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Glycyrrhetinic acid-modified poly(ethylene glycol)–b-poly(γ-benzyl L-glutamate) micelles for liver targeting therapy

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ABSTRACT

Liver targeted micelles were successfully constructed via self-assembly of glycyrrhetinic acid (GA)-modified poly(ethylene glycol)–b-poly(γ-benzyl L-glutamate) (GA–PEG–PBLG) block co-polymers, which were fabricated via ring opening polymerization of γ-benzyl L-glutamate N-carboxyanhydride monomer initiated by GA-modified PEG. The in vivo biodistribution and the in vitro cellular uptake of these micelles were investigated. The results showed that the relative uptake of doxorubicin (DOX)-loaded micelles (DOX/GA–PEG–PBLG) in liver was much higher than in other tissues, and the resulting DOX concentration in liver was about 2.2-fold higher than that from the micelles without modification by GA. Moreover, the cellular uptake study demonstrated that the introduction of GA to the micelles could significantly increase the affinity for human hepatic carcinoma 7703 cells, which induced a 3.7-fold higher endocytosis than unmodified ones. The cytotoxicity of DOX/GA–PEG–PBLG micelles (IC_{50} 47 ng ml^{-1}) was much higher than that of free DOX (IC_{50} 90 ng ml^{-1}). These results indicate that GA-modified micelles have great potential in liver targeting therapy.

1. Introduction

Recently, polymeric micelles have gained considerable attention as promising anticancer drug delivery carriers because of their remarkable advantages, such as small size and narrow size distribution, long-term survival in the blood circulation and solubilization of hydrophobic drugs [1–4]. These nanosized particles, especially those formed from the self-assembly of amphiphilic block co-polymers, possess a unique core–shell architecture in aqueous solution. The hydrophobic core acts as a nano-reservoir for therapeutic agents and can protect drugs from possible degradation during transportation in vivo, while the hydrophilic shell maintains a hydration barrier that can effectively stabilize the drug-loaded micelles in the blood circulation [5,6]. Various block co-polymers, such as poly(aspartic acid)–b-polylactide [7], poly(ethylene glycol)–b-poly(lactide–co-glycolide) [8], poly(ethylene glycol)–b-poly(γ-benzyl L-glutamate) [9] have been widely studied in drug delivery research.

Despite their numerous advantages – such as drug solubilization, prolonged stability in the blood circulation and reduced toxic side-effects of anticancer drugs, one major limitation of these micelles is their inability to achieve high targeting efficiency at desirable sites. In addition, insufficient cell uptake of these micelles results in an inadequate drug concentration within cells, resulting in a low therapeutic efficacy of the administered drugs [10,11]. Targeted drug delivery systems have emerged as novel nanomedicine platforms in recent years, since they offer the potential for site-specific cell targeting and increase drug accumulation [12]. One effective approach to construct a targeted drug delivery system is to modify the outer shell of the micelles with a ligand that can be specifically recognized by the receptor present on the desired site [13]. Targeting ligands that can serve this purpose include peptides [14], galactose compounds [15], folic acid [16] and glycyrrhiza [17,18]. For instance, Zhao et al. prepared folic acid-functionalized poly(ethylene glycol)–poly(lactic–co-glycolic acid) micelles (FA–PEG–PLGA). Compared with PEG–PLGA micelles, significantly enhanced endocytosis of FA–PEG–PLGA micelles by KB cells (folate receptor-positive cells) was detected via a receptor-mediated interaction, resulting in a 5-fold higher cytotoxicity [19]. The results indicate that the introduction of a specific ligand to micelles can greatly increase the drug concentration within the cell, thus significantly enhancing the efficacy of drugs.

Among various targeted drug delivery systems, liver targeted drug delivery systems (LTDDS) are particularly important because liver cancer has become one of the most common fatal cancers worldwide and the morbidity has increased year by year [20]. Traditional pharmacotherapy is limited by the toxic side-effects of the

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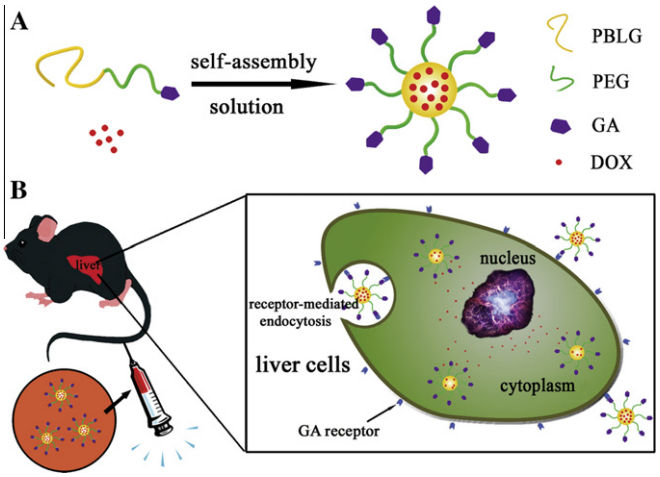
anticancer drugs used. Thus, the construction and development of an effective LTDDS is a highly desirable strategy to improve drug efficacy and reduce systematic side-effects. A number of scholars have studied liver targeted drug delivery [15,21–23]. Liang et al. prepared galactosamine conjugated poly(γ-glutamic acid)–poly(lactide) nanoparticles which mainly accumulated in the rat liver after intravenous injection. In addition, a significant delay in hepatoma tumor growth was observed, indicating potential application for targeted drug delivery to liver cancers [22]. Wang et al. also found that micelles surface modified with galactosamine had a specific affinity for hepatoma cells, and further work showed remarkable cytotoxicity as compared with non-targeted counterparts [23].

