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The effect of hyperbranched polyglycerol coatings on drug delivery using degradable polymer nanoparticles

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ABSTRACT

A key attribute for nanoparticles (NPs) that are used in medicine is the ability to avoid rapid uptake by phagocytic cells in the liver and other tissues. Poly(ethylene glycol) (PEG) coatings has been the gold standard in this regard for several decades. Here, we examined hyperbranched polyglycerols (HPG) as an alternate coating on NPs. In earlier work, HPG was modified with amines and subsequently conjugated to poly(lactic acid) (PLA), but that approach compromised the ability of HPG to resist non-specific adsorption of biomolecules. Instead, we synthesized a copolymer of PLA–HPG by a one-step esterification. NPs were produced from a single emulsion using PLA–HPG: fluorescent dye or the anti-tumor agent camptothecin (CPT) were encapsulated at high efficiency in the NPs. PLA–HPG NPs were quantitatively compared to PLA–PEG NPs, produced using approaches that have been extensively optimized for drug delivery in humans. Despite being similar in size, drug release profile and *in vitro* cytotoxicity, the PLA–HPG NPs showed significantly longer blood circulation and significantly less liver accumulation than PLA–PEG. CPT-loaded PLA–HPG NPs showed higher stability in suspension and better therapeutic effectiveness against tumors *in vivo* than CPT-loaded PLA–PEG NPs. Our results suggest that HPG is superior to PEG as a surface coating for NPs in drug delivery.

1. Introduction

Over the past decade, nanotechnology has been extensively applied to improve bioavailability, lower side effects, and enhance targeting of therapeutic agents for a wide variety of diseases [1–4]. When agents are administered systemically, the therapeutic effect is always lowered by rapid clearance through enzymatic digestion, renal filtration, and mononuclear phagocytic system (MPS) uptake [5]. Encapsulating the agent in NPs is a promising approach to modulate these factors, as the precisely engineered NPs can protect the agent from rapid clearance but also help it reach the target site more efficiently and preferentially [6]. Widely used materials for producing NPs include polymers, lipids and some inorganic materials [7,8]. However, encapsulation of therapeutic agents in NPs does not ensure successful delivery. In fact, particulates are often more efficiently cleared from the blood by MPS uptake, particularly by phagocytic cells in the liver, leading to rapid loss of NPs and their associated drugs from circulation, which limits their ability to reach non-liver targets [9].

It is well-known that surface modification of NPs with substances that prevent non-specific adsorption can reduce their interaction with serum proteins and increase the blood circulation of the NPs [10]. An ideal surface coating resists non-specific adsorption of proteins and facilitates the attachment of other functionalities, such as targeting ligands, to the particle [2]. To resist non-specific adsorption in physiological conditions, materials for coating are usually charge neutral, hydrophilic, and stable in physiological environments. Among the few materials used as coating for NPs, PEG has become ubiquitous. The advantages of PEG as a coating of NPs for drug delivery include its low toxicity, low immunogenicity, and resistance to non-specific adsorption of biomolecules. PEG has so dominated the field of surface coatings that—with one recent exception [11]—new approaches are rarely investigated.

But PEG has considerable limitations [7,12]. For instance, it is known that PEG chains can adopt a variety of configurations on the

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surface—depending on PEG surface density—and the most effective densities are often difficult to achieve. Hyperbranched polyglycerol (HPG) has been used as a substitute for PEG in other settings since its synthesis was improved by Sunder et al., in 1999 [13–17]. Compared to PEG, HPG has many potential advantages. First, HPG is more hydrophilic than PEG. Second, the hyperbranched structure enables the HPG to cover the surface more efficiently than PEG, leading to improved resistance to adsorption [12]. Third, the multiple hydroxyl groups enable the attachment of multiple ligands on an HPG. Importantly, because of the branching structure, the ratio of the number hydroxyl groups on HPG to molecular weight is always 1/74, regardless of molecular weight; in contrast, the ratio of hydroxyl groups to molecular weight on PEG drops sharply with molecular weight (2/Mw of PEG).

