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Sterically stabilized liposomes *: a hypothesis on the molecular origin of the extended circulation times

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Therapeutic applications of intravenously injected liposomes have been limited by their rapid clearance from the bloodstream and their uptake by the macrophage cells of the liver and spleen (RES). Recently, however, liposomes which substantially evade the rapid uptake by the RES have been introduced. Since these liposomes exhibit dramatically different pharmacokinetics and biodistribution, new therapeutic opportunities have appeared. These include enhanced efficacy of antineoplastic agents against tumors, sites of inflammation, and targeting ligand-coupled liposomes to extravascular targets. Despite extensive experimental work, the mechanism underlying the ability of liposomes to avoid the rapid uptake by the RES is still not fully understood. Our approach is an alternative to seeking the answers in complex differential interactions of liposomes with various components of blood. We believe that the effect can be easily explained, at least in qualitative terms, by the fundamental principles of colloid stability. In this communication, we propose that steric stabilization of liposomes is responsible for their prolonged circulation times. We propose that stabilization results from local surface concentration of highly hydrated groups that sterically inhibit both electrostatic and hydrophobic interactions of a variety of blood components at the liposome surface.

Introduction

The utility of liposomes for delivering encapsulated therapeutic agents to systemic sites of disease following intravenous administration [1–4] is severely limited by the rapid recognition and removal of these carrier particles from the bloodstream by specialized phagocytic cells residing primarily in the liver and spleen (known collectively as mononuclear phagocytic system, MPS or the reticuloendothelial system, RES). Despite many attempts to improve the situation, the circulating halflife ($t_{1/2}$) of a typical liposome preparation such as those used in recent clinical trials is measured in minutes rather than hours or days [4–7].

In the late 70's and early 80's it was found that the stability of liposomes in biological fluids (such as plasma) is dramatically improved by using formulations composed of neutral long-chain saturated phospholipids and cholesterol [6–8]. In vivo, optimal results are obtained using small neutral liposomes with very rigid membranes, i.e. ones composed of lipids with high gel-liquid crystal phase transitions (T_c) and cholesterol [7,8]. Membranes containing high level of cholesterol are, however, in the ordered fluid state over a wide temperature range and can be, therefore, better characterized as tightly packed, rather than rigid. With respect to pharmacokinetics, typical formulations containing distearoylphosphatidylcholine (DSPC) or sphingomyelin and 30–50 mol% of cholesterol with a mean diameter of less than 100 nm have been reported to have $t_{1/2}$ values on the order of several hours [7,8].

A class of new liposome formulations has been developed over the last few years, which yield $t_{1/2}$ values of up to 12 h following a single bolus injection. This behavior is dependent upon the presence of specific glycolipids, such as ganglioside GM_1 [9,10] or hydrogenated phosphatidylinositol (HPI) in the liposome membrane [10–12]. A characteristic long circulat-

* The term 'sterically stabilized' liposomes is referring to liposomes that have long circulation time in blood irrespective of the bilayer fluidity and surface charge density and in addition show a $t_{1/2}$ which is independent of dose. Such liposomes contain lipids with special headgroups such as GM_1, PI, PEG-PE that contribute to their long blood residence time.

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ing liposome composition would be HPI or GM_1 in combination with DSPC or sphingomyelin and cholesterol. The term Stealth * has been proposed for such liposomes [13]. Based on empirical observations, a hypothesis for three necessary conditions for liposomes with long circulation time has been proposed [10]. These include: (i) partially shielded (or absent) surface negative charge, (ii) rigid bilayer structure and (iii) small size (< 100 nm).

More recently, it was shown that the inclusion of a new class of synthetic diacyl lipids with bulky polyoxyethylene glycol (PEG) headgroups [14–16] into liposome bilayers results in a further prolongation of blood circulation times for liposome formulations [14–17]. Surprisingly, several of these PEG-containing liposome systems were found which violated the proposed rules for long blood circulation time. In the case of using liposomes containing PEG-derived phospholipid, the fluidity and the surface charge of the bilayers do not effect the circulation time [17,18]. The ability to remain in circulation for prolonged times is correlated with liposome diameters below 200 nm, and especially below 100 nm. Above this size range the uptake increases. The polymer coated liposomes, however, exhibit still longer circulation times than the conventional liposomes.

