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A proof-of-concept for folate-conjugated and quercetin-anchored pluronic mixed micelles as molecularly modulated polymeric carriers for doxorubicin

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ABSTRACT

Pluronic, F127 (PEG–PPO–PEG, Mn = 12,500 g/mol) and reverse pluronic, 10R5 (PPO–PEG–PPO, Mn = 2000 g/mol) were molecularly modulated to reach multifunctional mixed micelle systems aiming to overcome some of the inherent weaknesses of pluronic based drug delivery systems. Targeting function was introduced by covalent attachment of folic acid to F127 (F127-FA), while quercetin was anchored to 10R5 (P-Q). The successful syntheses were evidenced by ^{1}H NMR, FTIR, DSC and UV–Vis. The proof-of-concept for the mixed micelles prepared from the drug anchored pluronics was demonstrated through reduced CMCs, slower release rates and increased Doxorubicin (DOX) encapsulation capacity from ~19% to ~43%. Quercetin therefore boosted the interactions of DOX with the hydrophobic core of the micelles. This was further evidenced by colloidal probe AFM which demonstrated almost doubled adhesion forces between the DOX coated probe and the quercetin modified pluronic as compared to the plain pluronic. The pre-biological essay of the DOX-modulated mixed micelle demonstrates promising properties. In addition quercetin has previously been proposed as combinatory drug to DOX enhancing its therapeutic function and reducing the side effects to normal cells.

1. Introduction

Amphiphilic polymers with ability to self-assemble into micelles in aqueous solutions are highly promising drug delivery systems [1–3]. Advantage of polymeric micelles as drug carriers include the relatively easy structural and functional modification [4] at the same time as biodistribution and bioavailability of the drugs can be improved and the drugs can in the best case be targetfully delivered to correct locations such as tumor cell [5]. Micelles can incorporate hydrophobic bioactive molecules due to their unique core–shell structure, where a hydrophilic outer shell stabilizes the hydrophobic core. The drug loading capacity of the micelles depends mainly on the secondary interactions between the micelle core and the drug molecules to be incorporated [6].

Doxorubicin (DOX) is a potent cytotoxic, antineoplastic, anthracycline antibiotic agent with a range of antitumor activities. DOX can intercalate with DNA and RNA in cancerous cells and block their replication and transcription [7]. This phenomenon can lead to death of the cancerous cells in the tumor as well as normal cells at the other parts of body, which is a severe side effect [8]. As a neoplastic agent DOX has been used for treatment of wide spectrum of cancers including leukemia (hematological malignancy), breast cancer (solid tumor carcinoma) and fibrosarcoma (Soft-tissue sarcoma). Upon conventional DOX systematic administration, free DOX can diffuse in the whole body and cause serious side effects, myocardial toxicity and cardiotoxicity in the heart [9]. Investigations have revealed different mechanism of DOX-induced cardiomyopathy [10], where free radical generation (oxygen radical and lipid peroxidation) appears to be one of the most important mechanisms. Therefore co-delivery of some antioxidant agents (radical scavengers), which can counteract redox cycling in quinolone–semiquinolone ring of DOX, could be a possible strategy mitigating DOX cardiotoxicity [11].

Quercetin, [2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-4H-1-benzopyran 4-one], is a hydrophobic flavonoid synthesized by ferns and flowering plants presenting interesting properties due to its unique structure [12]. As its multiple role in plants acting as antioxidant, antimicrobial, photoreceptor, visual attractor and feeding repellant investigations have revealed varieties of biological activities such as: anti-carcinogenic, antiallergenic, antiviral,

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anti-inflammatory, and vasodilation action. Other studies suggested that quercetin could function as chemo-preventive or/and chemotherapeutic agent against e.g. prostate [13] and breast [14] cancer. Interestingly, some surveys have emphasized on possible synergistic combination of quercetin and DOX on the therapy of the highly invasive cancerous cells [15,16]. It was reported that interference of quercetin with cell metabolism, GST activity, cytoskeleton and invasive properties in breast tumor cells, but not non-tumoral breast cells, could strengthen the antitumor effects of DOX [15]. At the same time proteomic analysis and a cell biology assay revealed that the presence of quercetin could protect cardiomyocytes in a doxorubicin-induced cardiotoxicity and heart malfunctions [17]. Therefore it seems that the combined administration of DOX with quercetin not only enhances the therapeutic index in cancerous cells but also decreases the side effects of DOX for normal cells.

Additionally, polymeric micelles as efficient targeting drug carriers to tumors [18–20] have demonstrated great potential for reducing the systematic cytotoxicity of DOX through feasibility of surface chemical modification for active targeting ligands such as folate tagged micelles [21], prolonged circulation avoiding reticuloendothelial systems (RES) [22] and passive accumulation in solid tumor via enhanced permeability and retention (EPR) [23]. Pluronic-based micelles as an FDA approved polymeric carrier [24–26], are one of the most promising vehicles with potential to not only carry bioactive molecules but also to transport them proficiently across cellular barrier by overcoming multiple drug resistance (MDR) and efflux from cancerous cell [27]. It was observed that pluronics could inhibit P-glycoprotein and sensitize MDR cells as a result of ATP depletion which consequently decreases drug resistance in the cancerous cells [28]. Pluronics are readily available as biocompatible amphiphilic polymers, but they suffer from low capacity to encapsulate large amounts of hydrophobic drugs, which decreases the encapsulation efficiency of the final micelles, and they have high critical micelle concentration (CMC) decreasing the micelle stability in the body because of the dilution in the blood after administration [28]. Introduction of new chemical functional groups [29–31] for efficient interaction with hydrophobic drugs and mixing with boosting components [32] have been utilized as the most practical strategies to overcome these limitations of the pluronics as carrier systems.

