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Circulation kinetics and biodistribution of dual-labeled polymersomes with modulated surface charge in tumor-bearing mice: Comparison with stealth liposomes

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ABSTRACT

Polymersomes (Ps) based on poly(ethylene glycol)-b-poly(*D,L*-lactide) (PEG-PDLLA), with similar sizes (90–100 nm), but different zeta potentials (−7.6 to −38.7 mV) were prepared to investigate the effect of surface charge on blood circulation time and tissue distribution in tumor-bearing mice. For the *in vivo* studies dual labeled Ps were applied, which were obtained by encapsulating ^{3}H-dextran 70 k in the aqueous core of Ps and by post-coupling of ^{14}C-thioglycolic acid onto acrylated PEG chains of the Ps. Stealth liposomes (103 nm, −6 mV) were used as a control. A substantial longer half lifetime ($\tau_{1/2}$) (47.3 h) and a reduced liver uptake (27.9% of injected dose (% ID)) of Ps with a zeta potential of −7.6 mV were observed as compared to those of stealth liposomes (10.6 h, 39.8% ID) most probably due to the presence of a relatively thicker and denser PEG brush of the Ps as compared to the liposomes. As a result of their longer circulation times a high tumor accumulation of 18.6% ID was obtained for these Ps after 3 d circulation in mice while only 11.2% ID of stealth liposomes accumulated in the tumors as a result of their relatively short $\tau_{1/2}$ in blood. By increasing the zeta potential on Ps, more rapid clearance of Ps from the blood circulation was found due to an enhanced uptake by the liver. Importantly, co-localization of the two labels of Ps was observed during circulation indicating that dual labeled Ps were colloidally stable in blood without leakage of ^{3}H-dextran. In conclusion, the results show that Ps with a slightly negative surface charge (zeta potential −7.6 mV) are stable in the circulation and have longer circulation times and a higher tumor accumulation in mice than Ps with more negative zeta potentials or the stealth liposomes used as a control.

1. Introduction

Polymersomes (Ps), synthetic polymer-based vesicles, have attracted rapidly growing interest as a novel class of nanocarriers. Ps based on amphiphilic block copolymers with a relatively high molecular weight (MW) have relatively thick and stable membranes (up to 40 nm) [1–3]. In order to design Ps with long circulation times, amphiphilic PEG block containing polymers can be applied. The presence of PEG at the surface of the Ps will reduce interactions with blood components [4] and increase their biological stability (stealth effect) [5]. These effects are dependent on the MW of the PEG and its surface concentration. The protein resistant character of a PEG brush on the surface of Ps is due to the conformation of PEG in aqueous media, minimization of the interfacial free energy and steric repulsion [6–8]. The influence of PEG on the surface of vesicular carriers on their circulation times has been previously demonstrated by comparing the circulation times of pegylated and non-pegylated liposomes. Non pegylated liposomes have very short blood circulation times due to a fast uptake by the reticulo-endothelial system (RES) [9]. Stealth liposomes, which are coated with PEG exhibit much longer blood circulation times than non-pegylated liposomes. The PEG coating reduces protein adsorption onto the surface of the liposomes during the circulation [10].

Blood clearance of nanocarriers can be mediated by plasma proteins such as opsonins and dysopsonins that are adsorbed onto the surface of the carriers. Although there is little known about their effects on the opsonization process due to the complexity of the biological events, the surface charge and size of nanocarriers are undoubtedly also playing an important role in the adsorption of proteins to the surfaces [11–15]. It has been reported that the introduction of a slightly negative or positive charge on the surface of nanocarriers enhanced their blood circulation times and reduced the accumulation in the liver [16,17]. However, carriers with either a high negative or high positive charge were cleared more rapidly from the

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blood circulation [17–19]. Based on these results it seems likely that there may be an optimal surface charge for a specific nanocarrier to reach the longest circulation time and lowest hepatic uptake. Likewise, a range of optimal sizes for specific nanocarriers has been suggested to establish long circulation times. For example, pegylated liposomes with diameters larger than 200 nm showed a significant accumulation in the spleen as a result of mechanical filtration, which was followed by phagocytosis [20]. On the other hand pegylated liposomes with diameters below approximately 70 nm showed an increased accumulation in the liver, possibly also due to changes in protein adsorption related to the high curvature of such small liposomes [21]. It has to be realized that only particles with diameters below the cut-off value of the fenestrated sinusoidal endothelia will accumulate in the liver [22].

