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Effects of PEG tethering chain length of vitamin E TPGS with a Herceptin-functionalized nanoparticle formulation for targeted delivery of anticancer drugs

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ABSTRACT

Drug formulation by ligand conjugated nanoparticles of biodegradable polymers has become one of the most important strategies in drug targeting. We have developed in our previous work nanoparticles of a mixture of two vitamin E TPGS based copolymers PLA-TPGS and TPGS-TOOH with the latter for Herceptin conjugation for targeted delivery of anticancer drugs such as docetaxel to the cancer cells of human epidermal growth factor receptor 2 (HER2) overexpression. In this research, we investigated the effects of the PEG chain length in TPGS, which is in fact a PEGylated vitamin E, on the cellular uptake and cytotoxicity of the drug formulated in the Herceptin-conjugated nanoparticles of PLA-TPGS/TPGS-COOH blend (NPs). Such NPs of PEG1000, PEG2000, PEG3350 and PEG5000, i.e. the PEG of molecule weight 1000, 2000, 3350 and 5000, were prepared by the nanoprecipitation method and characterized for their size and size distribution, drug loading, surface morphology, surface charge and surface chemistry as well as *in vitro* drug release profile, cellular uptake and cytotoxicity. We found among such nanoparticles, those of PEG1000, *i.e.* of the shortest PEG tethering chain length, could result in the best therapeutic effects, which are 24.1%, 37.3%, 38.1% more efficient in cellular uptake and 68.1%, 90%, 92.6% lower in IC_{50} (thus higher in cytotoxicity) than the Herceptin-conjugated nanoparticles of PLA-TPGS/TPGS-COOH blend of PEG2000, PEG3350 and PEG5000 respectively in treatment of SK-BR-3 cancer cells which are of high HER2 overexpression. We provided a theoretical explanation from surface mechanics and thermodynamics for endocytosis of nanoparticles.

1. Introduction

Formulation of anticancer agents by nanoparticles of biodegradable polymers (NPs) alleviate side effects of the drugs by targeting tumor via enhanced permeability and retention (EPR) effect [1]. Active targeting improves the efficacy further by surface modification of the NPs with targeting ligand. The ligand assists the NPs in penetrating into cancer cells which have overexpression of the corresponding receptor [2]. The physicochemical characteristics which have been shown to affect the efficacy of the NPs formulation of anticancer agents include size and size distribution, surface charge, surface chemistry as well as surface modification by ligand through the tethering chain of the biodegradable polymer itself or a linker molecule such as Polyethylene glycol (PEG) and Vitamin E TPGS, a PEGylated vitamin E [3].

Nanoparticles of poly (lactide)-D-α-tocopheryl polyethylene glycol 1000 succinate copolymer (PLA-TPGS) have been proven to have higher drug encapsulation efficiency (EE) and cellular adhesion than traditionally used poly (D,L-lactide-co-glycolide) (PLGA) NPs [4]. TPGS is a water soluble derivative of vitamin E. Its amphiphilicity enables its usage as efficient emulsifier, solubilizer, and bioavailability enhancer for both hydrophobic drugs and colloidal nanocarriers [5]. In addition, it has been found that co-administration of TPGS with anticancer drugs could enhance their efficacy by inhibiting P-glycoprotein mediated multi-drug resistance (MDR) [6]. Furthermore, TPGS was demonstrated to have intrinsic toxicity for cancer cells, which could synergistically enhance the therapeutic efficacy of drug that was encapsulated in the TPGS micelles [7]. Compared with TPGS emulsified PLGA NP, including TPGS as part of the matrix material in the form of PLA-TPGS copolymer prevents desorption of TPGS from the particle

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surface, generating particles with improved EE and more desirable release rate [8]. Furthermore, TPGS with functional groups can be easily synthesized to provide functional groups for conjugation of targeting ligands. It has been proven feasible to blend carboxyl group terminated TPGS (TPGS-COOH) with PLA-TPGS to form carboxyl group functionalized NPs for conjugation with Herceptin for targeting cancer cells of HER 2 overexpression [9].

