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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 adsorptions 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.

Keywords

Surface coating; nanoparticles; camptothecin; hyperbranched polyglycerol; polylactic acid

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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 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 a 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 ($2/M_w$ 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 a 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 [19, 20]. 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, 21–24]. 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 nonspecific adsorption [25–27]. 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

Poly(lactic acid) (M_w = 20.2 kDa, M_n = 12.4 kDa) was obtained from Lactel. H₂N-PEG(5000)-OCH₃ was obtained from Laysan. Anhydrous dimethylformamide, dichloromethane, diisopropylcarboimide, dimethylaminopyridine, potassium methoxide, camptothecin, poly(vinyl 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'-Di(octadecyl-3,3,3',3'-Tetramethylindodicarbocyanine,4-Chlorobenzenesulfonate Salt (DiD) and DAPI stain were ordered from Invitrogen. Super frost 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) (Manassas, 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% CO₂ 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 hours. 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 (0.5–1k cut-off) and dialyzed against DI water. The water was replaced two times every 12 hours. HPG was precipitated with acetone and then dried under vacuum at 80°C for 12h.

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 diisopropylcarbodiimide (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 1.5–3.0ml 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 sec each. The resulting emulsion was diluted in 20ml DI water under stirring. It was stirred for at least 5 hours or hooked up to a rotavapor to evaporate the ethyl acetate and then applied to an Amico ultra centrifuge filtration unit (100k 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 [28]. 50mg PLA-PEG copolymer dissolved in 1.5–3.0ml 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 sec each. The resulting emulsion was diluted in 20ml DI water with 0.1% Tween 80 under stirring. The emulsion was stirred for at least 5 hours or hooked up to a rotavapor to evaporate the ethyl acetate and then the solution was applied to an Amico ultra centrifuge filtration unit (100k cut-off). The NPs were washed by filtration for 2 times then suspended in a 10% sucrose solution.

2.6 NMR method

¹H NMR spectra for HPG and PLA-HPG block-copolymer was recorded on a 400MHz Agilent instrument using DMSO-d₆ as solvent. Inverse gated ¹³C NMR spectra for HPG were recorded on a 600MHz Agilent instrument with methanol-d₄ as solvent.

The $\overline{DP}_n$ (number-average degree of polymerization) for HPG is calculated according to the inverse gated ¹³C 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 M_n of HPG is calculated with the following equation:

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

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 laser 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 (1N 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 (10K cut-off) was dialyzed against 40 ml PBS. At each time point, 970 μL solution was removed and the rest was replaced with 40 ml fresh PBS. To quantify the CPT in the 970 μL dialyte, 30 μL of quantification fluid (DMSO: 10% SDS: 1 N HCL = 1:1:1, volume ratio) was added and the CPT concentration was quantified at EX/EM 370/428 nm with a plate reader.

The release of DiD dye was measured following a previously published protocol [29]. Briefly, microdialysis tubes were filled with 100 μL of NPs loaded with DiD and placed on a floater in a large beaker with 4L PBS at 37°C. Tubes were removed in triplicates at different time points. The PBS was changed every 12 hours. The dye left in the dialysis tube was quantified by fluorescence.

180 μL of LLC cells was plated in each well of a 96 well plate at a density of 5,000/well and left in a 37°C incubator overnight. 20 μL of free CPT or NPs in medium were added to each well. The cells were incubated for 72 h and cell viability was quantified with Cell Titer Blue.