Despite an abundance of experimental data, the mechanism or mechanisms that determines blood circulation time remain uncertain. As an alternative to the conventional wisdom [10,18] that the circulation time is related to a variety of complex interactions with specific blood components (or lack thereof), we propose that the effect can be explained in relatively simple terms based on factors which contribute to the stability of colloid systems in biological fluids. The aim of this report is to offer a qualitative explanation of the delayed clearance effect while detailed theoretical and experimental studies are in progress. The first part discusses possible differences in the kinetics of blood clearance of various liposomes and their interaction with blood components, while the second tries to address possible steric constraints contributed by the bulky hydrophilic headgroups [19,20].

Materials and Methods

Sources of materials and liposome preparation were used as previously described [10]. Briefly, liposomes were produced by hydration of a thin lipid film and subsequent extrusion through double stacked polycarbonate membranes of defined pore size [21] using a commercially available high pressure extrusion device (Lipex, Vancouver, Canada). Liposomes were radiolabelled by an 'afterloading' procedure leading to the encapsulation of deferoxamine-^{67}Gallium complex [22]. All liposome preparations had a mean particle diameter of 70–100 nm (Gaussian mean distribution) as established by dynamic light scattering (Nicomp model 200). Tissue distribution studies of radiolabelled liposomes were carried out in 8 week-old Swiss Webster female mice (Simonsen) using an i.v. dose of 1 μmole phospholipid per mouse. Animal dissection protocol, data acquisition and tissue correction factors for residual blood content were as previously reported [10].

Results

Liposome distribution in blood and the RES

Table I shows the disposition of liposome-encapsulated ^{67}Ga-Desferal at 24 h following iv injection for a variety of lipid compositions. We are presenting the data as percent injected dose present in blood, RES (liver and spleen combined), the ratios between these tissues, and the total amount of material recovered in all tissues. We can distinguish two main classes of liposomes, using the behavior of PC:C (sample 3) as a baseline: Samples 1 and 2, represent a class where the addition of a negatively charged phospholipid enhances liposome clearance and uptake by RES (Ratios from 8.6 to 188). Samples 4–10 represent a class where the inclusion of a charged lipid component (PI or GM_1 or PEG-DSPE) inhibit liposome clearance and RES uptake (ratios from 8.6 to approx. 0.8). As reported before, the recovered dose in blood and RES were inversely proportional. The various samples shown in Table I were selected in order to make the following comparisons, which will help illuminate the ensuing discussion of possible mechanism of clearance:

Effect of negative charge. A comparison of samples 2 and 5, obtained with fluid bilayer liposomes differing only in the acidic phospholipid (PG vs. PI) indicate that the surface charge density which is similar in these two cases [23], is not enough to explain the large difference in clearance rate. It was postulated that steric hindrance of the negative charge (as in the case of PI by the inositol group, and in GM_1 ganglioside by the configuration around the carboxyl group) may inhibit specific interactions with plasma proteins [10] or cell surface proteins [24] which are necessary for liposome clearance. This point is reinforced by a comparison of samples 9 and 10, where the presence of PG now does not have an effect, presumably because of the steric hindrance of the bulky PEG group in these liposomes.

Effect of membrane fluidity. A comparison of samples 2 and 4 or 6 and 7 illustrates that rigid bilayers

* The term 'Stealth ®', has been proposed [13] for liposomes that evade the early uptake by the RES and circulate for a long time. 'Stealth' is a registered trademark for Liposome Technology, Inc., Menlo Park, CA 94025, U.S.A.

