A modified spectroscopic method for the determination of the transbilayer distribution of phosphatidylethanolamine in soya-bean asolectin small unilamellar vesicles

Paolo SARTI,*†‡ Agnese MOLINARI,‡ Giuseppe ARANCIA,‡ Antonella MELONI*† and Gennaro CITRO§

*Institute of Biological Chemistry, University of Cagliari, Cagliari, Italy, †Centre of Molecular Biology (CNR), Rome, Italy, ‡Istituto Superiore di Sanita', Ultrastructure Laboratory, Rome, Italy, and §Regina Elena Cancer Institute, Laboratory of Experimental Preclinic Chemotherapy, Rome, Italy

A spectroscopic kinetic approach for determining the relative concentrations of phosphatidylethanolamine (PE) exposed on the external and internal layers of small unilamellar vesicles (SUVs) used as a model system and prepared by sonication of purified soya-bean asolectin is proposed, based on the use of 2,4,6-trinitrobenzenesulphonic acid (TNBS) and *N*-succinimidyl 3-(2-pyridyldithio)propionate (SPDP). The known reactions between PE and TNBS and/or SPDP were used, separately or in combination, to derivatize PE in preformed vesicles. We have observed that mixing SUVs with excess TNBS results in a biphasic time course. Kinetic analysis of the data supports the conclusion that external PE is rapidly derivatized (fast phase) with a half-time of 2 min. In the next (slow) phase (half-time

70 min), TNBS permeates the vesicle membrane and also reacts with PE molecules facing the internal liposomal compartment. Under the experimental conditions chosen, SPDP reacted with only the external PE molecules. The reaction of SUVs first derivatized with SPDP and then with TNBS further demonstrates that the two phases, observed with TNBS, are due to modification of external and internal PE. Approx. 30 % of PE was found to be facing the external bulk phase, thus confirming the asymmetric distribution of the molecules in SUVs. The maximum number of thiol arms covalently linked by means of SPDP modification of PE on the surface of a single liposome was estimated at about 10^2 .

INTRODUCTION

Liposomes are microvesicles composed of one or more membrane lipids [1]. They have proved to be important tools for basic and biopharmacological research [2–4]. Drugs and effectors can be released into targeted tissues or cells by loading them into liposomes [5–7]. The choice of the type of liposome is crucial. The loaded liposome must represent the best compromise between stability, trapping efficiency and ability to fuse with cells, ideally *in vivo* and with suitable pharmacokinetics [8–10]. By varying lipid composition and preparation procedure, liposomes can be formed in a number of structures of different size, single or multilamellar, from small to large [3,10,11]. In the present study we have used small unilamellar vesicles (SUVs) prepared by sonication of a equimolar mixture of phosphatidylethanolamine (PE) and phosphatidylcholine (PC) [12,13] purified from soya-bean asolectin. The liposomes produced are SUVs of about 30–40 nm diameter. They are currently prepared for reconstitution of mitochondrial membrane proteins [14–16]. Owing to their low scattering properties when suspended in water medium, SUVs represent an ideal liposome model system for light-spectroscopic studies ([15] and refs. therein). We have concentrated on the role played by PE in SUVs because, when inserted into the external layer of the vesicle, it provides a reactive amino group. This characteristic has been extensively used to covalently link liposomes to ligands specific for cell receptors in order to achieve selective cell targeting [17,18]. We here report on an experimental approach which enables the

relative concentrations of PE exposed on the external and internal layers of SUV liposomes to be determined. It makes use of two well-known reagents that are reactive with amino groups, 2,4,6-trinitrobenzenesulphonic acid (TNBS) [19] and the heterobifunctional cross-linker *N*-succinimidyl 3-(2-pyridyldithio)propionate (SPDP) [20]. The latter has also been used to covalently insert thiolated proteins such as immunoglobulins [21,22] and other specific cell-surface ligands ([23] and refs. therein) on the surface of the liposome.

