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Formulation of Docetaxel by folic acid-conjugated D-α-tocopheryl polyethylene glycol succinate 2000 (Vitamin E $TPGS_{2k}$) micelles for targeted and synergistic chemotherapy

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ABSTRACT

Although high efficacy has been showed, Paclitaxel and Docetaxel cause serious side effects due to the adjuvant used in their clinical formulation Taxol® and Taxotere®. We developed a micelle system with a newly synthesized $TPGS_{2k}$ polymer, which shows lower CMC of 0.0219 mg/ml compared with 0.2 mg/ml for traditional micelles with TPGS involved, to achieve sustained and controlled drug delivery with Docetaxel used as a model anti-cancer drug. The $TPGS_{2k}$ micelles were further conjugated to folic acid (FA) for targeted drug delivery. The Docetaxel-loaded $TPGS_{2k}$ micelles with and without FA conjugation were found of desired size and size distribution, high drug encapsulation efficiency and favorable drug release. *In vitro* studies using MCF-7 cancer cells demonstrated significantly the higher cellular uptake of the formulated drug for $TPGS_{2k}$ micelle formulation than that for Taxotere®. The targeting effects for the FA conjugated $TPGS_{2k}$ micelles are also demonstrated. The IC_{50} value, which is the drug concentration needed for 50% cell viability in the designated time period, is 103.4, 1.280 and 0.1480 μg/ml for MCF-7 cancer cells after 24, 48, and 72 h treatment respectively, which is greatly decreased to be 0.526, 0.251 and 0.233 μg/ml, *i.e.* a 99.5%, 80.4% decrease and 57.5% increase for the $TPGS_{2k}$ micelle formulation, and further decreased to be 0.1780, 0.1520 and 0.1140 μg/ml, *i.e.* a 99.8%, 88.1% and 23.0% decrease for the folic acid conjugated micelles, respectively. A synergistic effect between $TPGS_{2k}$ and Docetaxel is also achieved. The present work represents a new concept in the design of drug delivery systems – the carrier materials of the drug delivery system can also have therapeutic effects, which either modulate the side effects of, or promote a synergistic interaction with the formulated drug.

1. Introduction

Low solubility in water and most pharmaceutical agents is always considered as a main disadvantage for most of the potent anti-cancer drugs such as Paclitaxel and Docetaxel due to their bulky polycyclic structure. Various drug delivery systems, such as polymeric or inorganic nanoparticles, dendrimers, liposomes and micelles, have been developed to solve this problem and further to promote sustained, controlled and targeted delivery of anti-cancer drugs [1–5]. Among them, micelles are used most often due to their technical ease, high biocompatibility and high efficiency in drug delivery. Micelles can encapsulate a drug of poor solubility in their hydrophobic core and stabilize the drug within the hydrophilic corona, thus enhancing its bioavailability [6–8]. Moreover, compared with other delivery systems, micelles show advantages to passively target tumor area through the leaky vasculature because of the enhanced permeability and retention (EPR) effect due to its small size ranging from 10 nm to 100 nm [9]. Long circulation time of micelles can also be achieved because of the steric hindrance caused by the presence of a hydrophilic shell and the small scale of micelles. The use of micelles often achieves favorable biodistribution and higher therapeutic effects and lower side effects of the drug [10,11]. However, traditional micelles have limitations such as high critical micelle concentration (CMC) which decreases the stability of micelles in the plasma, low encapsulation efficiency, which increases the amount of the micelles for a given drug dose, and the lack of active targeting effects which lowers therapeutic efficiency and causes side effects [12,13].

D-α-tocopheryl polyethylene glycol succinate (Vitamin E TPGS or TPGS) is a water-soluble derivative of natural vitamin E, *i.e.*

