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The effect of surface charge on *in vivo* biodistribution of PEG-oligocholic acid based micellar nanoparticles

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ABSTRACT

To systematically elucidate the effect of surface charge on the cellular uptake and *in vivo* fate of PEG-oligocholic acid based micellar nanoparticles (NPs), the distal PEG termini of monomeric PEG-oligocholic acid dendrimers (telodendrimers) are each derivatized with different number (n = 0, 1, 3 and 6) of anionic aspartic acids (negative charge) or cationic lysines (positive charge). Under aqueous condition, these telodendrimers self-assemble to form a series of micellar NPs with various surface charges, but with similar particle sizes. NPs with high surface charge, either positive or negative, were taken up more efficiently by RAW 264.7 murine macrophages after opsonization in fresh mouse serum. Mechanistic studies of cellular uptake of NPs indicated that several distinct endocytic pathways (e.g., clathrin-mediated endocytosis, caveolae-mediated endocytosis, and macropinocytosis) were involved in the cellular uptake process. After their cellular uptake, the majority of NPs were found to localize in the lysosome. Positively charged NPs exhibited dose-dependent hemolytic activities and cytotoxicities against RAW 264.7 cells proportional to the positive surface charge densities; whereas negatively charged NPs did not show obvious hemolytic and cytotoxic properties. *In vivo* biodistribution studies demonstrated that undesirable liver uptake was very high for highly positively or negatively charged NPs, which is likely due to active phagocytosis by macrophages (Kupffer cells) in the liver. In contrast, liver uptake was very low but tumor uptake was very high when the surface charge of NPs was slightly negative. Based on these studies, we can conclude that slightly negative charge may be introduced to the NPs surface to reduce the undesirable clearance by the reticuloendothelial system (RES) such as liver, improve the blood compatibility, thus deliver the anti-cancer drugs more efficiently to the tumor sites.

1. Introduction

Different types of nanoparticles (NPs), including liposomes, polymeric NPs, micellar NPs, albumin-based particles, inorganic or other solid particles (gold, iron oxide, quantum dots and carbon nanotubes) have been widely used as drug delivery vehicles for the diagnosis and targeted therapy of cancers. Early clinical results suggest that some nanoparticle therapeutics can enhance the therapeutic efficacy of delivered drugs while reducing their side effects, which can be explained by the preferential delivery of loaded drugs to tumor sites via the enhanced permeability and retention (EPR) effect [1]. The physicochemical characteristics of NPs such as composition, particle size, surface charge and surface hydrophobicity may affect their interaction with plasma proteins (opsonins) and blood components (hematocompatibility), uptake and clearance by macrophages, and hence potentially influence their biodistribution and targeted delivery of payload to the intended target sites [2]. The desired particle size of NPs for passive tumor targeting has been reported to be around 10–100 nm [3]. Hydrophilic polymers such as polyethylene glycol (PEG) have been widely used to coat the surface of NPs, in order to minimize the rapid opsonization and subsequent sequestration of NPs by

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macrophages in the reticuloendothelial system (RES). PEG surface coating can counteract the hydrophobic and electrostatic interactions between NPs and plasma proteins or macrophages, resulting in less RES uptake and prolonged blood circulation time [4–6]. Surface charge is usually introduced onto certain types of NPs (such as iron oxide and gold) to improve stability and prevent from further aggregation in aqueous solution via the electrostatic repulsion [7,8]. It has been reported that surface charge is a very important factor to determine the efficiency and mechanism of cellular uptake, and the *in vivo* fate of NPs [6,9–12]. However, the optimum surface charges (e.g. positive, neutral or negative) and charge densities were reported differently for different nanoparticle systems, in order to prolong the blood circulation time, minimize the non-specific clearance of NPs and prevent their loss to undesired locations. For example, Juliano et al. [10] reported that neutral and positively charged liposomes were cleared less rapidly than negatively charged ones, which could be explained by the tendency of negatively charged liposomes to coalesce in the presence of proteins and calcium ion in blood plasma. Conversely, Yamamoto et al. [13] demonstrated that both neutral and negatively charged PEG-PDLLA micelles exhibited no remarkable difference in their blood clearance kinetics; however, negatively charged micelles significantly reduced the non-specific uptake by liver and spleen, compared with neutral micelles, which was attributed to the electrostatic repulsion between negatively charged micelles and cellular surface. The inconsistent results from the above studies may be due to the difference of nanoparticle types, variation in stability of NPs resulted from surface charge, the nature of charged groups, and other confounding factors such as inhomogeneous particle sizes.