MATERIALS AND METHODS

Preparation of SUVs

SUV liposomes were prepared by sonication as previously reported [16]. Briefly, soya-bean phospholipids (PLPs) (L- α -phosphatidylcholine type IV S; Sigma, St. Louis, MO, U.S.A.) were purified by acetone/ether fractionation cycles. As stated by the supplier and independently verified [12], the purified PLPs contained 40 % PE, 40 % PC, 9 % diphosphatidylglycerol, 11 % phosphatidylserine and phosphatidylinositol, in different proportions depending on the preparations [24]. A suspension of liposomes (typically 10 ml) was prepared by dissolving PLPs (9 mg/ml) in 0.1 M K⁺/Hepes buffer, pH 7.6, and this is referred to as a standard suspension. The suspension was stirred gently for 1 h at room temperature. The multilamellar vesicles formed were sonicated for 10 min, i.e. 1 min/ml, using a probe sonicator

Abbreviations used: TNBS, trinitrobenzenesulphonic acid; SPDP, *N*-succinimidyl 3-(2-pyridyldithio)propionate; TNP, trinitrophenyl; PD, 2-pyridyldithio; PE, phosphatidylethanolamine; PC, phosphatidylcholine; PLP, phospholipid; SUV, small unilamellar vesicle.

* To whom correspondence should be addressed at: Dipartimento di Scienze Biochimiche, Universita' La Sapienza Roma, 00185 Roma, Italy.

(MSE; Soniprep 150) equipped with a 7 mm titanium probe, with power output set at 60 W [3,16]. Sonication was carried out in the cycling mode (1 min sonication followed by 1 min interval), and the suspension was cooled in an ice/water bath. SUVs were used as standard or diluted in the same Hepes buffer at the concentrations given in the text. The experiments presented here mainly deal with the reactivity of the amino groups of PE, the total concentration of which in the standard SUV suspension was 4.3 mM, as independently determined after solubilization of the liposomes with Triton X-100. In SUVs PE molecules are found both in the outer and inner layer of the membrane, so PE will be defined as total ($PE_{tot.}$), external ($PE_{ext.}$) or internal ($PE_{in.}$). Pure PE [dipalmitoyl-*N*-dansyl (catalogue no. P-3656)] was from Sigma.

Reaction with TNBS

Formation of the trinitrophenyl (TNP) derivative was detected spectrophotometrically using a Cary 219 spectrophotometer (Varian; Techtron, Mulgrave, Vic, Australia). Either liposomes were used as such (concentration of PLP given in the text) or they were solubilized with 2% (w/v) Triton X-100, generally for 30 min unless otherwise stated. The stock solution of TNBS (0.1 M) was freshly prepared in the same Hepes buffer used to prepare the liposomes. TNBS was purchased from Pierce (Rockford, IL, U.S.A.). Kinetic and thermodynamic parameters of the reaction were calculated according to the following minimal reaction scheme:

The rate constants k_1 and k_2 account for the bimolecular collision between TNBS and PE (step 1), and their ratio k_1/k_2 represents the true equilibrium (affinity) constant K_{eq} . The final product is stabilized by a covalent bond, thus its formation (step 2) is indicated as irreversible, and the apparent association constant $K_{ass} = K_{eq} \times k_3$. The rate constant k_3 is considered to be the rate-limiting step of the reaction it was measured experimentally and reported in the text as k_{fast} or k_{slow} . The value of k_3 was calculated by fitting the time courses of the reaction to a single- or a double-exponential process. The fitting procedure was carried out using a 486 dx PC, running the analytical program MATLAB (The Math Works Inc., South Natick, MA, U.S.A.).

Reaction with SPDP

Stock solutions of 20 mM SPDP were prepared by dissolving the compound in 95% (v/v) ethanol. The extent of the reaction of PE with SPDP was calculated after removal of excess SPDP (see below), by directly reducing the 2-pyridyldithio (PD) derivative (PD-liposomes) with 25 mM dithiothreitol. The $\Delta\epsilon$ of the transition (343 minus 380 nm) was calculated as $6.7 \text{ mM}^{-1} \cdot \text{min}^{-1}$. The fraction of amino groups that reacted with SPDP was determined directly by allowing the PD-liposomes to react further with TNBS. The amount of PD-PE formed was calculated by subtracting the contribution of TNP-PE from the total amount of PE, as independently determined before the reaction with SPDP. Excess SPDP was removed by extensive dialysis or by gel chromatography on a Sephadex G-25 column. In both cases dilution of the sample that resulted from the removal procedure was carefully controlled and taken into account in the calculations.