In 1991, Negishi et al. showed that there were specific binding sites for glycyrrhizin (GL) and glycyrrhetinic acid (GA) on the cellular membrane of hepatocytes, and that the number of GA binding sites on the hepatocyte surface was much higher than those for GL [24]. Since then, growing interest has focused on GA/GL-mediated LTDDS. Lin et al. found that GL-modified chitosan nanoparticles were preferentially accumulated in hepatocytes via a ligand–receptor interaction [18]. Mao et al. successfully prepared GA surface-modified liposome nanoparticles (GA–LP NPs) using a simple ethanol injection method. The in vitro uptake of GA–LP NPs by rat hepatocytes was markedly higher (3.3-fold) than that of unmodified ones [25]. However, several factors limit the application of liposomes as effective drug carriers, such as leakage of contents, rapid clearance from the blood stream and uptake by macrophages [26]. Based on this research it could be concluded that GA/GL can be effectively used as specific ligands for liver targeting. It is now necessary to develop a superior dosage GA/GL-mediated drug delivery system.

Polymeric micelles have turned out to be a promising tool for drug delivery due to their characteristics, as previously mentioned [1–4]. In this study glycyrrhetinic acid-modified poly(ethylene glycol)–b-poly(γ-benzyl L-glutamate) (GA–PEG–PBLG) micelles were fabricated as drug carriers for liver targeting. The in vivo distribution of DOX-loaded micelles in rats was investigated to evaluate their targeting ability. The cytotoxicity of DOX-loaded micelles and cellular endocytosis by human hepatic carcinoma 7703 cells were also studied. The self-assembly of DOX-loaded GA–PEG–PBLG micelles in aqueous solution and specific internalization by liver cells after intravenous administration in rats are both illustrated in Scheme 1.



Scheme 1. (A) Schematic representation of the core–shell micelles self-assembled from GA–PEG–PBLG block co-polymers and encapsulated with free base DOX. (B) Liver targeting by GA-receptor-mediated endocytosis.

2. Materials and methods

2.1. Materials

For synthesis of the block co-polymers GA was purchased from Fujie Pharmaceutical Co. (Xi'an, China). γ-Benzyl glutamate (BLG) was supplied by Sichuan Tongsheng Amino Acids (Sichuan, China). Dicyclohexylcarbodiimide (DCC), N-hydroxysuccinimide (NHS), methoxy poly(ethylene glycol)-amine (mPEG-NH_2) (molecular weight 5000), poly(ethylene glycol)-bis-amine (ATPEG) (molecular weight 3350), doxorubicin hydrochloride (DOX·HCl), fluorescein isothiocyanate (FITC) and t-butoxycarbonyl hexamethylenediamine were all purchased from Sigma–Aldrich (St. Louis, MO). Tetrahydrofuran (THF) was dried by distillation over potassium–sodium alloy. Dichloromethane (DCM) was dried by calcium hydride distillation (CaH_2). All other regents were used as received without further purification.

For cell culture and the animal experiments human hepatic carcinoma 7703 cells and female Wistar rats (200 ± 20 g) were kindly supplied by Tianjin Medical University Cancer Institute and Hospital. Care and handling of the animals were in strict compliance with the "Guide for the Care and Use of Laboratory Animals". 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), RPMI-1640 medium and fetal bovine serum (FBS) were all purchased from Sigma–Aldrich.

2.2. Synthesis of GA–PEG–PBLG block co-polymers

GA–PEG–PBLG co-polymers were prepared via a two-step procedure: synthesis of glycyrrhetinic acid-modified poly(ethylene glycol)–bis-amine (GA–PEG-NH_2) as a macro-initiator and ring opening polymerization of N-carboxyanhydride (NCA) initiated by it. The routes of synthesis are illustrated in Scheme 2.

2.2.1. Synthesis of the macro-initiator

An aliquot of 470 mg (1 mmol) of GA was dissolved in 5 ml of DCM and then added drop-wise into 10 ml of DCM containing ATPEG, DCC and NHS (molar ratio GA:ATPEG:DCC:NHS = 1:5:1.2:1.2). The mixture was stirred under a nitrogen atmosphere at room temperature (r.t.) for 24 h and the insoluble by-product dicyclohexylurea (DCU) was removed by filtration. A precipitate was obtained by the addition of ice-cold diethyl ether, while unreacted GA was not precipitated due to its solubility in diethyl ether. The product was further purified by SP-Sephadex C-25 cation exchange (Biosciences, Sweden) according to the literature procedure [27]. Finally, a pale yellow solid was obtained after freeze drying, herein denoted GA–PEG-NH_2.

2.2.2. Synthesis of γ-benzyl L-glutamate N-carboxyanhydride (BLG-NCA)

BLG-NCA was synthesized according to the method of Daly and recrystallized from ethyl acetate/hexane prior to use [28].

2.2.3. Synthesis of block co-polymers

The GA–PEG–PBLG block co-polymers were prepared via the ring open polymerization (ROP) of BLG-NCA initiated by GA–PEG-NH_2 in DCM. After reaction at r.t. for 72 h the mixture was poured into a large excess of diethyl ether to precipitate the GA–PEG–PBLG co-polymers. The resultant product was washed thoroughly with diethyl ether and then dried under vacuum. A thin ninhydrin assay was employed to confirm the absence of unreacted GA–PEG-NH_2.

PEG–PBLG block co-polymers without glycyrrhetinic acid were also synthesized by the ROP method using mPEG-NH_2 as a macro-initiator.

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