2.9 Evaluation of NPs in blood circulation and biodistribution

All animal care and studies were approved by Yale's Institutional Animal Care and Use Committee (IACUC). Both NPs were loaded with 0.2% fluorescence dye (DiD from Invitrogen). 14 C57BL/6 mice (n=7 per group) received tail vein injection of 150 μ L DiD loaded NPs (3 mg/ml in PBS solution). At 5min, 15min, 30min, 1hr, 2hr, 4hr, 8hr, 24hr, 48hr and 72 hr 10–20 μ L blood were collected from each mouse by tail snipping. The blood was lyophilized. To quantify the fluorescence in the blood, 100 μ L of DMSO and 1ml of acetonitrile were added and homogenized with a homogenizer. The homogenized solution was spun on a benchtop centrifuge at 13,000RPM and then 0.8 ml supernatant was removed and added to an eppendorf tube. All the acetonitrile was evaporated with a SpeedVac and the dye in the DMSO was quantified at Ex/Em 644/670 nm with a plate reader.

The biodistribution of DiD-loaded NPs was evaluated in Balb/C mice bearing subcutaneous LLC tumors. LLC cells (1×10^6 cells, 0.1 ml) were injected subcutaneously into Balb/C female mice (6 week old, Charles River Laboratories), and nanoparticle administration was started after 7 days, a time when the average tumor volume reached approximately 100 mm³. Thirty mice were divided into four groups with 7–8 animals in each group. The average size and size variation of the tumors in all groups were comparable. 150 μ L DiD loaded NPs (3 mg/ml in PBS solution) were administered intravenously through tail vein. At 12h and 24h, the mice were euthanized and blood was collected with cardiac puncture. After perfusion through the left ventricle with PBS, the organs were collected. The blood and organs were lyophilized. To quantify the dye in the organs and tumors except lung and spleen, 1ml DMSO was added and homogenized. The homogenized solution was spun on a benchtop centrifuge at 13,000RPM and then 0.1 ml of the supernatant was added to a 96 well plate and the dye in the DMSO was quantified at Ex/Em 644/670 nm with a plate reader. To quantify the dye in lung and spleen, 1ml of DMSO was added and homogenized. The homogenized solution was spun on a benchtop centrifuge at 13,000RPM and then 0.1 ml supernatant was added to 1ml acetonitrile. The solution was spun on a benchtop centrifuge at 13,000RPM and then 0.8 ml supernatant was removed and added to an eppendorf tube. All the acetonitrile was evaporated with a SpeedVac and the dye was quantified in the DMSO at Ex/Em 644/670 nm with a plate reader. One-way analysis of variance (ANOVA) was performed to determine the statistical significance of the dose distribution in organs and blood, $p < 0.05$ was considered to be significant.

2.10 Immunohistochemistry

Nine balb/c mice (n=3 per group) bearing LLC tumors underwent tail vein injection of 150 μ L NPs (3 mg/ml in PBS solution) or PBS control. At 12 hrs, animals were sacrificed. After perfusion through the left ventricle with PBS the tumors were dissected and frozen in OCT. The tumors were sectioned at 10 μ m thickness and immobilized onto SuperFrost Microscope slides. The tumor sections were fixed in 4% paraformaldehyde in PBS for 30 min and then washed with TBS (20 mM Tris PLA-HPG 7.6, 140 mM NaCl) 3 times 5min each. Samples were blocked in TBS with 1% BSA and 5% donkey normal serum for 1 h and then incubated with Rabbit-anti-CD31 antibody (1:50 dilution in TBS with 1% BSA and 5% donkey normal serum). The sections were washed with TBS 3 times 5min each and then incubated with Donkey-anti-rabbit secondary antibody tagged with Alexa488 fluorophore

(Invitrogen, 1:200 dilution in TBS with 1% BSA and 5% donkey normal serum) for 1 hour and washed again with TBS 3 times, 5min each. Several drops of DAPI were placed on each slide and the slides were covered with a coverslip. Images were taken with a Zeiss fluorescence microscope.