Electron microscopy

For negative staining, a drop of the liposome suspension was floated on the surface of a 300-mesh copper grid, immediately removed and stained using 2% phosphotungstic acid, pH 7.2. For freeze-etching, the suspension of liposomes on the carrier was quickly frozen using freon 22 and liquid nitrogen. Frozen samples were then transferred to a Balzers BAF 300 apparatus and cleaved at -100°C and 200–400 pa; samples were shadowed using 2.5 nm Pt/carbon, and replicated with 20 nm carbon. Pt/carbon evaporation (angle 45°) and carbon evaporation (angle 90°) were carried out using an electron-beam gun. The thickness of the deposit was evaluated using a quartz-crystal thin-film monitor. Liposomes were digested from the replica for 2 h at room temperature in Clorox. Replicas were mounted on naked 300-mesh grids. Micrographs were taken using a Zeiss EM 10 C electron microscope operating at 60 kV.

RESULTS

Liposomes were subjected to electron microscopy. Micrographs showing the shape and size of the liposomes after negative staining and their freeze-fracture patterns are shown in Figure 1; the diameter distribution of the vesicles is also reported. The penetration pattern of the electron-dense staining material inside the liposomes reveals that vesicles with a diameter of 40 nm or less are unilamellar. This observation is clearly supported by the freeze-fracture pattern showing that most of the vesicles are cross-fractured as expected for single lipid bilayers (Figure 1). Quantitative analysis was performed on negative-staining images, by screening five fields and about 500 vesicle profiles. It reveals that vesicle diameter ranges from 15 to 70 nm, with a mean value of 34.12 ± 13.39 nm and a median of 30 nm. About 70% of the vesicles display a diameter less than 40 nm (Figure 1).

Pure PE (0.1 mM) solubilized with $T \times 100$ was mixed with an increasing amount of TNBS, up to 0.8 mM. After 1 h incubation at room temperature A_{420} was measured. As shown in Figure 2, A_{420} increased with increasing concentration of TNBS, reaching a maximum (0.77) when the concentration of TNBS was about 0.4 mM. A Scatchard plot of the data shows a linear correlation between data points. The calculated apparent association constant, K_{ass} , is $6.5 \times 10^4 \text{ M}^{-1}$, and the independently measured rate constant limiting the derivatization process (see below) allows calculation of the true collisional equilibrium constant between PE and TNBS [$K_{eq} = 2.5 \times 10^7 \text{ M}^{-1}$ (see also the Materials and methods section)]; the intercept on the x -axis (0.1 mM) confirms that 100% of PE is derivatized (inset A to Figure 2). The extinction coefficient of the TPN derivative was independently determined under similar conditions. The A_{420} values in the range of concentration of amino groups, 0–0.3 mM, are reported in inset B to Figure 2, and compared with those obtained using glycine, in the presence or absence of the same amount of Triton X-100. Linear regression analysis of the data allows us to calculate ϵ ($7.2 \text{ mM}^{-1} \cdot \text{cm}^{-1}$). When Triton X-100 concentration was increased above 2% (w/v), a tendency for the specific absorbance to decrease was observed (not shown).

A suspension of SUVs (0.22 mg/ml PLP; [PE] 0.1 mM), as such or after solubilization in Triton X-100, was incubated for 30 min with increasing amounts of TNBS. As shown in Figure 3, the A_{420} of detergent-solubilized liposomes (0.73) was already about 95% of the maximal (0.77) at the lowest TNBS concentration used (0.2 mM). SUVs not treated with detergent were also incubated with increasing amounts of TNBS for 2 and 4 h, and within the same concentration range. In both cases the absorbance increased with the concentration of TNBS and length of incubation, tending to (but not reaching) the same maximal

Figure 1 Electron micrographs of liposomes and analysis of diameter distribution

Negatively stained (a) and freeze-fractured (b) liposomes are shown along with a histogram of diameter distribution (c). Bars = 100 nm.

value as observed in the presence of Triton X-100. The Scatchard plot shows that in both cases the data points cannot be feasibly linearized (see inset to Figure 3).

Suspensions of SUVs containing different amounts of PLP, from 9 to 0.42 mg/ml corresponding to 4.3 and 0.2 mM $PE_{tot.}$,

Figure 2 Spectroscopic titration with TNBS of pure PE solubilized in $T \times 100$

Pure PE (0.1 mM), solubilized with 2% (w/v) Triton X-100, was incubated for 1 h at room temperature (20 °C) with TNBS, and absorbance was recorded. Inset (A): Scatchard plot; b , bound TNBS; b/f , bound/free; binding in this context includes covalent derivatization (see also the Materials and methods section). Inset (B): determination at 420 nm of the ϵ of the TNP-derivative; glycine minus ($\blacktriangle$) and plus ($\triangle$) Triton X-100; pure PE ($\bullet$).