Some recent findings suggested the potential for HPG as a coating for NPs. Zhao et al. reported that HPG is more hydrophilic than PEG, resulting in significant improvements in the dispersion of superparamagnetic iron oxide particles in an aqueous phase [18]. High molecular weight HPG has been demonstrated to resist the adsorption of proteins onto gold surfaces better than PEG [12]. On its own, HPG has a long blood circulation time, which depends on its molecular weight, as well as low immunogenicity [15,16]. Because of its multi-functionality and biocompatibility, HPG has been widely used as a coating for medical devices, imaging reagents and drug delivery vehicles [14,19–22]. For instance, HPG has been used as a core to conjugate with multiple PLGA molecules to load hydrophilic drugs or modified with amines and subsequently conjugated to PLA to load proteins and drugs. It is noteworthy that the introduction of amines on the HPG surface make the HPG positively charged, compromising the ability of HPG to resist non-specific adsorption [23–25]. Surprisingly, however, none of these prior studies have examined the ability of HPG to function as a coating for NPs to reduce their interaction with serum proteins and increase their blood circulation. Here, we report the analysis of HPG as a coating for NPs and compare it directly to highly optimized systems based on PEG.

2. Materials and methods

2.1. Materials

Polylactic acid (Mw = 20.2 kDa, Mn = 12.4 kDa) was obtained from Lactel. H_2N-PEG(5000)-OCH_3 was obtained from Laysan. Anhydrous dimethylformamide, dichloromethane, diisopropylcarboimide, dimethylaminopyridine, potassium methoxide, camptothecin, polyvinyl alcohol, paraformaldehyde, Tween 80, and 1,1,1-trihydroxymethyl propane were obtained from the Sigma–Aldrich. Anhydrous dry ether, methanol, acetonitrile and dimethylsulfoxide were obtained from J.T. Baker. The 1,1′-Dioctadecyl-3,3,3′,3′-Tetramethylindodicarbocyanine,4-Chlorobenzenesulfonate Salt (DiD) and DAPI stain were ordered from Invitrogen. Superfrost microscope slides were obtained from Thermo Scientific. Donkey normal serum and Rabbit-anti-CD31 antibody was ordered from Abcam and the Donkey-anti-rabbit secondary antibody tagged with Alexa488 fluorophore was from Invitrogen. Cell titer blue was obtained from Promega. Microdialysis tubing was from Thermo Scientific. Phorbol 12-myristate 13-acetate (PMA) was from Abcam.

2.2. Cell lines

Lewis lung carcinoma (LLC) cell line was obtained from the American Type Culture Collection (ATCC) (Manassa, VA). LLC cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin at 37 °C under 5% CO_2 humidified atmosphere. *U937 was maintained in RPMI1640* supplemented with 10% FBS. Differentiation of U937 to macrophage was induced by PMA (50 ng/ml).

2.3. Synthesis of HPG

HPG was synthesized by anionic polymerization [13]. Briefly, 4.6 mmol 1,1,1-trihydroxypropane (THP) was added into an argon protected flask in 95 °C oil bath and 1.5 mmol KOCH3 was added. The system was hooked up to a vacuum pump and left under vacuum for 30 min. The system was refilled with argon and 25 ml glycidol was added by a syringe pump over 12 h. The HPG was dissolved in methanol and precipitated by addition of acetone. HPG was purified with 2–3 times methanol/acetone precipitation. To further remove the low molecular weight HPG, 2–5 ml HPG was placed in a 10 ml dialysis tube (0.5–1 k cut-off) and dialyzed against DI water. The water was replaced two times every 12 h. HPG was precipitated with acetone and then dried under vacuum at 80 °C for 12 h.

2.4. Synthesis of PLA-HPG and PLA-PEG copolymers

PLA (5 g) and 2.15 g HPG were dissolved in DMF and dried with molecular sieve overnight. 0.06 ml diisopropylcarboimide (DIC) and 10 mg 4-(N,N-dimethylamino) pyridine (DMAP) was added and the reaction ran for 5 days at room temperature under stirring. The product was precipitated by pouring the reaction into cold ether and collecting precipitate down by centrifugation. The product was redissolved in DCM and precipitated again with a cold mixture of ether and methanol. The product was washed with the cold mixture of ether and methanol. The polymer was dried under vacuum for 2 days. To synthesize PLA–PEG, 2.6 g PLA and 1.0 g MPEG-NH2 were dissolved in DMF and dried with molecular sieve overnight. 0.038 ml DIC was added and reaction ran for 2 days at room temperature under stirring. The product was precipitated by pouring the reaction into cold ether and collecting the precipitate by centrifugation. The product was redissolved in DCM and precipitated again with cold ether, washed with a cold mixture of ether and methanol and dried under vacuum for 2 days.