2.11 Therapeutic studies

Forty C57BL/6 mice (6 weeks old, Charles River Laboratories) were subcutaneously injected 1×10^6 LLC cells. After 7 days, a time when the average tumor volume reached approximately 100 mm^3 , 40 mice were equally divided into 5 groups with comparable average size distribution of tumors. After grouping the mice, drug treatments were started immediately with PBS control, blank PLA-HPG NPs control and 3 CPT formulations: 1) PBS (PLA-HPG=7.4); 2) blank PLA-HPG NPs in PBS; 3) CPT DMSO solution (2.5mg/ml, DMSO: PBS10x =9:1 volume ratio); 4) PLA-PEG/CPT NPs in PBS; 5) PLA-HPG/CPT NPs in PBS. Treatments were administrated 2 times at 7 and 11 days with a dose of CPT (5mg/kg) each time. The tumor volumes and body weights of the mice were measured and recorded every two days. The tumor volumes were calculated with the formula $\text{Volume} = \text{LW}^2/2$, where L and W are the long and short diameter of a tumor respectively. Animals were euthanized when the tumor size exceeded 2000 mm^3 , total body weight loss exceeded 20%, or when other signals of sickness, such as breathing problems, failure to eat and drink, lethargy or abnormal posture, were observed. One-way ANOVA analysis was performed to determine the statistical significance of treatment-related changes in tumour volume of animals and $p < 0.05$ was considered to be significant.

3. RESULTS

3.1 Synthesis of NPs

To demonstrate that HPG can be used as a substitute for PEG as a surface coating, we made NPs from both PLA-HPG and PLA-PEG. Both particles have a biodegradable PLA core, which can be used to load hydrophobic agents, and a hydrophilic shell of HPG or PEG. HPG was made by anionic polymerization and characterized by ^1H NMR and ^{13}C NMR (Fig. S1). PLA-HPG copolymer was synthesized by esterification and the conjugation of PLA-HPG was confirmed by ^1H NMR (Fig. S2). The weight percentage of HPG in PLA-HPG was about 29% as calculated from the ^1H NMR results. PLA-HPG NPs were made from a single emulsion as described in Methods. PLA-PEG copolymer was synthesized by the conjugation of PLA-COOH with amine terminated mPEG and also characterized with ^1H NMR (Fig. S3). The weight percentage of PEG was about 26% as calculated from the ^1H NMR result.

3.2 Characterization of NPs

Transmission electronic microscopy (TEM) confirmed the spherical shape of the PLA-HPG and PLA-PEG NPs (Fig. 1a–d). The hydrodynamic diameter of NPs was 100 nm as measured by dynamic light scattering (DLS) (Fig. 1e). In this study, CPT loading of both PLA-HPG and PLA-PEG NPs is 5%. The NPs loaded with CPT have a larger fraction in the upper size range and larger size of hydrodynamic diameters by TEM imaging. When the NPs were loaded with CPT and incubated in buffered water, the agent was released over a period of ~1 week (Fig. 2a). Both NPs showed similar patterns of CPT release. After 24 hr

of incubation, over half of the CPT was released from PLA-HPG (59%) and PLA-PEG (56%) NPs. The rest of the encapsulated drug was slowly released over a period of 1 week. PLA-HPG/CPT NPs remain suspended in solution significantly longer than PLA-PEG/CPT NPs, indicating greater stability of PLA-HPG/CPT NPs in suspension (Fig. 2b).

3.3 *In vitro* evaluation of NPs

The effect of HPG on NPs was evaluated *in vitro* by measuring cell uptake by macrophages. Both PLA-HPG NPs and PLA-PEG NPs showed significantly lower macrophage uptake compared to that of the plain PLA NPs (Fig. 3). PLA-HPG/CPT NPs were evaluated for cell toxicity to LLC cells. The controls chosen for this study were PLA-PEG/CPT NPs and free CPT. Both NP formulations showed the significantly improved cytotoxicity profile (Fig. 4a). To show that this toxicity is due to the CPT, and not the polymers, we examined the effect of blank NPs on LLC cells: both blank NP formulations showed no toxicity (Fig. 4b).