Figure 3 Spectroscopic titration with TNBS of liposomal PE

Standard SUVs, diluted to 0.22 mg/ml PLP, were incubated with TNBS for 2 ($\blacksquare$) and ($\square$) 4 h, or preliminarily solubilized with Triton X-100 and thereafter allowed to react with TNBS for 30 min ($\bullet$). Increasing the incubation time to 60 min had no effect. Inset: Scatchard plot of the 2 and 4 h incubation sets of data.

were also allowed to react with SPDP maintaining the SPDP/ $PE_{ext.}$ molar ratio at 6. As shown in Figure 4, the amount of PE titrated increased linearly with the concentration of $PE_{tot.}$ and therefore with liposome concentration; from the linear regression analysis the slope $S = 0.27$ was calculated. Aliquots of SUVs (0.36 mg/ml PLP) were incubated with a constant amount of SPDP for different times, from 15 min to 6 h. After incubation the excess SPDP was removed chromatographically or by dialysis (see the Materials and methods section). The amount of PD derivative formed was measured kinetically by difference, mixing with TNBS the PD-liposomes solubilized with Triton X-100. The results are shown in Figure 5. The percentage of amino groups that react with SPDP increased with incubation time,

Figure 4 Extent of formation of PD-SUVs at different SUV concentrations

Liposomes were diluted to the given values and mixed with 6 molar excesses (over PE_{ext}). The concentration of SUVs is reported as equivalents of PE_{tot} to simplify the calculation of the fraction of PE reacted. The concentration of PD derivative was determined spectroscopically after reduction with dithiothreitol of the sulphide bond (details in the Materials and methods section). From the linear regression analysis, slope $S = 0.27$, and $\chi^2 = 0.0083$.

reaching a plateau value corresponding on average to $0.05 \text{ mM} = 0.17 (PE_{tot}) - 0.12 (PE_{TNBS})$, indicating that approx. 30% of PE_{tot} was PD-derivatized. The time-dependence of the extent of derivatization is better resolved when excess SPDP is removed by gel chromatography (Figure 5).

A standard suspension of SUV liposomes was diluted to 0.36 mg of PLP/ml and mixed with 4 mM TNBS. As shown in Figure 6(a), the reaction is biphasic showing a fast and slow phase, and is completed in about 4.5 h . The total absorbance change is 1.2 . The amplitude of the fast phase is $\Delta A_{420} = 0.3$. The calculated values of the first-order rate constants of the fast and slow phase are respectively $k_{fast} = 5.3 \times 10^{-3} \text{ s}^{-1}$ and $k_{slow} = 1.6 \times 10^{-4} \text{ s}^{-1}$. The time course of the reaction between TNBS and the same liposomes after solubilization with $T \times 100$ is also shown in Figure 6(a). The reaction is completed in about 30 min , and is monophasic. The absorbance recovery (ΔA) is 1.12 , and the calculated first-order rate constant k' is $2.6 \times 10^{-3} \text{ s}^{-1}$ (Table 1). The difference between the total absorbance change in the presence and absence of Triton X-100 is within approx. 7% ($1.12/1.20$), and is compatible with the dilution of the sample produced by the addition of Triton X-100. An aliquot of the same

Figure 5 Time-dependence of the reaction between PE_{ext} and SPDP

Aliquots of SUVs (0.36 mg/ml PLP) were incubated with 0.2 mM SPDP. Removal of excess SPDP, by dialysis (□) or gel chromatography (●), was started at the indicated time (0). Left ordinate, % of PE_{tot} not derivatized; right ordinate, $[PE_{tot}]$ (mM).

Figure 6 Time courses of the reaction between TNBS and SUVs or PD-SUVs

(a) Trace 1, SUVs; trace 2, solubilized in Triton X-100 SUVs. Solubilization of SUVs was preliminarily achieved by adding $100 \mu\text{l}$ of a solution 20% Triton X-100 to 0.9 ml of liposomes, and leaving the mixture at room temperature for 30 min . (b) PD-SUVs, before (trace 3) and after (trace 4) solubilization in Triton X-100, were prepared by allowing SUVs (0.36 mg/ml PLP), to react with 0.2 mM SPDP; excess SPDP was removed by dialysis. Solid lines represent the best fit to a double (trace 1) and a single (all other traces) exponential process; rate-constant values are reported in the text. Concentrations of reactants after mixing were: standard SUVs diluted to 0.36 mg/ml PLP, equivalent to 0.17 mM PE_{tot} ; 4 mM TNBS; 0.1 M Hepes buffer, pH 7.6. The temperature was 20°C . See also Table 1.