He et al. systematically studied the effects of particle size and surface charge on cellular uptake and biodistribution of chitosan derivative polymeric NPs [11]. However, the NPs applied in this study had large particle sizes (150–500 nm), which led to significant high liver uptake regardless the surface charges. We have recently developed a novel micellar nanocarrier with desired narrow-dispersed particle sizes of 20–60 nm for effective tumor targeting drug delivery with minimum liver uptake [14–16]. These NPs are formed by the self-assembly of novel linear-dendritic block copolymers (named as telodendrimer) with engineerable and well-defined structures, comprising polyethylene glycol (PEG) and dendritic cholic acids (CA). PEG^{5k}-CA_8 is a representative telodendrimer with optimal properties, where "5 k" represents the molecular weight of PEG (5000 Da) and "8" indicates the number of CA subunits in the telodendrimer. PEG^{5k}-CA_8 micelles exhibited high drug loading capacity, outstanding stability, preferential tumor accumulation via EPR effects, and superior anti-tumor effects when loaded with paclitaxel (PTX) in the human ovarian cancer (SKOV-3) xenograft mouse model [14].

To optimize our nanocarriers for efficient *in vivo* cancer drug delivery, we systematically studied the effects of particle surface charges on their *in vitro* cellular uptake by macrophages, cytotoxic effects, hemolytic properties and *in vivo* biodistribution in xenograft models. Different number (n = 0, 1, 3 and 6) of anionic D-aspartic acids (d) or cationic D-lysines (k) were conjugated onto the distal end of PEG chain in PEG^{5k}-CA_8 telodendrimer (the micellar subunit) to modulate the surface charge of the micellar NPs. This allowed us to systematically evaluate the effect of surface charge on the cellular uptake and *in vivo* biodistribution of NPs under the identical conditions, e.g. the same composition and similar particle sizes. The particle sizes and surface charges (zeta potential) of aspartic acids or lysines derivatized NPs were characterized by transmission electron microscopy (TEM) and dynamic light scattering (DLS), respectively. The uptake efficiencies, pathways and intracellular fates of different charged PEG^{5k}-CA_8 NPs were examined in RAW 264.7 murine macrophages. The hemolytic properties and *in vitro* cytotoxicities against RAW 264.7 cells of these nanoparticle preparations were also evaluated. Finally, the *in vivo* biodistribution and tumor targeting efficiency of different charged PEG^{5k}-CA_8 NPs after intravenous administration were investigated in nude mice bearing SKOV-3 human ovarian cancer xenograft via NIRF optical imaging.

2. Methods

2.1. Materials

Diamino polyethylene glycol (Boc-NH-PEG-NH_2, MW = 5000 Da) was purchased from Rapp Polymere (*Tübingen*, Germany). Fmoc-D-Asp(Otbu)-OH, Fmoc-D-Lys(Boc)-OH, and Fmoc-Lys(Fmoc)-OH were purchased from Anaspec, Inc. Hydrophobic NIRF dye DiD (1,10-dioctadecyl-3,3,30,30-tetramethylindodicarbocyanine perchlorate, D-307), 4′, 6-diamidino-2-phenylindole (DAPI) and LysoTracker® Green DND-2 were purchased from Invitrogen. Paclitaxel (PTX) was purchased from AK Scientific Inc. (Mountain View, CA). Cholic acid, MTT [3-(4,5-dimethyldiazol-2-yl)-2,5 diphenyl tetrazolium bromide], endocytosis inhibitors including chlorpromazine hydrochloride, amiloride hydrochloride hydrate, filipin III and all other chemicals were purchased from Sigma–Aldrich.