3.4 *In vivo* evaluation of NPs

To demonstrate the effect of the HPG coating *in vivo*, NPs were injected intravenously and blood was analyzed periodically for the presence of particles. To permit quantification of NP concentration in the blood, NPs were loaded with 0.2% 1,1'-Diiodododecyl-3,3',3'-Tetramethylindodicarbocyanine, 4-Chlorobenzenesulfonate Salt (DiI). DiI is a hydrophobic dye, which has been widely used as a marker for NPs in other studies [29, 30]. DiI-loaded NPs release a minimal amount of dye (~20%) over 5 days of continuous incubation in PBS (Fig. S4). Both PLA-HPG and PLA-PEG NPs were loaded with equivalent amounts of DiI. Comparing the PLA-PEG NPs, the PLA-HPG showed a longer time of circulation after administration (Fig. 5a). The elimination half life of PLA-HPG NPs (10.3 hr) was significantly longer than PLA-PEG NPs (6.8 hr): half lives were determined by fitting with a two-compartment model [31] (Fig. 5a, inset). A negligible amount of either NP was found in circulation 2 days after administration.

To further demonstrate the effect of the PLA-HPG NPs, we studied the biodistribution of the NPs in mice with subcutaneous LLC tumors. Fluorescence measured in tissues was normalized to percent dose/gram tissue (Fig. 5 b & c). There was no significant difference in the accumulation of either PLA-HPG or PLA-PEG NPs in brain, heart, kidney, lung and spleen at 12 and 24 hr. However, compared to the PLA-PEG NPs, the PLA-HPG NPs were present in significantly higher concentration in the tumor and blood, but significantly lower concentration in the liver at 12 hr after injection. These differences persisted at 24 hr (although were not statistically significant in blood and tumor). To better understand the impact of overall NP distribution we calculated the total mass of NPs in each organ, tumor and the whole blood by multiplying the percent dose/gram tissue with tissue weight (Fig. 5 d & e). The majority of NPs were in either liver or blood at both time points. At 12 hours, two times as many PLA-HPG NPs were present as PLA-PEG NPs in whole blood and one third as many PLA-HPG NPs, compared to PLA-PEG NPs, were present in the liver. At 24 hours, PLA-HPG accumulation in the liver was half of PLA-PEG though both NPs were present in similar quantities in blood.

3.5 Immunohistochemistry

To visualize the penetration of NPs in tumors, we performed immunohistochemistry on cryo-sections of tumors that were treated with PLA-HPG NPs. NPs (red) were found beyond the boundaries of blood vessel lumens (green), indicating that the NPs penetrated deep into the tumor tissue after extravasating through the tumor vascular after intravenous administration (Fig. 6).

3.6 Therapeutic studies

To compare the therapeutic effect of PLA-HPG NPs with the optimized PLA-PEG NPs, we injected CPT-loaded NPs intravenously in mice bearing LLC subcutaneous tumors at a CPT dose of 5 mg/kg at 7 and 11 days after tumor inoculation (Fig. 7a). The growth rate of tumors treated with the blank PLA-HPG NPs was indistinguishable from that of tumors treated with PBS control, indicating that PLA-HPG alone has no effect on the tumor growth, as we anticipated. The tumor growth rate in mice given PLA-HPG/CPT NPs was significantly lower than that of the mice treated with PBS, free CPT, or PLA-PEG/CPT NPs. Interestingly, no significant *in vivo* toxicity was observed for all formulations (Fig. 7b).

4. Discussion

In much of the literature published during the past few decades, PEG is attributed a unique, almost magical, ability to resist non-specific adsorption of biomolecules, particularly serum proteins. As a result, PEG has been widely used as coating in biomaterials and drug delivery systems. It is commonly accepted that the properties of PEG result from a combination of its neutral charge, molecular flexibility, and hydrophilicity. The use of PEG has become so dominant in the field of particulate drug delivery, that alternatives are rarely examined.