Table 1 Total absorbance change and pseudo-first-order rate constant values of TNBS derivatization

Sample	$10^3 \times k'_{fast}$ (s^{-1})	$10^4 \times k'_{slow}$ (s^{-1})	ΔA_{tot}
SUV	5.3	1.6	1.20
SUVs dissolved in Triton X-100	2.6	—	1.12
PD-SUVs	1.2*	0.8	0.84
PD-SUVs dissolved in Triton X-100	2.0	—	0.80

* Rate constant value of the reaction after addition of 2% Triton X-100 (trace 4 in Figure 5; see the text).

suspension of liposomes was also incubated for 2 h with SPDP, before being mixed with TNBS. Figure 6(b) shows the progress curves for formation of the TNP derivative observed when TNBS is mixed with the PD-liposomes. A_{420} increased slowly and monophasically; after 100 min Triton X-100 was added directly to the sample in the cuvette, and allowed to equilibrate with (dissolve) the liposomes for about 10 min : a rapid increase in reaction rate

was observed. The overall change in absorbance is 0.84; the calculated first-order rate constant values, before and after the addition of detergent, are $0.8 \times 10^{-4} \text{ s}^{-1}$ and $1.2 \times 10^{-3} \text{ s}^{-1}$. The time course of the reaction between TNBS and PD-liposomes preliminarily solubilized with Triton X-100 is also shown. The time course is monophasic, absorbance recovery is 0.80, and the calculated first-order rate constant is $2.0 \times 10^{-3} \text{ s}^{-1}$. Table 1 summarizes the kinetic parameters relevant to the experiments shown in Figure 6 (see also the Materials and methods section).

DISCUSSION

It is difficult to quantify the transbilayer distribution of PE in liposomes without an independent measurement of the number of bilayers forming the vesicles. For this reason liposomes were observed by electron microscopy directly or after freeze-fracturing. The majority had the structure of SUVs, i.e. they were about 30 nm in diameter. Measurement of the transbilayer distribution of PE in SUVs prepared from purified soya-bean asolectin was initially performed statically using TNBS, by making use of the typical absorption maximum of the TNP derivative centred at 420 nm [19]. As expected, pure PE dispersed in detergent reacts with TNBS, and the amount of TNP derivative formed can be easily titrated. The reaction yield was carefully controlled, as both the nature of the compound bearing the amino group(s) and the experimental conditions are known to affect the specific absorption of the TNP derivative [19,25]. For this reason, the extinction coefficient of the derivative at 420 nm was determined in the same buffer used for the experiments, and in the presence or absence of Triton X-100. The detergent, by dissolving the liposomes, was necessary to gain full access to PE_{in} . The value of ϵ ($7.2 \text{ mM}^{-1} \cdot \text{cm}^{-1}$) was found independently of the experimental conditions used and was compatible with other determinations, although it was in the lower range of the absorbance value [19]. The results of static titration of PE_{ext} in SUVs, however, could not be interpreted on the basis of the same simple covalent derivatization observed with pure PE or SUVs dissolved in Triton X-100. The static titration basically showed that the amount of TNP derivative formed at any concentration of TNBS (i) always increases with time (on the time scale of a few hours), (ii) is not limited by PE availability until 100 % of PE_{tot} is derivatized; moreover (iii) Scatchard plots of the data are not linear. These findings are not compatible with the unique derivatization of PE_{ext} , and therefore with the assumption that TNBS reacts only with the external fraction of PE. In agreement with findings of a measurable permeation of biological membranes by TNBS [26,27], these results rather suggest that, when soya-bean SUVs are used, TNBS reacts, although on different time scales, with all PE molecules regardless of whether they are inside or outside the vesicle.