Here we designed multifunctional micelles by anchoring active compounds at the chain ends of common FDA proved pluronics, F127 and reverse pluronic 10R5. In addition to targeted delivery of combinatory drugs, our primary aim was to improve the weak-spots of plain pluronic micelles including low drug-loading capacity, high CMC and fast release rate. To do this components acting complementary to each other, both structurally and characteristically were selected. Quercetin was chosen for chemical conjugation to hydrophobic PPO part of 10R5 (P-Q) to i) boost the interaction of final micelles with DOX (giving enhanced encapsulation efficiency and more controlled release), ii) stabilize the micelles and decrease the CMC by adding a hydrophobic molecule to pluronic 10R5) and iii) co-deliver quercetin as complementary/combinatory drug to DOX to enhance cytotoxicity in tumor cells and reduce the side effects in normal cells. This molecular design at the hydrophobic core was accompanied by folate decoration at the hydrophilic shell via anchoring of folic acid to F127 (F127-FA molecule) mixed with P-Q to form the final micelles.

2. Experimental

2.1. Materials

Pluronic®F-127 (Mn ~ 12,600) and Pluronic®10R5 (Mn ~ 2000), calcium hydride, triethylamine (TEA), folic acid (FA), quercetin (Q), adipoyl chloride (AC), N,N′-dicyclohexylcarbodiimide (DCC) and 4-dimethylaminopyridine (DMAP) were obtained from Sigma–Aldrich. Dimethyl sulfoxide (DMSO) was purchased from EMD Millipore Company. Chloroform and acetone were from Fisher Chemical. Doxorubicin hydrochloride was purchased from VWR international LLC. DMSO was dehydrated (using calcium hydride, vacuum distillation and water adsorption by 3 Å molecular sieve) before use, all other reagents were of analytical grade and used as received without further purification. Polystyrene particles (PS06N) containing 2% DVB were purchased from Bangs Laboratories, Inc. and used without further purification.

2.2. Synthesis of folate-conjugated pluronic F127 (F127-FA)

F127-FA was synthesized based on the Steglich esterification method [33,34] (Scheme 1a). In brief, 0.21 g of folic acid, 0.058 g of DMAP and 0.098 g of DCC were dissolved in 25 ml of anhydrous DMSO (molar ratios of 1:1:1). The solution was stirred for 30 min under the dark environment with continuous nitrogen flow at 30 °C to activate the folic acid. Then 1 g of Pluronic®F-127 (1:6 M ratio of F-127 to FA) was added to the stirred solution and the reaction was kept additional 24 h under the same conditions. The formed solid dicyclohexyl urea (DCU) was removed by centrifugation. Then, the solution was dialyzed against deionized water for 3 days (MWCO = 3500 Da). Deionized water was renewed every 3 h during the first 12 h and once in every 12 h in the remaining 60 h to remove all the unreacted small molecules such as excess of FA, DCC and DMAP. Finally fine powder of F127-FA was obtained through lyophilization of the aqueous dispersions inside dialysis tubes.

2.3. Synthesis of quercetin-conjugated pluronic 10R5 (P-Q)

P-Q was synthesized based on a two-step reaction, as a first step the acylation of the pluronic 10R5 and as a second step the nucleophilic substitution of quercetin (Scheme 1b). Primarily, 0.201 g of adipoyl chloride (AC) as acylating agent was added continuously to 5 ml of chloroform solution of 10R5 (concentration = 200 mg/ml with ratio 1:2.2 of 10R5:AC) at a round bottom flask. The reaction was started from 15 °C with gradual increase of temperature to 60 °C under reduced pressure to evaporate added chloroform and generated HCl. The reaction was kept for another 6 h under solvent free conditions until no more HCl was released. Then10 ml of anhydrous acetone was added to dissolve the acid chloride end functionalized 10R5 product (chemical 1 in the Scheme 1b). Subsequently this solution was injected drop-wise to a 250 ml three-necked round bottomed flask containing 20 ml of acetone solution with 0.15 ml of TEA and 0.453 g of quercetin (1:3:2 M ratio of 1:quercetin:TEA). This step was processed in the room temperature under nitrogen gas for 24 h while the level of the solution was kept constant by addition of acetone. The synthesized P-Q was further purified by centrifugation–precipitation of the produced triethylammonium chloride salt. Then the solution was dialyzed against deionized water for 3 days (MWCO = 1000 Da) to remove the remaining salt and unreacted quercetin. Finally the purified aqueous solution of P-Q was lyophilized and a viscous liquid of pure P-Q was obtained.

2.4. DOX free micelles by nanoprecipitation method

All the DOX free nanometric micelles including mixed micelles (FF/PQ from F127-FA and P-Q and FF/PL from F127-FA and 10R5) and single component micelles (from F127, 10R5, F127-FA and P-Q) were prepared by nanoprecipitation method [35–37]. Briefly, 25 mg of the polymers (50:50 M ratio of the two polymers in the