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a PEGylated Vitamin E (Vitamin E conjugated with polyethylene glycol (PEG)), which has amphiphilic structure comprising lipophilic alkyl tail and hydrophilic polar head portion. Its bulky structure and large surface area makes it an excellent emulsifier, solubilizer, and bioavailability enhancer of hydrophobic drugs. It has also been found that vitamin E TPGS could inhibit P-glycoprotein mediated multi-drug resistance and thus greatly enhance oral drug delivery [14–16]. One of the most sophisticated designs of drug delivery systems is that the carrier materials can also be of therapeutic effects which may either treat the side effects caused by, or promote synergistic effects with, the encapsulated anti-cancer drug. So far there has been no such drug delivery system available in the literature except for one example, *i.e.* the Poly(D,L-lactide-co-glycolide)/montmorillonite (PLGA-MMT) nanoparticle formulation of Paclitaxel. MMT is a kind of medical clay. MMT is a potent detoxifier for various toxins that may result in a host of common symptoms, including nausea, vomiting, and diarrhoea, most of which are typical symptoms of the side effects caused by anti-cancer drugs. The PLGA-MMT nanoparticle drug delivery system thus represents a new concept in developing drug delivery systems, formulating the drug carrier from a material, which can also have therapeutic effects to mediate the side effects of the drug to be delivered [17]. Our present work provides another example, in which the material of the drug delivery system itself also possesses therapeutic effects and further promotes synergistic effects with the encapsulated drug. In fact, vitamin E analogues such as TPGS are the so-called 'mitocans' which have been found to have selective cytotoxicity for cancer cells. TPGS acts by destabilizing the organelles, unleashing their apoptogenic potential, resulting in the death of malignant cells and thus suppressing the tumor growth [18].

Nevertheless, one of the disadvantages of TPGS in micelle formulation is its high CMC (0.2 mg/ml), which decreases the stability of micelles in physiological environment. Also, the chain length of PEG in TPGS is not long enough to ensure the micelles to extend blood circulation times to reduce liver accumulation. Thus, till now, TPGS was usually used together with other lipids or synthetic copolymers to form micelles and the presence of TPGS in the micelles has greatly improved the drug encapsulation efficiency and stability of the micelles [18–20]. In this work, we synthesized a new $TPGS_{2k}$ amphiphilic polymer via reaction between tocopheryl succinate and PEG of molecular weight 2000 (mPEG2000). The mPEG2000 used here was considered as a prototype to demonstrate the concept since other TPGS with longer chain length can also be developed in a similar way. The new synthesized $TPGS_{2k}$ showed much lower CMC value compared with the traditional TPGS. This change makes it possible to form stable micelles by TPGS alone without any other polymers or lipids. In fact, long chain PEG helps the micelles escape from being recognized and eliminated by the reticuloendothelial system (RES). Furthermore, through the strategy of synthesis, reactive functional groups can be easily induced by selecting different functionalized PEG at the polymer termini for further attachment of targeting moieties such as folates, aptamers, anti-bodies or peptides. For example, our group used folic acid (FOL), an oxidized form of folate, as a model targeting probe, which is efficiently internalized into the cells through the receptor-mediated endocytosis even conjugated with a wide variety of bioactive molecules. Folate receptors are over-expressed in most types of human cancer cells such as ovarian, breast and prostate cancers while only minimally distributed in normal tissues [21–23]. We thus further synthesized the $TPGS_{3350}$-FOL conjugates which are mixed with $TPGS_{2k}$ to form micelles for targeted Docetaxel delivery to the cancer cells of folate receptor overexpression. It can be predicted that such a new design of the micelles drug delivery system should achieve much higher drug encapsulation efficiency, much higher cellular uptake of the formulated drug, much longer circulation time in the plasma, and thus much higher therapeutic effects and much lower side effects.

Docetaxel was used in this work as a model anti-cancer drug, which is a semi-synthetic analogue of Paclitaxel. Docetaxel has been shown superior to Paclitaxel in a number of preclinical models due to its improved cellular uptake and increased potency of promoting the assembly of microtubules as an inhibition of the disassembly process of tubulin [24]. However, the nonionic surfactant Tween 80® (polysorbate 80) and ethanol used in its current clinical dosage form Taxotere® has been found to cause serious side effects such as neurotoxicity, fluid retention and musculoskeletal toxicity [25–27]. We hope that our new design of micelles, *i.e.* those consisting of $TPGS_{2k}$ and $TPGS_{3350}$-FOL conjugates, would provide an ideal solution for the adjuvant problems of Docetaxel as well as other anti-cancer drugs and further, to realize a sustained, controlled and targeted drug delivery. Moreover, TPGS micelles formulation may also achieve synergistic effects with the formulated Docetaxel [28].