Here, we tested the hypothesis that HPG can provide NPs with properties that are superior to PEG. HPG is a hyperbranched, hydrophilic polymer with a high density of hydroxyl groups on its surface: it is more hydrophilic than PEG and has been demonstrated to have better compatibility and non-specific resistance to biomolecules than PEG in certain applications [12, 14, 32]. HPG is well known to have a lower intrinsic viscosity than linear PEG, which decreases the possibility of red cell aggregation when present in the circulation [20] [33]. While HPG has been explored in a variety of biomedical settings—principally for coatings on implanted materials—it has never before been tested as a surface coating for drug delivery systems. In our studies, we demonstrate that HPG has substantially improved properties compared to PEG when conjugated to NPs: these properties result from its higher hydrophilicity leading to a better effect and more stability in suspension. We conclude that NPs coated with HPG are likely to be more effective in clinical medicine than NPs coated with PEG.

Certain properties of the PLA-HPG conjugate are important for the observed effects. Because high molecular weight HPG has better resistance of non-specific adsorption to biomolecules [12], we removed the low molecular weight components from the HPG we synthesized by multiple solvent precipitations and dialysis. PLA was selected as the hydrophobic core material because it is biodegradable, has a long history of clinical use, and is the major component of a NP system that is advancing in clinical trials [34]. To covalently

attach the PLA to HPG, the previous approach is to first functionalize the HPG with an amine and then conjugate the carboxylic group on PLA to the amine. This approach is efficient [27] but cannot be used to make HPG as surface coatings since any amines that do not react with PLA will lead to a net positive charge on the neutral HPG surface and reduce the ability of HPG to resist non-specific adsorption of proteins and other biomolecules. To avoid this, our approach used a one-step esterification between PLA and HPG, which maintained the charge neutral state of the HPG.

Blood circulation and biodistribution are standard methods used to examine the surface effect *in vivo*. NPs coated with certain coatings tend to escape the MPS and circulate longer in blood. Although it is known that PEG coatings can significantly enhance blood circulation of NPs and reduce accumulation in the liver [34], the majority of injected PEG-coated NPs still accumulate in the liver [35]. In contrast, the HPG coating on PLA NPs produces much lower NP accumulation in the liver. With different markers, labeling and detection methods, the absolute value of biodistribution of NPs varies. This makes it difficult to compare our data to other NP formulations. However the liver to blood ratio of PLA-HPG NPs, $\sim 1/3$ at 12 hr and ~ 1 at 24 hr, is comparable to that of BIND-014[34], a PLA-PEG NP formulation in clinical trials, that was optimized from more than 100 NP formulations. Surprisingly, the spleen to blood ratio of PLA-HPG NPs, $\sim 2/3$ at 12h and ~ 1 at 24 h, is even lower than that of BIND-014.

Nanoparticles accumulate in tumors through the enhanced permeability and retention (EPR) effect, which results from leaky vasculature of the tumor. Both PLA-HPG NPs and PLA-PEG NPs have a hydrodynamic diameter of 100nm. Notably, tumor accumulation of PLA-HPG NPs is ~ 3 times greater than accumulation of PLA-PEG NPs (Fig. 5b–e). This enhanced tumor accumulation of PLA-HPG NPs over PLA-PEG NPs must be due to the enhanced blood circulation time, which is conferred by their surface coatings. Penetration of the PLA-HPG NPs was further confirmed by immunohistochemistry (Fig. 6). To demonstrate that PLA-HPG NPs were improved carriers for drugs, we performed the therapeutic study of PLA-HPG NPs encapsulated chemotherapy drug on mice bearing subcutaneous LLC tumors. CPT was selected because it is known to be effective against a wide variety of tumors, but it is limited in clinical use by very low solubility and side effects. Here, PLA-HPG/CPT NPs provided significantly better tumor treatment than PLA-PEG/CPT NPs (Fig. 6a). In most respects, these two NPs were similar: PLA-HPG and PLA-PEG NPs had similar weight percentage of surface coating; both PLA-HPG/CPT NPs and PLA-PEG/CPT NPs had enhanced *in vitro* cytotoxicity (Fig. 4a); PLA-HPG/CPT NPs were similar in size PLA-PEG/CPT NPs (Fig. 1); PLA-HPG/CPT and PLA-PEG/CPT NPs showed similar *in vitro* release profiles of CPT (Fig. 2a). The most notable difference between the two NP formulations is the presence of HPG versus PEG. Therefore, we believe that the improved therapeutic effectiveness of PLA-HPG/CPT NPs is due to the greater emulsion stability, improved blood circulation time, improved biodistribution, and improved tumor penetration that result from HPG. We conclude that HPG is superior to PEG as a surface coating for NPs.

