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Inhibition of HeLa cell growth by doxorubicin-loaded and tuftsin-conjugated arginate-PEG microparticles

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In order to improve the release pattern of chemotherapy drug and reduce the possibility of drug resistance, poly(ethylene glycol amine) (PEG)-modified alginate microparticles (ALG-PEG MPs) were developed then two different mechanisms were employed to load doxorubicin (Dox): 1) forming Dox/ALG-PEG complex by electrostatic attractions between unsaturated functional groups in Dox and ALG-PEG; 2) forming Dox-ALG-PEG complex through EDC-reaction between the amino and carboxyl groups in Dox and ALG, respectively. Additionally, tuftsin (TFT), a natural immunomodulation peptide, was conjugated to MPs in order to enhance the efficiency of cellular uptake. It was found that the Dox-ALG-PEG-TFT MPs exhibited a significantly slower release of Dox than Dox/ALG-PEG-TFT MPs in neutral medium, suggesting the role of covalent bonding in prolonging Dox retention. Besides, the release of Dox from these MPs was pH-sensitive, and the release rate was observably increased at pH 6.5 compared to the case at pH 7.4. Compared with Dox/ALG-PEG MPs and Dox-ALG-PEG MPs, their counterparts further conjugated with TFT more efficiently inhibited the growth of HeLa cells over a period of 48 h, implying the effectiveness of TFT in enhancing cellular uptake of MPs. Over a period of 48 h, Dox-ALG-PEG-TFT MPs inhibited the growth of HeLa cells less efficiently than Dox/ALG-PEG-TFT MPs but the difference was not significant ($p > 0.05$). In consideration of the prolonged and sustained release of Dox, Dox-ALG-PEG-TFT MPs possess the advantages for long-term treatment.

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1. Introduction

Chemotherapy drugs possess the ability to inhibit the growth of tumor cells. However, the efficacy of conventional chemotherapy was limited due to inefficient cellular uptake of drug and drug resistance induced by large drug dosages [1]. The latter challenges can be partially overcome through optimization of drug delivery vehicles. Biomedical scientists have made numerous efforts to enable efficient drug trafficking through modifying the chemical and physical properties of drugs and their polymeric carriers, or adding biological features of tumor cells such as surface receptors onto delivery vehicles. Alginate (ALG) has been widely used in biomedical fields owing to its outstanding biosafety and biodegradability [1,2]. Furthermore, it is naturally anionic because of the presence of a large amount of carboxyl groups [3–6]. The anionic property enables the electrostatic interactions with cationic chemotherapy drugs and formation of ALG/drug complexes. In order to further prevent burst release of drug, polymers were employed to modify ALG [4,5,7]. In recent research, ALG has been modified with different types of polyethylene glycol (PEG) to improve its bioactivity and biocompatibility in emulsification [2,5,8,9], self-assembled polymeric micelles [10], microcapsule [11], hybrid microspheres, and cell microencapsulation [8,12,13]. PEG-modified ALG in these formulations exhibited improved

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pharmacokinetic properties of Dox through providing physical encapsulation. Actually, PEG-modified ALG (ALG-PEG) can be further improved by adding functional units with pH-sensitivity, photosensitivity or other responsive linkages and thus achieve improved release pattern of chemotherapy drugs [1,2,14,15].

Tuftsin (TFT), a Thr-Lys-Pro-Arg tetrapeptide, is a natural immunomodulation peptide formed by enzymatic cleavage of the Fc portion of immunoglobulin (IgG). TFT has been shown to interact with neuropilin-1 (NRP-1) receptors on macrophages, and around 72,000 binding sites are available on the surface of macrophages for this peptide [16]. As NRP-1 expression level has been shown elevated in a number of human patient tumor samples, including brain, prostate, breast, colon, and lung cancers [17–19], exogenous TFT would tend to be at a higher chance of binding tumor cells than normal cells. As such, TFT is of promise in delivering chemotherapy drugs in terms of targeting NRP-1 on tumor cell surface and mediating cellular uptake.

This study focused on the roles of Dox loading mechanisms (covalent binding and electrostatic attraction) and TFT conjugation in ALG-PEG microparticle-mediated chemotherapy. Particularly, PEG-ALG microparticles (ALG-PEG MPs) were modified in two ways: 1) with different types of linkages: electrostatic attractions between Dox and ALG-PEG (forming Dox/ALG-PEG), and chemical reaction between amino groups in Dox and carboxyl in ALG by EDC-chemistry (forming Dox-ALG-PEG); 2) with or without TFT conjugation (Dox/ALG-PEG, Dox/ALG-PEG-TFT, Dox-ALG-PEG and Dox-ALG-PEG-TFT). The release rates of Dox from different types of complexes in neutral and acidic medium were characterized. Furthermore, relative inhibition efficiencies of different MPs in HeLa cell growth over a period of 48 h were investigated.