Herceptin (trastuzumab) is a humanized monoclonal antibody, which has been approved by US FDA as the first-line treatment of human epidermal growth factor receptor type 2 (HER2) positive breast cancer [10]. The potential antitumor mechanism of action includes activation of antibody-dependent cellular toxicity, inhibition of extracellular domain cleavage, abrogation of intracellular signaling, reduction of angiogenesis, and decrease in DNA repair [11]. However, patients treated with Herceptin monotherapy usually have disease progresses within one year. Combination of Herceptin and other traditional cytotoxic drugs such as paclitaxel and docetaxel demonstrated synergistic effect and has been used clinically as the first-line treatment for women with HER2 positive metastatic breast cancer (MBC) [12]. Docetaxel, a semisynthetic analog of paclitaxel, demonstrates superior efficacy in terms of overall survival and median time to regression than paclitaxel in a phase III clinical trial for the treatment of MBC [13]. Its combination with Herceptin showed significant benefits than docetaxel alone in a phase II clinical trial [14]. The benefits of combining docetaxel and Herceptin could be maximized by modifying docetaxel encapsulated nanoparticle surface with Herceptin [9].

Therefore, we developed a drug delivery system of nanoparticles of a mixture of PLA-TPGS and TPGS-COOH at a designated blend ratio as matrix materials, which was loaded with docetaxel (as a model hydrophobic anticancer drug) by physical entrapment, and modified with Herceptin by chemical conjugation. PEG, the hydrophilic component of TPGS, can make the particle "stealth" after being injected into the circulation. In commercially available TPGS, the PEG component is of molecular weight 1000. However, it has been reported in the literature that when PEG molecular weight was beyond a threshold (e.g. 1500 [15], 2200 [16], 3000 [17], 3500 [18]), the plasma protein resistance could be maximized, and the nanocarrier could have prolonged circulation time [19]. Although there is less argument on whether PEG coating could extend the circulation half-life of nanocarriers, there is a debate on whether PEG coating could increase drug accumulation in tumor site. Wang and Dormidontova established a nanocarrier model which had a hard core and a shell consisting of flexible polymers, the end of which was attached with a monomeric ligand for targeting [20]. Using Monte Carlo simulation, design parameters such as nanoparticle core size, ligand-receptor binding energy, ligand density, tethering chain length and density were examined. The simulation results suggested that in close proximity of cell surface, either increasing the number of tethering chains or increasing the tethering chain length without increasing the number of ligands could increase the conformational penalty (entropy) due to the stretching or compression of the flexible polymer chains, and hence lead to a decrease in targeting effect. On the other hand, however, larger number or longer tethering chains could increase the probability of ligand-receptor interaction in far distance over larger cell surface area. In addition, two independent *in vivo* experiments showed that compared with non-PEG containing liposomes, PEG containing formulations have equal or even less accumulation of drugs in tumors, due to either reduced particle cell interaction or hindered payload release [21,22]. Kawano and Maitani evaluated the targeting effect of folic acid modified PEGylated liposomes at PEG (the tethering chain) molecular weight of 2000, 3400, and 5000 on KB cells which have an overexpression of folate receptor. Folic acid modification was achieved through post-insertion of folic acid conjugated PEG-distearoylphosphatidylethanolamine (FA-PEG-DSPE) into the liposomes. The flow cytometer results showed that at low surface ligand density (0.01–0.3 mol% FA-PEG-DSPE), the highest cellular uptake occurred at longest PEG chain length (MW 5000).

Therefore, effects of PEG chain on the performance of the PLA-TPGS NPs for drug delivery is an issue of importance, which is due in the literature. In this study, Herceptin decorated PLA-TPGS/TPGS-COOH nanoparticles are used as platform to investigate how length or molecule weight of the tethering chain TPGS-COOH (or the PEG portion of TPGS-COOH) could affect the physicochemical characteristics of the PLA-TPGS NPs and their interactions with cancer cells. We speculate that at the given particle size, matrix material (PLA-TPGS), targeting ligand type (Herceptin) and density, TPGS-COOH with PEG MW of 1000 (TPGS-$COOH_{1000}$) will result in the highest cellular uptake efficiency and cytotoxicity of the formulated drug among the various PLA-TPGS NPs consisting of TPGS-$COOH_{1000}$, TPGS-$COOH_{2000}$, TPGS-$COOH_{3350}$, and TPGS-$COOH_{5000}$ due to their smallest conformational penalty.