5. Conclusion

HPG-coated PLA NPs were successfully fabricated and characterized. The blood circulation time and biodistribution reveal a unique effect of HPG over PEG, including extended blood circulation time and significantly reduced accumulation in the liver. Improved delivery of CPT to tumors further confirms the greater effectiveness of HPG over PEG, and indicates the translational potential of HPG coating as a substitute for PEG in drug delivery.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Characterization of NPs using TEM and DLS. The size distribution and TEM image of NPs comprising PLA-HPG (a), PLA-HPG/CPT (b), PLA-PEG (c) and PLA-PEG/CPT (d). The size of NPs was analyzed by image J on lower resolution images with much larger population of NPs and plotted as histograms. The scale bar represents 200nm. (e) Z-average hydrodynamic diameter of NPs measured by DLS.

Fig. 2.
Release profile and emulsion stability of PLA-HPG/CPT and PLA-PEG/CPT NPs. (a) Controlled release profile of the PLA-HPG/CPT and PLA-PEG/CPT NPs. Data are shown as mean $\pm$ SD. (b) Emulsion appearance of the PLA-HPG, PLA-HPG/CPT and PLA-PEG/CPT NPs (from left to right) at different time points 30min, 1day and 10 days.

Fig. 3. Cellular uptake of PLA-HPG, PLA-PEG and PLA NPs detected by confocal microscopy. Macrophage derived from U937 were incubated with NPs overnight. The colors of the NPs and nuclei are red and blue respectively. The scale bar represents 25 μm .

Fig. 4.

In vitro cytotoxicity of CPT loaded NPs. (a) Cell viability of LLC cells incubated with free CPT, PLA-HPG/CPT and PLA-PEG/CPT NPs for 3 days. (b) Cell viability of LLC cells incubated with PLA-HPG and PLA-PEG NPs for 3 days.

Fig. 5.

Comparison of PLA-HPG and PLA-PEG NPs *in vivo*. (a) Blood concentration of DiD loaded PLA-HPG NPs, PLA-PEG NPs recorded as a function of time after intravenous administration. The fluorescence of NPs was converted to dose and the dose was plotted against the time point of the blood collection and fitted with a two-compartment model (a, inset). Data are shown as mean $\pm$ SD ($n = 7$). Biodistribution of DiD loaded PLA-HPG and PLA-PEG NPs after intravenous administration recorded as percent dose per gram tissue at 12h (b) & 24h (c) and as dose per organ at 12h (d) & 24 h (e). Data are shown as mean $\pm$ SD ($n = 6$).

Fig. 6. Histological study of the DiD loaded NPs in tumors. Tumor cryosections 12 hours after injection of PLA-HPG NPs, PLA-PEG NPs, and PBS were visualized in Dapi, GFP and Cy5 channels at 20x ($420\ \mu\text{m} \times 320\ \mu\text{m}$). The colors of the NPs, blood vessels and nuclei were red, green and blue respectively.

Fig. 7.

Tumor growth inhibition and animal body weight change with therapeutic treatments. (a) Tumor growth on LLC bearing C57BL/6 mice treated with PBS, PLA-HPG NPs, free CPT, PLA-HPG/CPT and PLA-PEG/CPT NPs at 7 and 11 day after tumor inoculation. Data are shown as mean $\pm$ SD ($n = 7$). (b) The body weight of the mice after receiving the therapy.