So far, only one paper has been published on the blood circulation times and the biodistribution of Ps [5]. Discher and his colleagues injected fluorescently labeled Ps based on PEG-poly(1,2-butadiene) (PEG-PBD) with varying lengths of the copolymer chains into rats. They found that the Ps had half life circulation times ($\tau_{1/2}$) up to 28 h and that the Ps accumulated primarily in the liver and the spleen. However, it should be noted that it cannot be excluded that some of the label is released during blood circulation and transferred to blood components (e.g. albumin).

In this study we have evaluated the effect of the surface charge of PEG-PDLLA based Ps with an average diameter of 100 nm on the circulation kinetics, organ distribution and tumor accumulation in tumor-bearing mice. In order to exclude possible transfer of labels, Ps dual labeled with ^{3}H and ^{14}C were used for the *in vivo* studies. ^{14}C-thioglycolic acid was used to label the Ps membrane and ^{3}H-dextran with a high molecular weight (70,000 g/mol) was co-localized with the Ps in the aqueous core. The zeta potential of the Ps was varied from −7.6 to −38.7 mV by coupling of thioglycolic acid onto Ps containing different molar ratios of acrylated PEG. Ps were prepared by using combinations of PEG-PDLLA's of which one of the block copolymers had PEG blocks with methoxy end groups [23] and the other copolymer PEG blocks with acrylamide end groups. Dipalmitoyl phosphatidylcholine (DPPC)/cholesterol based stealth liposomes with 7.5% of PEG distearoyl phosphatidylethanolamine (PEG-DSPE) were used as a reference. Ps containing non-labeled dextran and thioglycolic acid model systems were used for optimizing the dual labeling process and for characterizing the dual-labeled Ps with respect to the hydrodynamic diameter, polydispersity index (PDI) and zeta potential. The cytotoxicity of non-radioactive Ps for human umbilical vein endothelial cells (HUVEC) and HeLa cells was also evaluated.

2. Materials and methods

2.1. Materials

D,L-lactide (DLLA) was obtained from Purac Biochem b.v. (The Netherlands) and recrystallized from toluene. Monomethoxy poly (ethylene glycol) with a molecular weight of 5000 g/mol (mPEG, Iris Biotech, Germany) was dried by dissolution in anhydrous toluene followed by azeotropic distillation under N_2. Stannous octoate, $Sn(Oct)_2$ (Sigma, U.K.) and mercapto PEG (5000 g/mol, Iris Biotech, Germany) were used as received. FITC-dextran (70,000 g/mol) (FD70), thioglycolic acid (TG), *N,N*-methylenebis(acrylamide) (MBA) and 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) were obtained from Sigma (U.S.A.). ^{3}H-dextran (^{3}H-D70, 70,000 g/mol) and ^{14}C-thioglycolic acid (^{14}C-TG) were purchased from American Radiolabeled Chemicals (U.S.A.). DPPC and PEG-DSPE (2750 g/mol) were provided by Lipoid GmbH (Germany). Cholesterol was purchased from Sigma-Aldrich (The Netherlands). ^{3}H-cholesteryl oleylether was a product of Amersham (The Netherlands). Hionic-Fluor™ and Soluen®-350 were obtained from Perkin Elmer BioScience BV (The Netherlands). Deionized water (DI water), obtained from a Milli-Q water purification system (Millipore, The Netherlands) and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, Sigma, U.S.A.) were used for cell and animal studies. EGM® endothelial cell growth medium and EX-CELL® medium were supplied by Lonza Ltd (U.S.A.) and used for HUVEC and HeLa cells, respectively. CellTiter 96® Aq_{ueous} Non-Radioactive cell Proliferation Assay kit was purchased from Promega Benelux BV (The Netherlands) and used for the MTS assay. All other reagents used in the study were of analytical grade.

