PEG BRUSH INHIBITION KINETICS IN OPSONIZATION-CLEARANCE: MW-SCALING FROM SELF-ASSEMBLED POLYMER VESICLES *IN VIVO*

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ABSTRACT

Although it is well established that PEG prolongs liposome circulation time in vivo, the extent to which these circulation times can be extended has not been adequately explored. This is due in part to the narrow range of PEGylation possible with stealth liposomes: PEG2000-lipid is a highly asymmetric amphipihle and can only be incorporated at <10% in liposomes without inducing vesicle micellization [Beddu-Addo, 1996]. Extension of the PEG molecular weight further reduces this critical percentage (for PEG5000, this limit is only about 3-4% [ibid]), thereby restricting, in both brush length and density, the extent to which PEGylation effects can be explored. Polymersomes offer a unique possibility in exploring such a dependence of PEG brush parameters on in vivo circulation time, since they are purely synthetic and are therefore only limited by chemical synthesis. Composed of diblock copolymers, these vesicles have a PEG chain on every amphiphile, giving an essentially 100% PEGylated vesicle. Importantly, the hydrophobic block of each molecule (either poly(ethylethylene) or poly(butadiene)) is synthesized proportionately longer such that micellization is not induced. It is therefore possible to tune both the PEG brush length and PEG density (through hydrophobic block chemistry) on polymersomes to separately elucidate the in vivo effects on PEG brush properties.

INTRODUCTION

As potential drug delivery vehicles, polymersomes extend the class of bilayered vesicles that include stealth liposomes. Because the hydrophobic/hydrophilic volume fraction of their constitutive diblock copolymers is similar to those of lipid molecules, polymersomes form spontaneously in aqueous solutions, facilitating the encapsulation of aqueous solutions of proteins or drugs potentially unstable in organic solvents. Additionally, the extended yet proportional hydrophobic core provides not only additional membrane stability and toughness, but also the potential for increased lipophilic loading, as well as the possibility for a hyper-stable cross-linked membrane.

The PEG on polymersomes is in the 'brush' conformation, and therefore likely to behave in vivo like stealth liposomes whose PEG density is also above the mushroom-to-brush transition point. It has been our goal to determine what the effect of the denser and/or thicker polymersome brush is. Vesicle clearance in vivo is generally dependent on two processes: (1) deposition on the vesicle surface by plasma proteins, and (2) uptake by phagocytes of the RES. Since the brush density on polymersomes is considerably higher than that on stealth liposomes, the resistance to opsonin deposition on the polymer layer (process 1) should be greater. We indeed find in rats that polymersomes circulate longer than stealth liposomes, but are otherwise cleared by similar mechanisms.

TABLE 1. Structural properties of vesicle-forming super-amphiphiles with copolymers compared to a prototypical lipid. For the copolymers, the number-average molecular weight, M_n, and the hydrophilic volume fraction, $f_{hydrophilic}$, were obtained by gel permeation chromatography and NMR, respectively [eg. Hillmyer and Bates, 1996]. Cryo-TEM was used to measure hydrophobic core thickness, d_{core}.

	Amphiphile	Mn (g/mol)	$f$hydrophil (v/v)	$d$core (±1 nm
SOPC	C_{18} phospholipid	790	0.31	4 nm
OE7	EO_{40}–EE_{37}	3900	0.39	8 nm
OB2	EO_{26}–BD_{46}	3600	0.28	9 nm
OB18	EO_{80}–BD_{130}	10,400	0.29	14 nm
X-OB18	E O $_{80}$ –(crosslinked-BD_{130})	$>10^6$	0.29	14 nm

From this in vivo study of PEGylated polymer vesicles, a new perspective is obtained on stealth liposomes. Combining the data from the stealth liposome literature with data on polymersomes, we deduce scaling laws that predict in vivo behavior of a generic polymer-brush vesicle. Comparison of these trends to theoretical calculations suggests that the rate-limiting step to in vivo clearance is protein

deposition on the polymer brush surface, rather than other physiological processes such as vesicle fragmentation or dissolution.

METHODS AND MATERIALS

1) Vesicle preparation: Polymersomes were prepared with a method analogous to liposomes [3]. The self-assembled vesicles were sized down to ~100 nanometer through freeze-thaw/extrusion processes, and fluorescently labeled. Final vesicle size was determined by DLS.
2) In vivo studies with polymersomes: Polymersome suspensions were injected into the tail veins of adult rats. At pre-set time points, the rats were bled via orbital bleeds; no more than 10% of the total blood volume was removed per animal. Plasma containing polymersomes was then separated and quantified by fluorescence microscopy.

