tissues. Non-specific drug delivery leads to significant complications that represent a serious obstacle to effective anticancer therapy. The ideal delivery carrier for anticancer drug should be able to transport the drug specifically to the cancerous tissues and release the drug molecules inside the tissues [1]. Polymeric nanoparticles (NPs) to be developed as anticancer drug carriers is recently emerging as a result of their promise in both they protect the drug from rapid metabolism or clearance and they selectively accumulate in cancerous tissues via the enhanced permeability and retention effect (EPR) induced by the defective vascular architecture of cancerous tissue and a poor lymphatic drainage system [2].

Among different polymeric NPs for targeted delivery of anticancer drugs, there has been rising interest in those NPs based on natural polysaccharides, such as heparin [3], dextran [4], curdlan [5], xyloglucan [6], arabinogalactan [7], hyaluronic acid [8–10], alginate [11–14], pullulan [15–19], and chitosan [20–27]. In particular, alginate has received our increasing attention due to its specific structure and outstanding biological properties, such as biocompatibility, biodegradability, low immunogenicity, non-toxicity, and water-solubility. Alginate is a linear anion polysaccharide consisting of two kinds of 1,4-linked hexuronic acid residues, namely β-D-mannuronate (M) and α-L-glucuronate (G) residues, arranged

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in blocks of repeating M residues (MM blocks), blocks of repeating G residues (GG blocks), and blocks of mixed M and G residues (MG blocks). The plentiful hydroxyl and carboxyl groups on alginate provide great opportunity for chemical modification.

Phytosterols which occur in plants are steroid compounds similar to cholesterol. Phytosterols can decrease serum cholesterol concentration and reduce the risk of cardiovascular disease. Preliminary evidence indicates that phytosterols may help prevent ovarian, breast, prostate, colon, stomach, and lung cancer [28]. In this study, self-assembled core/shell NPs of phytosterol-alginate (PA) were synthesized from water-soluble alginate with phytosterols as hydrophobic moieties. Folate, being widely used as a targeting ligand for delivery of anticancer drugs, was subsequently attached to PA NPs in order to target folate receptors-overexpressing cancer cells. The physicochemical characteristics of folate-phytosterol-alginate (FPA) NPs were studied using nuclear magnetic resonance (NMR), transmission electron microscopy (TEM), dynamic light scattering (DLS), electrophoretic light scattering (ELS), and fluorescence emission spectroscopy (FES). Doxorubicin (DOX), an anticancer drug displays the broad spectrum of anticancer activity, was encapsulated in the prepared NPs by simple dialysis method and its release characteristics were studied. The active targeting and therapeutic potential of the DOX/FPA NPs to folate receptors-overexpressing cancer cells was assessed by cytotoxicity and folate competition assays in vitro. The cellular uptake and internalization of pure DOX and DOX/FPA NPs was confirmed by confocal laser scanning microscopy (CLSM) image. It is anticipated that this synergistic combination of passive and active targeting will provide a promising platform for future application in vivo.

2. Materials and methods

2.1. Materials and reagents

Sodium alginate, folate, dicyclohexyl carbodiimide (DCC), 4-dimethylaminopyridine (DMAP), *N*,*N*-dimethyl acetamide (DMAc), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, 98%), propidium iodide (PI) and hoechst 33342 were purchased from Sigma Chemical Co., USA. Phytosterols were supplied by Xian Lantian Bioengineering Co. Ltd., China. DOX hydrochloride (DOX·HCl) was acquired from Zhejiang Hisun Pharmaceutical Co. Ltd., China. KB cells (folate receptor (+)) were obtained from the Fourth Military Medical University, China. RPMI 1640 folate-free culture medium was purchased from Invitrogen technologies (Carlsbad, CA). All other chemical reagents obtained from commercial sources and used in the study were analytical grade.