2. Materials and methods

2.1. Materials

Sodium ALG was purchased from Sangon Biotech (Shanghai, China). Poly(ethylene glycol amine) (PEG, 4 arms, M_w = 10 kDa) was supplied by Sinopeg Biotech (Xiamen, China). Tuftsin, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were obtained from Sigma Aldrich. Doxorubicin hydrochloride (Dox) was purchased from Sangon Biotech (Shanghai, China). Triethylamine (TEA), N-dimethylformamide (DMF), and dimethyl sulfoxide (DMSO) were obtained from J&K Scientific (Beijing, China). N-ethyl-N′ (3-dimethylaminopropyl) carbodiimide hydrochloric acid (EDC), N-hydroxysuccinimide (NHS) and phosphate buffered saline (PBS) were purchased from Aladdin Bio-Chem Technology (Shanghai, China). Penicillin, streptomycin and Trypsin EDTA were purchased from Gibco. All the chemicals were of analytical grade. Deionized water was used throughout the experiments. Human cervical cancer (HeLa) cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplied by Gibco, supplemented with 10% fetal bovine serum (FBS) and antibiotics (Penicillin-Streptomycin solution) under standard cell culture conditions (humidified, 37 °C, 5% CO_2).

2.2. Preparation of ALG-PEG and Dox-ALG-PEG

ALG-PEG was synthesized via EDC reaction [20]. Briefly, 0.25 mg of ALG was dissolved into 25 mL DMSO and mixed with 15 mL distilled water in a 100-mL conical flask, followed by magnetic stirring for 12 h at room temperature [14]. Then, EDC·HCL and NHS were added into the emulsion at molar ratio of 1/1.2/1.2 (ALG/EDC·HCL/NHS) under continuous stirring [15]. The solution was divided into two equal parts and PEG was added into both solutions at a final concentration of 2 mg/mL under continuous stirring. In the first solution, the solution was stirred overnight at room temperature and precipitated by centrifugation under 19,750 g for 5 min to remove the leftover PEG and ALG [1]. Then, the solution was loaded into a dialysis bag (MWCO 8000–14000 Da) and dialyzed against distilled water for 2 days to remove residual reagents before obtaining ALG-PEG [10,14]. The second solution was incubated for 2 min under magnetic stirring, followed by adding Dox to a final concentration of 1 mg/ml, and then stirred overnight at room temperature. The mixture was precipitated by centrifugation under 19,750 g for 5 min to remove free Dox. Then, the solution was placed into a dialysis bag (MWCO 8000–14000 Da) and dialyzed against distilled water for 2 days to remove the leftover substances before Dox-ALG-PEG conjugates were obtained. ALG-PEG and Dox-ALG-PEG microparticles (MPs) were lyophilized before characterizations analysis (Scheme 1).

2.3. Preparation of ALG-PEG-TFT and Dox-ALG-PEG-TFT

0.4 mg of lyophilized ALG-PEG and Dox-ALG-PEG MPs, respectively were added into 20 ml of TFT solution (1 mg/mL). The carboxyl groups in TFT were activated beforehand via EDC reaction overnight under magnetic stirring at room temperature. The mixture was stirred for 12 h at room temperature, then transferred into a dialysis bag (MWCO 8000–14000 Da) and dialyzed against distilled water for 2 days to remove free TFT and other reagents before Dox-ALG-PEG-TFT and ALG-PEG-TFT were obtained by lyophilization (Scheme 1).

2.4. Preparation of Dox/ALG-PEG-TFT and Dox/ALG-PEG MPs

Dox·HCl (1 mg/mL) was dissolved in DMF·TEA (DMF/TEA molar ratio = 2/3) under stirring to remove HCl [1,14,15]. The solution was then added to ALG-PEG-TFT and ALG-PEG solution respectively and stirred overnight. Subsequently, the suspension (in red color) was precipitated by centrifugation under 19,750 g for 5 min to remove free Dox, and then transferred into a dialysis bag (Mw. 8000–14000 kDa) and dialyzed against distilled water for 2 days to remove leftover reagents. The medium was changed frequently, and the dialyzed solution was lyophilized to obtain Dox/ALG-PEG-TFT and Dox/ALG-PEG MPs, respectively (Scheme 2).

2.5. Characterization of the MPs

The structures of ALG-PEG, Dox/ALG-PEG, Dox-ALG-PEG, ALG-PEG-TFT, Dox/ALG-PEG-TFT, and Dox-ALG-PEG-TFT were characterized using a UV–vis spectrophotometer (UV-4802H, Unic, US) and a FT-IR spectrometer (Bio-Rad FTS-6000, US). The size and surface charge (zeta potential) of the samples were measured at room temperature using Zetasizer Nano ZS90 (Malvern Instruments, UK). Before measurement, the samples were suspended in distilled water and subjected to ultrasound sonication (Branson 2510, 100 w) for 10 min. In order to obtain the loading capacity of Dox on Dox/ALG-PEG, Dox-ALG-PEG, Dox/ALG-PEG-TFT and Dox-ALG-PEG-TFT, absorbance of the samples at 480 nm were measured by Micro spectrophotometer (NSPL3488A/NSPL3276, Zeiss, US) and the absorbance of respective carrier (ALG-PEG or ALG-PEG-TFT) was subtracted, then the corresponding Dox concentration was calculated based on a standard curve of pure Dox at 480 nm.

2.6. Drug release

In vitro release of Dox from different MPs was monitored by a dialysis method [21]. Briefly, a specific amount of MPs were loaded into a Spectro/Por dialysis membrane bag (MWCO: 2000 Da) and

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