RESULTS

The initial design of the vesicle-forming copolymer OE7 [Discher et al, 1999] has been extended to a new series of 'OB' vesicle-forming diblocks (Table 1) that also allow for crosslinked membranes. The total M_n of the largest copolymer, OB18, is more than 15-fold greater than typical M_n's of phospholipids such as SOPC. The listed copolymers, as well as a growing list of other vesicle-forming super-amphiphiles (VFSA), do have one trait in common with their natural lipid counterparts: they have a hydrophilic volume fraction in the range of $f \sim 20 - 40\%$ [reviewed in Discher et al, 2000]. Using the OE and OB chain chemistry as an archetype, it has been previously found that cylindrical micelles occur between $f \sim 45$ and 55% [Won et al, 1999] and spherical micelles when $f > 55$ % PEO [Won et al, 2000], which is consistent with the micellization phenomenon of over-PEGylated lipids.

Polymersomes can readily be made with the tabulated copolymers by a number of methods and in a very broad range of strictly aqueous media [Lee et al, 2001] (Fig. 2a). Polymersomes of 100 – 350 nm diameter and a small polydispersity of ~10-20% are readily made by liposome-based techniques. In particular, extrusion through nano-porous filters is found to efficiently fragment vesicles to a size only slightly larger than the filter pore diameter. DLS and cryo-TEM confirm vesicle size and uniformity, including unilamellar membrane thickness. Literature (Neulsen and Marks, 2000; Scherpereel et al.2001; R.Wiewrodt et al, 2001) data generally shows that this ~200 nm size carrier permits relatively prolonged circulation in the bloodstream with minimal non-specific uptake by tissues as well as permitting internalization if needed, the latter of which is particularly important to the intracellular delivery of therapeutic cargoes such as enzymes and genes. Although larger polymersomes, in sizes of 500 to 2000 nm were not studied directly, they might also be anticipated, based on the following results, to be rapidly taken up by the reticuloendothelial system.

On short time scales, the dense PEO brush on the polymersomes is expected to behave somewhat like the passive component of the glycocalyx on cellular exofaces, preventing the deposition of phagocytic ligands such as plasma antibodies or C3a/b as well as repelling less specific adhesion. Indeed, the protein-rejecting properties of PEO-coatings, including those of nanoparticles [Peracchia et al., 1999], are well appreciated. The interaction of polymersomes with the main cellular components of blood was assessed on a single-vesicle basis for extended times. Earlier experiments were only done for short times of ~1 hr or less [Lee et al, 2001]. Such experiments are done by manipulating vesicles into contact with cells in autologous plasma (diluted 1:1 with PBS). After incubations of a few hours or less, polymersomes do not exhibit *any* adhesion or elicit any other obvious cellular response when placed in contact with either a red cell or a neutrophil. Control experiments with manipulated yeast cells show strong adhesion with spreading and active engulfment being generally complete within 3 – 5 minutes. Almost identical results were obtained with macrophages, which are generally considered, as part of the RES to constitute a major clearance pathway for circulating liposomes.

After 10's of hours in plasma, however, vesicles become adhesive to blood cells, depending on PEG MW. This clear indication that some of the many soluble components of plasma are integrating into the polymersome membrane suggests a basis for clearance *in vivo*.

Injection of 100 nm polymersomes into rats was done both with and without fluorescent labeling with the cell-tracking dye PKH-26 (Sigma-Aldrich, St. Louis, MO), the unlabeled having no obvious ill-effect. As a hydrophobic dye, PKH-26 partitions into membranes. From previous studies, giant polymersomes are stable for a week or more in both size and number in plasma [Lee et al, 2001], and dye-labeled, extruded polymersomes are likewise stable when diluted into whole blood for a similar length of time. The small, fluorescent vesicles also showed no tendency to adhere to cells and remained suspended in plasma. Following injection, quantitative analysis of dye-labeled polymersome by fluorescence showed an obvious but gradual decrease in the number of circulating polymersomes. From the limited but thorough studies to date, polymersome half-lives in the circulation are generally very close to 20 hours, consistent with the in vitro studies. Initial searches for fluorescent polymersomes in major organs suggest that liver, spleen, and kidney are the primary sites of uptake, with an initial, perhaps incorrect, impression being that vesicles lodge in interstitial spaces between cells. A primary control we have employed for our relatively novel method of tracking is to follow PKH-26-labeled red cells: with these we find only a gradual decrease in labeled cell number over a period of a week.

CONCLUSIONS: From these *in vivo* studies it seems clear that the circulation time and even organ localization of our first generation polymersomes is slightly longer than that of polymer-decorated commercial stealth liposomes and is lso significantly longer than that of unmodified liposomes. Trends in the stealth liposome literature suggest a scaling exponent of circulation half-life in PEG density to be ~0.15, and in PEG length to be about ~0.46. This data suggests, for example, that extending the PEG brush by a factor of two (OB19) would circulate for ~28 hours in rats and therefore upwards of 80 hours in humans.

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