2. Materials and methods

2.1. Materials

Herceptin (21 mg/ml, 4.76 ml) was purchased from National Cancer Centre, Singapore. Docetaxel (anhydrous, 99.56%) was obtained from Shanghai Jinhe Bio-Technology Co. Ltd., China. Lactide (3,6-dimethyl-1,4-dioxane-2,5-dione, $C_6H_8O_4$) was purchased from Aldrich. Vitamin E TPGS (D-α-tocopheryl polyethylene glycol 1000 succinate, $C_{33}O_5H_{54}(CH_2CH_2O)_{23}$) was from Eastman Chemical Company, USA. Polyethylene glycol (PEG), stannous octoate ($Sn(OOCC_7H_{15})_2$), acetone, dimethyl sulfoxide (DMSO), coumarin-6, phosphate buffered saline (PBS, pH 7.4), sodium borate, Bradford reagent (for 1-1400 ug/ml protein), N,N′-dicyclohexylcarbodiimide (DCC), 4-Dimethylaminopyridine (DMAP), N-(3-Dimethylaminopropyl)-N′-ethyl-carbodiimide hydrochloride (EDC), N-Hydroxysuccinimide (NHS), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay, trypsin-ethylenediaminetetraacetic acid (EDTA), triethylamine (TEA), α-tocopheryl- succinate (TOS) and propidium iodide (PI) were purchased from Sigma–Aldrich (St. Louise, MO, USA). Ethanol was obtained from VWR Singapore Pte Ltd. Tween-80 was from ICN Biomedicals, Inc. (OH, USA). Triton X-100 was from USB Corporation (OH, USA). Fetal bovine serum (FBS) and penicillin–streptomycin solution were provided by Invitrogen. Dulbecco's Modified Eagle's Medium (DMEM) was purchased from Thermo Scientific Hyclone (South Logan, USA). SK-BR-3 and MCF7 breast cancer cells were obtained from American Type Culture Collection (ATCC). Water was treated with the Milli-Q Plus System (Millipore Corporation, Bedford, USA).

2.2. Methods

2.2.1. Synthesis of PLA-TPGS

PLA-TPGS was synthesized from lactide and TPGS by ring-opening polymerization with stannous octoate as a catalyst based on previous publications from our group [4]. Briefly, weighted amount of freeze-dried lactide and TPGS (85:15 w/w) and 0.5 wt% stannous octoate (in distilled toluene) were added to a round bottom flask (RBF). The RBF was purged three times with liquid nitrogen and connected to a vacuum pump via a condenser. The reaction was carried on at 145 °C for 12 h under reflux. After the reaction, the product was dissolved in dichloromethane (DCM) and precipitated in excess cold methanol to remove the unreacted lactide monomers and TPGS. The final product was collected by filtration and vacuum dried at 45 °C for two days. The product formed was analyzed by ^{1}H NMR at 500 Hz (Bruker AMX500). The number average molecular weight was calculated from the NMR spectrum.

2.2.2. Synthesis of TPGS-COOH of different molecule weights

TOS, PEG, DCC and DMAP were weighed and dissolved in DCM with a stoichiometric ratio of 1:1:2:0.1 and concentrations of TOS and PEG of 0.2 mol/L. The resulting reaction mixture was then left to stir in a nitrogen environment overnight. After reaction, the solution was filtered to remove by-products such as N,N-dicyclohexylurea (DCU). The filtrate which carried the TPGS synthesized was purified by three times precipitation in cold anhydrous ethyl ether. This entire procedure was carried out for TPGS with PEG MW of 2000, 3350 and 5000.

TPGS, glutaric acid, DCC and DMAP were weighed and dissolved in DMSO with a stoichiometric ratio of 1:1:1:0.1. The resulting reaction mixture was then left to stir in a nitrogen environment overnight. After reaction, the solution was filtered to remove by-products such as DCU. The filtrate which carried the TPGS-COOH synthesized was dialyzed against DMSO to remove excess DCC. It was then dialyzed against ultrapure water to remove DMSO before it was freeze dried and collected. The procedure was carried out for $TPGS_{1000}$, $TPGS_{2000}$, $TPGS_{3350}$, and $TPGS_{5000}$.