TABLE I

Liposome distribution in blood, liver and spleen (RES), 24 h after injection [a]

Lipid composition [b]	% Injected dose (S.D.) [c]			Ratio RES/blood
	blood	RES	total	
1 PS/EPC/C	0.2(0.0)	37.6(1.2)	43.3(0.6)	188
2 PG/EPC/C	0.3(0.1)	37.0(3.6)	49.8(3.9)	123
3 EPC/C	3.0(0.8)	25.8(1.3)	44.4(2.9)	8.6
4 DPPG/DSPC/C	6.5(0.8)	31.2(3.2)	59.9(5.5)	4.8
5 PI/EPC/C	4.1(1.3)	14.8(3.4)	37.4(6.3)	3.6
6 GM_1/EPC/C	6.0(0.4)	17.9(4.4)	42.3(1.5)	3.0
7 GM_1/DSPC/C	16.0(3.8)	16.5(1.4)	66.3(4.2)	1.0
8 PEG-DSPE/DSPC/C	23.6(3.1)	27.7(2.2)	68.1(3.4)	1.2
9 PEG-DSPE/EPC/C	29.3(2.8)	20.6(2.6)	63.4(3.3)	0.7
10 PEG-DSPE/PG/EPC/C	22.1(1.6)	18.1(0.5)	56.4(3.3)	0.8

[a] Liposomes contained encapsulated ^{67}Ga-Desferal, as a marker of liposome contents. Tissues were dissected at 24 h, and blood corrections were supplied as before [10].

[b] Mole ratios were as follows: 1, 1:10:5; 2, 1:9:5; 3, 10:5; 4, 1:10:5; 5, 1:9:5; 6, 1:9:5; 7, 1:10:5; 8, 1:10:5; 9, 1:10:5; 10, 1:3:7:5.

[c] First two columns show the percent of injected dose recovered in blood (column 1) and in liver, plus spleen, collectively referred to as RES (column 2). The total recovered in all tissues is given in column 3. Numbers in parenthesis give the standard deviation of the mean, from 3–5 different animals.

composed of long chain saturated acyl chains (rather than a variety of unsaturated and saturated ones as in the fluid bilayer of EPC) show a much lower ratio. The relatively long circulation time of rigid liposomes, has already been reported earlier [7,8] and was therefore expected. More interesting however, is the comparison between samples 8 and 9, where the change from a fluid (sample 9) to a rigid (sample 8) bilayer does not make a significant difference in the circulation time or uptake by the RES. This is presumably due to the steric hindrance effect of the PEG-head group, as will be discussed below.

Discussion

Clearance rate, tissue disposition and drug delivery

In contrast to conventional liposome formulations, the $t_{1/2}$ values of sterically stabilized liposomes do not depend on the lipid dose [12,17,18]. At similar values of $t_{1/2}$conventional and Stealth liposomes also differ with the respect to the extent of RES uptake. In the case of long circulating conventional liposomes this uptake is only delayed while in the case of Stealth liposomes it is actually reduced. We should add, of course, that lower values of $t_{1/2}$ do not necessarily mean higher uptake of liposomes by RES but may simply mean that an appreciable amount of small liposomes has extravasated. PEG-containing liposomes of fairly large size (> 200 nm), containing high surface negative charge, composed of fluid bilayers and no cholesterol were found to have a remarkably long $t_{1/2}$ values [14–16]. A typical Stealth composition of small vesicles with diameters ≈ 80 nm, containing about 5–10 mol% of a conjugate of distearoylphosphatidylethanolamine coupled to a 1900 dalton segment of methyl PEG may exhibit $t_{1/2}$ values above 24 h in dogs and above 40 h in humans (Gabizon, A., private communication). The species dependence of $t_{1/2}$ can be rationalized by different blood flows through the various organs, which could be modeled in a first approximation by pulse rates and blood/volume ratios.

Long circulating liposomes have expanded their potential in medical applications not only by their increased $t_{1/2}$ in blood and their decreased uptake by liver, but also by showing an increased accumulation in implanted tumors [10,12,17]. Obviously, Stealth liposomes exhibit different biodistribution and pharmacokinetics and therefore open new opportunities for the delivery of encapsulated pharmaceutical agents to non-RES sites. For example, they can serve as a micro-reservoir for controlled release of various drugs within the vascular compartment or directly deliver drugs to tumors or sites of infection by penetrating through capillary defects often found in such pathological situations [10,25,26]. The coupling of various ligands (such as antibodies) on the surface of liposomes for selective targeting is also made practical for in vivo applications now that liposomes which are not rapidly taken up by liver and spleen are available [27,28].