2.2. Synthesis of aspartic acids or lysines derivatized PEG^{5k}-CA_8 telodendrimers

Boc-NH-PEG^{5k}-CA_8 telodendrimer was first synthesized using Boc-NH-PEG-NH_2 (MW, 5000 Da), lysine and cholic acid as building blocks via solution phase condensation reactions as described previously [14]. Briefly, Fmoc peptide chemistry was used to couple Fmoc-Lys(Fmoc)-OH onto the unprotected amino group of PEG for three rounds to generate a third generation of dendritic polylysine. Cholic acid NHS ester was coupled to the terminal end of dendritic polylysine, resulting in Boc-NH-PEG^{5k}-CA_8 telodendrimer. Then, the Boc group on the PEG chain of the telodendrimer was deprotected with 50% (v/v) trifluoroacetic acid (TFA) in dichloromethane (DCM), and different number (n = 0, 1, 3 and 6) of Fmoc-D-Asp (Otbu)-OH (d) or Fmoc-D-Lys(Boc)-OH (k) were subsequently conjugated to the distal end of PEG chain of PEG^{5k}-CA_8 telodendrimer by using Fmoc peptide chemistry. The primary amine at the N-terminal of corresponding aspartic acids or lysines conjugated PEG^{5k}-CA_8 telodendrimer was acetylated by acetic anhydride. Finally, the Otbu groups of aspartic acids and Boc groups of lysines were removed to generate PEG^{5k}-CA_8 telodendrimer with different number of free carboxylic acids and primary amines, respectively. The telodendrimers were precipitated and washed three times with cold ether, dialyzed against water for 24 h and then lyophilized.

2.3. Preparation and characterization of aspartic acids or lysines derivatized PEG^{5k}-CA_8 NPs

Different number (n = 0, 1, 3 and 6) of D-aspartic acids (d) or D-lysines (k) derivatized PEG^{5k}-CA_8 telodendrimer was used to prepare the corresponding micellar NPs with various surface charge densities. The dry-down (evaporation) method was utilized as described previously [17]. Briefly, 10 mg telodendrimer were first dissolved in chloroform, mixed, and evaporated in rotavapor to obtain a homogeneous dry polymer film. The film was reconstituted in 1 mL phosphate buffered solution (PBS), followed by sonication for 30 min, allowing the polymer to self-assemble into micelles. Finally, the micelle formulation was filtered with 0.22-μm filter to sterilize the sample. The morphology of aspartic acids or lysines derivatized PEG^{5k}-CA_8 micellar NPs was observed by TEM. The particle size distribution and zeta potential of these NPs were measured by DLS using Zetatrac (Microtrac). The stability of these NPs was evaluated by monitoring the particle size of NPs in 50% fetal bovine serum (FBS) over time.

Hydrophobic near-infrared fluorescence (NIRF) dye DiD as drug surrogate was physically encapsulated in the core of micellar NPs, in order to track the *in vitro* cellular uptake and and *in vivo* biodistribution of these NPs. Briefly, 10 mg aspartic acids or lysines derivatized PEG^{5k}-CA_8 telodendrimer was dissolved in chloroform, along with 0.2 mg DiD dye and 1 mg paclitaxel (PTX). Then, the same procedure of blank micellar NPs preparation described above was followed. The fluorescence spectrum and intensities of DiD-labeled NPs with different surface derivations were characterized by fluorescence spectrometry (SpectraMax M2, Molecular Devices, USA). The *in vitro* release profiles of DiD dye from NPs were measured by the dialysis method. Briefly, DiD loaded NPs with different surface derivations were injected into a 500-μL dialysis cartridge (MWCO, 3.5 kDa, Thermo Scientific, Rochford, IL). The cartridges were dialyzed against 500 mL 10 mg/mL bovine serum albumin (BSA) solution in the presence of activated charcoal at 37 °C at the rotation rate of 100 rpm. The fluorescence intensities of DiD dye remaining in the dialysis cartridge at different time points were measured by the fluorescence spectrometer.

The surface charge properties of aspartic acids or lysines derivatized PEG^{5k}-CA_8 NPs were further characterized by the agarose gel electrophoresis. 15 μL DiD-labeled PEG^{5k}-CA_8 NPs displaying aspartic acids or lysines were loaded in 1% agarose gel and subjected to electrophoresis in the running buffer (pH 7.4) at 120 voltages for 30 min.