2.2. Synthesis of block copolymers

Two PEG-PDLLAs were synthesized by ring-opening polymerization (ROP) of DLLA using the hydroxyl groups of methoxy PEG (mPEG) or acrylamide PEG (aPEG) to initiate the polymerization. mPEG or aPEG (0.50 g, 0.1 mmol), DLLA (4.2 g, 29.2 mmol), $Sn(Oct)_2$ (0.04 g, 0.1 mmol) and toluene (30 ml) were charged in that order in a reaction vessel. The reaction was performed at 110 °C for 26 h under stirring. After cooling, a drop of HCl (37 wt.%) was added to the reaction mixture to hydrolyze the tin-oxygen bond. The copolymer was isolated by precipitation in methanol. After filtration and washing with methanol, the copolymer was dissolved in dichloromethane and precipitated in diethyl ether. Subsequently, the polymer was isolated by filtration, washed several times with diethyl ether, and dried under vacuum. The monomer conversion and the number average molecular weight of block copolymers were determined by ^{1}H NMR (Inova 300 MHz, Varian).

aPEG was prepared prior to ROP by reacting mercapto PEG with MBA. HO-PEG-SH (0.5 g, 0.1 mmol) was dissolved in 5 ml of DI water and MBA (0.154 g, 1 mmol) in a carbonate buffer solution (pH 10) was added drop wise to this solution. After gentle stirring for 1 h at room temperature, the resulting solution was ultrafiltrated through a membrane with a cut-off of 1,000 g/mol (Ultracel Ultrafiltration Disc, Millipore) for 2 d and lyophilized. An ELLMAN assay was carried out to determine the amount of unreacted thiol groups which indicated that less than 1% of mercapto PEG had not reacted.

2.3. Preparation and characterization of Ps

FD70 was encapsulated in Ps during the formation of Ps. mPEG-PDLLA and aPEG-PDLLA were used to prepare self-assembled Ps by injecting a THF solution of the block copolymers into an aqueous solution. Ps based on aPEG-PDLLA and mPEG-PDLLA with a weight ratio of 10:90, 50:50 and 100:0 were prepared and abbreviated as Ps10, Ps50 and Ps100, respectively. In brief, the block copolymer or the mixture (10 mg/ml) was dissolved in THF (3 ml) and FD70 (0.1 mg/ml) was dispersed in the solution. The THF dispersion was injected into HEPES (50 ml, pH 7.4, 10 mM) to spontaneously form Ps. After 15 min without shaking, the mixture was gently inverted several times resulting in a turbid dispersion. To remove the organic solvent, the dispersion of FD70 loaded Ps (FD70-Ps) was transferred into a dialysis bag (cut-off 50,000 g/mol, Spectra/Por), which was placed in a 4 l flask with DI water. The dialysis was performed for 1 d by replacing the DI water for at least 5 times. After dialysis, the dispersion of FD70-Ps was filtrated with a syringe filter (pore size 100 nm, PES, Acrodisc, Pall) and concentrated to 10 ml using Vivaspin20 (Sartorius stedim biotech.).

TG was coupled onto the acrylamide groups of PEG of Ps by a Michael addition reaction to introduce anionic charge on the Ps. For the reaction, carbonate buffer solution (1 ml, pH 10) was added to the dispersions (1 ml) of Ps10, Ps50 and Ps100. A stock solution of TG was prepared prior to the coupling by diluting TG (1 ml) with a carbonate buffer solution (9 ml). Different amounts of the stock solution were dropped into Ps dispersions (0.02 ml to Ps10, 0.20 ml to Ps50 and 1.00 ml to Ps100) under stirring and the reaction was carried out for 1 h at room temperature. Unreacted TG and free FD70 were removed by dialysis (cut-off 100,000 g/mol, Spectra/Por) in DI water at 37 °C