Interaction of liposomes with blood components

For the purposes of this discussion we will assume that there are three principal mechanisms responsible for the removal of liposomes from the bloodstream: (i) binding of soluble plasma proteins (opsonization) followed by macrophage uptake [29,30], (ii) lipid depletion/exchange during collision and interaction of liposomes with plasma lipoproteins, followed by possible liposome destabilization or disintegration [31,32], and (iii) direct recognition and binding to cell surface pro-

teins, followed by phagocytosis in liver and spleen macrophages [24]. Clearly, the reduction in the rate of any one of these reactions would result in enhanced blood circulation times.

For 'effective' opsonization to occur, a plasma (or cell surface) protein must not only collide with a liposome; in addition, it must bind in some way to the liposome surface: this could involve stable adherence or adsorption onto the surface liposome and/or penetration and anchoring into the bilayer membrane.

Model for steric stabilization

Most of the initial reactions of various proteins on the liposome surface are likely to be electrostatic (ionic) in nature. Clearly the absence of charged groups on the liposome surface reduces the likelihood of such interactions. This is reflected in the difference between samples 1, 2 and 3, Table I, and many literature citations which document less rapid uptake of neutral liposomes compared with their charged counterparts. It is also logical to expect that more tightly packed (rigid) bilayers, those with stronger intrabilayer attractive dispersion forces and better packing in the polar head group domain, will exhibit a greatly reduced tendency to anchor or adsorb plasma proteins and to allow their penetration and anchoring into the bilayer. This is a process which can be driven, in the absence of electrostatic forces, by hydrophobic, dipolar and van der Waals forces. (Compare samples 2 and 4, also, 6 and 7, Table I).

Although these arguments may apply to tightly packed, cholesterol-containing compositions of high T_c lipids, they do not explain why PEG-coated liposomes with high negative surface charge density and made from fluid bilayer forming lipids circulate for extended periods (samples 8–10, Table I). We propose that liposomes composed of practically any lipids that form stable bilayers in physiological buffer solutions (i.e., they do not aggregate or leak their contents in a balanced salt solution, for example) can be sterically stabilized in biological fluids by the presence of-water-compatible polar headgroups of appropriate molecular weight and surface density. Such a hydrophilic surface layer would be expected to reduce van der Waals attraction among liposomes and different macromolecules by increasing center-to-center separation upon collision, and would increase the hard core repulsion between the possible reactants. The steric repulsion can be simply imagined as literally brushing and pushing away the incoming macromolecules or lipoprotein complexes. It can be physically explained in terms of osmotic pressure, elastic restoring forces and spring constants of polymers [33,34]. This would slow-down protein absorption and practically eliminate lipid depletion or exchange (which presumably requires close juxtaposition of the liposome bilayer and the 'receiving' lipoprotein structure). At the molecular level, this steric repulsion can be described as a sum of hard core interaction, repulsive hydration force, osmotic force and entropic interaction. While the first two are well documented [35,36], the last two can be qualitatively explained as the consequence of high local concentration of polymer and other surface groups upon close approach of two particles. This, in turn, forces water to flow into this local cluster, separating the particles. Similarly, such intertangling of approaching particles would also be expected to reduce the mobility of the surface groups and their conformational degrees of freedom which would be therefore entropically unfavorable [33,37,38].

These qualitative arguments can be supported by various data from the literature, if we assume that the interaction between two equal bilayers is proportional to the interaction of a bilayer with a macromolecule, such as a plasma protein or a cell surface protein. For example, it has been shown recently that the inclusion of 10 mol% of GM_1 in PC bilayers increased the interbilayer separation for more than a factor of 2 as compared to pure PC bilayers [39] while preliminary experiments with 5 mol% of ^{1900}PEG-DSPE in the bilayer have shown even larger separations (Needham, D. and McIntosh, T.J., private communication). These measurements confirm earlier studies where it was shown that the inclusion of 10 mol% GM_1 in DPPC or DPPC bilayers doubles the hard wall separation in the force distance curves [40]. Similar increase in the bilayer separation was observed also in a study of DPPC bilayers doped with 25 mol% of PI [41].