2. Materials and methods

2.1. Materials

Docetaxel (anhydrous, 99.56%) was purchased from Shanghai Jinhe Bio-Technology Co. Ltd, China. Taxotere® was provided by National Cancer Center (Singapore). Poly(ethylene glycol) methyl ether (MW 2000), D-α-Tocopheryl succinate, polyoxyethylene bis (amine) (MW 3350), folic acid, dichloromethane (DCM), N,N′-dicyclohexylcarbodiimide (DCC), 4-dimethylaminopyridine (DMAP), N-hydroxysuccinimide (NHS), triethylamine (TEA), dimethyl sulfoxide (DMSO), coumarin-6, phosphate buffered saline (PBS, pH 7.4), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay, trypsin-EDTA solution and propidium iodide (PI) were all purchased from Sigma–Aldrich (St. Louise, MO, USA). Tween-80 was from ICN Biomedicals, Inc. (OH, USA). Triton X-100 was provided by USB Corporation (OH, USA). Fetal bovine serum (FBS) was purchased from Gibco Life Technologies (AG, Switzerland). Penicillin-streptomycin solution was from Invitrogen. Dulbecco's Modified Eagle's Medium (DMEM) was from Sigma. All solvents used in this study were HPLC grade. MCF7 breast cancer cells and NIH/3T3 fibroblast cells were provided by American Type Culture Collection (ATCC). The water used was pretreated with the Milli-Q® Plus System (Millipore Corporation, Bedford, USA).

2.2. Synthesis of $TPGS_{2k}$ and $TPGS_{3350}$-FOL

D-α-Tocopheryl succinate and $mPEG_{2k}$ was weighed and dissolved in DCM together with DCC and DMAP with stoichiometric ratio of 1:1:2:0.1 respectively and left to stir overnight in nitrogen environment at dark. The solution was then filtered to remove the by-products and precipitated in cold diethyl ether. The precipitate obtained was washed by diethyl ether again, and dissolved in water and dialyzed against water. The milky dispersion was filtered to remove impurities and the filtrate was collected. D-α-tocopheryl polyethylene glycol 2000 succinate ($TPGS_{2k}$) powder was obtained after freeze drying of the filtrate.

For amine terminated TPGS, tocopheryl succinate, PEG_{3350} bis-amine, DCC and NHS were weighed and dissolved in DCM separately with stoichiometric ratio of 1:1.2:2:2 respectively. Here the bis-amine PEG was selected such that its one end can react with the carboxyl group in tocopheryl succinate and the other end will be reserved to achieve the amine terminated TPGS. The solution was mixed with 20 μl of TEA and left to stir in a nitrogen environment at dark for 2 days. The solution was then filtered to remove by-products and precipitated in cold diethyl ether. The purifying procedure was in the same way as that for $TPGS_{2k}$. D-α-tocopheryl amino polyethylene glycol 3350 succinate ($TPGS_{3350}$-NH_2) powder was obtained after freeze drying the filtrate.

$TPGS_{3350}$-FOL was synthesized following the same procedure as above mentioned by weighing $TPGS_{3350}$-NH_2, folic acid, DCC and NHS with stoichiometric ratio of 1:1:2:2 respectively. The final product was purified and freeze-dried in the same way [29,30].

2.3. Preparation of micelles

Docetaxel (5 mg) or Coumarin-6 (1 mg) was dissolved in chloroform first and was then added into a chloroform solution of $TPGS_{2k}$ (50 mg). The organic solvent was removed by rotary vacuum evaporation. The film formed was additionally freeze-dried in vacuum, and then hydrated with 15 ml 1X PBS buffer, incubated at 37 °C for 30 min, and then sonicated for a few minutes. The resultant mixture was filtered through 0.2 μm polyethersulfone syringe filter in a sterile environment to remove the crystalline Docetaxel or Coumarin-6. The targeting micelles were prepared in a same way with $TPGS_{2k}$ replaced by $TPGS_{2k}$ and $TPGS_{3350}$-FOL mixture at a weight ratio of 9:1, which will be called FA micelles from now on. The total preparation process was shown in Fig. 1.