2.5. Fabrication of NPs

Fifty mg of PLA–HPG copolymer dissolved in 1.5–3.0 ml solvent mixture (Ethyl acetate:DMSO = 4:1) was added into 4 ml DI water under vortexing and then subjected to probe sonication for 3 cycles at 10 s each. The resulting emulsion was diluted in 20 ml DI water under stirring. It was stirred for at least 5 h or hooked up to a rotavapor to evaporate the ethyl acetate and then applied to an Amico ultra centrifuge filtration unit (100 k cut-off). The NPs were washed by filtration 2 times then suspended in a 10% sucrose solution. The NPs were kept frozen at −20 °C. The PLA–PEG NPs were made by single emulsion [26]. 50 mg PLA–PEG copolymer dissolved in 1.5–3.0 ml solvent mixture (Ethyl acetate:DMSO = 4:1) was added into 4 ml DI water with 2.5% PVA under vortexing and then subjected to probe sonication for 3 cycles of 10 s each. The resulting emulsion was diluted in 20 ml DI water with 0.1% Tween 80 under stirring. The emulsion was stirred for at least 5 h or hooked up to a rotavapor to evaporate the ethyl acetate and then the solution was applied to an Amico ultra centrifuge filtration unit (100 k cut-off). The NPs were washed by filtration for 2 times then suspended in a 10% sucrose solution.

2.6. NMR method

^{1}H NMR spectra for HPG and PLA–HPG block-copolymer was recorded on a 400 MHz Agilent instrument using DMSO-d_6 as solvent. Inverse gated ^{13}C NMR spectra for HPG were recorded on a 600 MHz Agilent instrument with methanol-d_4 as solvent.

The $\overline{DP_n}$ (number-average degree of polymerization) for HPG is calculated according to the inverse gated ^{13}C NMR spectra for HPG with the following equation [13]:

$$\overline{DP_n} = \frac{(T + L_{13} + L_{14} + D)}{(T - D)} f_c$$

The functionality of the core molecule (TMP)f_c, is 3.

The Mn of HPG is calculated with the following equation:

$$Mn = \text{Molecular weight of glycidol} \times \overline{DP_n} \text{ of HPG} + \text{molecular weight of THP}$$

2.7. Characterization of NPs

The NPs were characterized with TEM. A drop of nanoparticle suspension was applied on the top of carbon coated copper grids and most of the droplet was removed with a piece of filter paper. The thin layer of NPs suspension was dried for 5–10 min and then a droplet of uranyl acetate was applied. Most of the droplet was removed with a filter paper and left to dry for 5 min. The sample was mounted for imaging with TEM. The size distribution of NPs was analyzed in Image J. The hydrodynamic size of NPs was determined by dynamic light scattering (DLS). Briefly, NPs suspension was diluted with DI water to 0.05 mg/ml and 1 ml was loaded into the cell for detection.

To determine the concentration of the dye in NPs, 990 μL DMSO was added to 10 μL NPs in aqueous solution. The solution was vortexed and left in the dark for 10 min. The concentration of the dye was quantified with a plate reader by fluorescence of the DiD dye at 670 nm with an excitation wavelength at 644 nm.

The amount of CPT encapsulated in NPs was determined by fluorescence of CPT at 428 nm with an excitation wavelength at 370 nm. One volume of NP suspension was diluted in acidified DMSO (1 N HCl : DMSO = 1:100, volume ratio) at least 10 fold. The fluorescence of CPT was measured and the amount of CPT was determined by comparing to a standard curve.

2.8. Evaluation of NPs in vitro

A suspension containing 3 mg NPs in a dialysis tube (10 K cut-off) was dialyzed against 40 ml PBS. At each time point, 970 μL solution was removed and the rest was

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