2.2. Synthesis of PA and FPA

Phytosterols were coupled to alginate by DCC/DMAP-mediated esterification reaction. Briefly, to a solution of alginate (3 g) in 150 mL DMSO, 500 mg of DCC and 250 mg of DMAP were added in the stirring for activating the carboxyl groups on alginate. Subsequently, 1.0 g of phytosterols was added with stirring to the above mixture and allowed to react for 24 h at room temperature. Then, the reactant mixture was filtered and dialyzed against distilled water for 2 days using a dialysis tube (molecular cut-off: 12 kDa). Distilled water was changed at intervals of 4 h. Precipitation was recovered by filtration, washed thoroughly with diethyl ether and distilled water, followed by freeze drying to obtain dried PA.

FPA was synthesized by above similar method. Briefly, to a solution of folate (600 mg) in 20 mL DMSO, 400 mg of DCC and 200 mg of DMAP were added in the stirring for activating the carboxyl groups on folate. Subsequently, 130 mL of DMSO solution containing above lyophilized PA was mixed with the activated folate mixture and allowed to react with stirring for 24 h at room temperature. Then, the reactant mixture was filtered, dialyzed and washed, and finally freeze-dried to obtain dried FPA. The synthetic route of FPA was summarized in Scheme 1.

The structures of the alginate, synthesized FPA were analyzed by ^{1}H NMR spectroscopy for confirming the conjugation of phytosterols and folate to alginate.

2.3. Preparation of self-assembled NPs

Self-assembled NPs with a roughly spherical shape were prepared by probe sonication in aqueous medium. PA or FPA was dispersed in distilled water with gentle shaking at 37 °C for 2 days, followed by sonication using a probe-type sonifier at 100 W for 2 min. The sonication step was repeated three times until the desired size of self-assembled NPs had been attained. To prevent heat built-up in the sample solution during the sonication, the pulse function (pulse on 2.0 s, pulse off 2.0 s) was used. The solution of self-assembled NPs was then filtrated through a 1.0 μm Millipore filter to remove dust and impurities.

The morphology of the self-assembled FPA NPs was observed by TEM. The sizes and zeta potential of prepared NPs were determined by DLS and ELS.

2.4. Fluorescence spectroscopy of FPA

The critical aggregation concentration (CAC) and self-aggregation behavior of FPA were investigated by FES with pyrene as a hydrophobic fluorescent probe [29]. The pyrene solution (1.0×10^{-4} M) in acetone was added to a series of test tubes, and the solution was evaporated under a stream of nitrogen gas to remove the solvent. Then, FPA solutions of various concentrations were added to each test tube to bring the final concentration of pyrene to 6.0×10^{-7} M, which was nearly equal to the solubility of pyrene in water at 22 °C. The mixture solutions were sonicated for 30 min in an ultrasonic bath. Pyrene emission spectra were recorded by fluorescence spectrophotometry. The probe was excited at 343 nm, and the emission spectra were obtained in the range of 360–420 nm at an integration time of 1.0 s. The slit widths for excitation and emission were 10 and 2.5 nm, respectively. From the pyrene emission spectra, the intensity (peak height) ratio of the third band (386 nm, I_3) to the first band (374 nm, I_1) was plotted against the logarithm of the FPA concentration. The CAC value was taken from the intersection of two straight lines.

2.5. Preparation of DOX loaded self-assembled NPs

DOX, a hydrophobic anticancer drug, was physically entrapped in the prepared self-assembled NPs by dialysis method. Briefly, DOX·HCl (5 mg) was mixed with 3 equivalents of triethylamine in *N*,*N*-dimethyl acetamide (DMAc) (2 mL) to form the DOX basic adduct. Then a PBS suspension containing predetermined amount of PA or FPA (100–500 mg) was added to the DOX solution, and the mixture was stirred overnight at 4 °C in the dark. The mixture was then transferred to a dialysis tube (molecular cut-off: 12 kDa) and dialyzed against the phosphate buffered saline (PBS) solution (1/15 M, pH 7.4) for 3 days at room temperature. The dialysis medium was replaced with a fresh PBS solution every 4 h for the first 24 h, then daily. The mixture was filtered through a filter (1.0 μm, Millipore) to remove any polymer or drug that precipitated out of solution, and then lyophilized to obtain the DOX-loaded NPs. The sample (carrier/drug = 40) was used to carry out drug release and cellular experiments.

The lyophilized drug-loaded sample was dissolved in DMAc and the solution was vigorously stirred for 2 h followed by 3 min of

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