The same determination of PE_{ext} was therefore attempted kinetically. On mixing SUVs with excess TNBS, a biphasic time course was observed. A first rapid phase (half-time = 2 min) is followed by a (30–40 times) slower phase (half-time = 70 min). The overall change in absorbance accounts for derivatization of all PE molecules, the initial fast phase accounting for about 25 % of the reaction. On the basis of the relative absorbance recovery, the two phases have been assigned to the reaction of TNBS with PE_{ext} (rapid phase) and PE_{in} (slow phase). This conclusion is strongly supported by the finding that solubilization of the vesicles with Triton X-100 results in a monophasic time course with the same total amplitude as that observed for the reaction with SUVs, the reaction proceeding at the same rate as the initial rapid phase (observed with intact SUVs). The slow phase

observed with SUVs corresponds therefore to derivatization of the PE molecules (the majority) assembled in the internal layer of the vesicle membrane. The kinetics of derivatization of PE are slow, as they are limited by the rate of TNBS permeation. In addition, the SUVs previously modified by SPDP (PD-liposomes) react with TNBS following a monophasic time course, and the kinetics are comparable in amplitude and rate with those of the slow phase observed when TNBS and SUVs are mixed. As expected, in this case no rapid change in A_{420} is observed, confirming that all PE_{ext} was PD-derivatized. Consistently, using PD-liposomes, either solubilized in Triton X-100 or not, the amount of PE that reacted never exceeded 70–75 % of PE_{tot} , the latter being measured using the same SUVs, not allowed to react with SPDP. It may be worth noting that the slow derivatization of PE_{in} is in any case described by a single exponential, suggesting that, at least within the time window of 100 min, a time-dependent change in ϵ of the TNP derivative does not occur [19].

Assignment of the fast and slow phase to derivatization of PE_{ext} and PE_{in} , respectively has at least two implications: (i) the transbilayer distribution of PE in SUVs is asymmetric, and (ii) the TNBS molecule permeates the vesicle membrane. An asymmetric transbilayer distribution of PE in biological membranes is a common finding. It has previously been reported [28] in SUVs similar to those used here, although in that case asymmetry was only deduced on the basis of the buffering capacity expressed by the external and internal layer of the vesicle. Transbilayer asymmetry of PE_{in} may be explained in view of the difference between the bulk volume of the two headgroups of PE and PC; the less bulky head group of PE would fit better in the smaller crowded space of the internal surface of a small vesicle. In order to react with PE_{in} , TNBS has to permeate the vesicle membrane. This is not unlikely because although it bears a sulphonic residue that is probably deprotonated at the working pH (7.6) [19], it contains an aromatic backbone, which may account for hydrophobic interactions with the membrane. Moreover, the heterogeneity of the membrane lipid composition of these SUVs may also favour permeation to some extent.

The time course of PD derivatization of SUVs suggests that SPDP does not react with PE_{in} . At least on the time scale explored, a maximum yield of PD derivative is reached and is stable over time. The concentration of PD derivative formed corresponds to about 30 % of PE_{tot} , a value consistent with the amount of TNP derivative formed during the rapid phase observed with TNBS. The reaction between SPDP and SUVs was not stopped before the removal of (excess) SPDP, so it probably continued, at least during early stages of dialysis. This explains the different time course of PD derivatization observed when using dialysis instead of gel chromatography to remove excess SPDP. The gel chromatography column was run as fast as possible (a few minutes; see the Materials and methods section) so as to compete with the covalent derivatization rate. This allowed a better time resolution of the process, and a half-time of about 100 min ($k' = 1.16 \times 10^{-4} \text{ s}^{-1}$) was estimated. Using a stoichiometric ratio $\text{SPDP}/\text{PE}_{\text{ext}} = 6$, and 30 min incubation at 25 °C (excess SPDP was removed by dialysis), the amount of PE derivatized in SUVs is on average equal to 27 % of PE_{tot} . This value, as well as confirming the fraction of PE_{ext} , is moreover independent of the absolute amount of PLP, within a concentration factor of 20, suggesting that there is no liposome-concentration-dependent masking of amino groups accessible to SPDP.

Electron-microscopy measurements were consistent with previous observations [12–14], and allowed us to establish the size distribution of the SUVs generated. By keeping the vesicle to a sphere of about 30 nm diameter (see also the Introduction), and

taking into account the average area occupied by the PE and PC headgroups in the two layers of the membrane, the number of PLP molecules forming a single vesicle, and thus the molecular mass of a single liposome, can be calculated as about 1.5×10^3 – 2×10^3 kDa [12]; on this basis, the concentration of liposomes in the standard SUV suspension used for the experiments reported here is approx. $10 \mu\text{M}$. As the concentration of $\text{PE}_{\text{ext.}}$ in the standard suspension of SUVs is about 1 mM, about 10^2 amino groups are exposed on the vesicle surface and can be therefore derivatized. The distribution of amino groups on the liposomal surface is a useful parameter for standardizing liposome-derived systems; it may be essential, for instance, to optimize the ratio between liposomes, covalent arms generated on their surface and in turn bound ligands for cell targeting.

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