The model of a polymer extending over the liposome surface [33] can now also explain the finding that increased surface charge, by inclusion of PG into the bilayer, does not increase the blood clearance rates of liposomes composed of PEG-DSPE/EPC/C [14]. Theoretically, it is probable that increased surface charge density increases the degree of hydration and forces the polymer chains to extend even further from the surface. In support of this view, simple phase diagram studies of EPC and ^{1900}PEG-DSPE mixtures and ^{1900}PEG-DSPE micelles have shown that the polymer is not in a random coil conformation but is rather extended, reaching on average ≈ 6 nm normal to the surface [42]. It was also shown that inclusion of 3.3 wt% of PEG-like surfactants ($C_{12}E_n$) increased the interbilayer separation proportionally to n [43]. It seems therefore that the ability of grafted polymer to form a brush [33] above the liposome surface is closely correlated with the ability to evade rapid clearance. PEG chains are especially effective because of their strong interaction with water. The liposome surface prefers water to the polymer and a polymer depletion layer [33] is formed. This, in turn, helps in brush formation. Such considerations, however, do not include the possi-

bility of phase separation which may also influence the liposome clearance rate. It was recently observed that sterically stabilized liposomes containing phosphatidylserine exhibit fast blood clearance rates [44] which can be explained by the well documented interaction of phosphatidylserine with Ca^{2+} ions [45]. In addition, the difference in the position of the negative charge probably also correlates with their accessibility and reactivity, for example in PG vs. PS.

While measuring electrokinetic properties of PC vesicles with incorporated gangliosides, it was concluded that ganglioside head groups project 2.5 nm from the bilayer [46]. However, no significant variations among various gangliosides were found while, on the other hand, it is known that only GM_1 effectively prolongs blood circulation times [9,10]. This probably indicates that either electrokinetic measurements of the liposome drag coefficients are not a good test for the steric stabilization, or that different bilayer compositions may exhibit different mixing behavior, or that some receptor mediated uptake/processes may occur in vivo with some of the gangliosides, but not with GM_1. Electrophoretic measurements with PEG-DSPE containing liposomes have shown that their mobility is reduced despite the fact that the surface charge density was not altered when compared with other charged phospholipids. This was attributed to the increased hydrodynamic drag of the polymer chains in a brush-like conformation [19].

This qualitative model cannot, however, explain the size dependence of the clearance of sterically stabilized liposomes [20,47,48]. At present, we can only speculate that this may be due to the different hydrodynamics and mechanical stability of liposomes of different size distributions, increased van der Waals attraction or some immunogenic characteristics. For instance, random lateral diffusion of molecules in the bilayer can produce larger transient clusters of particular molecules. This can increase the local epitope density and decrease the steric shielding at particular patches on the liposome surface at particular times. Another possibility is the multi-valency of binding: in the case of weak multi-valent binding, one would expect, especially in the case of non-stabilized liposomes, that high curvatures reduce the binding to various macromolecules. A similar but irreversible phenomenon is also the already mentioned phase separation. It is a cooperative process and may therefore also depend on the size of the liposome. In addition, phase separation may lead into phase transition which normally results in the liposome destabilization [49].

In agreement with our model, recent work has shown that liver uptake does not depend on the dose of stabilized liposomes [17,50] but does depend on their particle size [48]. It was also found that the clearance of larger liposomes occurs predominantly in the spleen [48] where in concord with a complex function of blood clearance in spleen, other clearance mechanisms may operate.

Concluding remarks

The discussion presented here is in general agreement with earlier proposals [10] suggesting that steric hindrance of negative charges on the liposome surface inhibits interaction with plasma proteins and therefore is the primary origin of prolonged blood circulating times. In addition, we suggest that the origin of the stabilizing effect is different in the case of polymer-coated liposomes. We believe the polymer coating reduces the rate of interactions with blood components by increasing the free energy barrier of various specific and non-specific reactions which lead to the uptake/destruction of liposomes. The examples mentioned above support the notion that there are different ways to stabilize liposomes and we believe that by appropriate selection/optimization of liposome properties even longer blood circulation times can be achieved.

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