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External surface and lamellarity of lipid vesicles: a practice-oriented set of assay methods

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Abstract

Three methods are presented for the determination of external surface of large lipid vesicles of different lamellarity with 2% absolute accuracy. These methods (referred to as EPR, NBD and TNBS assays) use different marker lipids which provide signals (electron paramagnetic resonance, fluorescence of *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) residues, and UV absorption increase of 2,4,6-trinitrobenzenesulfonic acid after reaction with aminolipids, respectively). The signals change upon addition of different membrane-impermeant reagents due to reaction with marker lipids at the external vesicle surface. They were applied to the same vesicle samples, including unilamellar and multilamellar vesicles, both at two different lipid compositions. External surface data matched for the three assays within 2%, but only after appropriate redesign or adaptation of so far published procedures. Main improvements related to slow influx of reagents (TNBS and NBD assays) or to redistribution of marker lipids (EPR assay), obscuring determination of outer vesicle surface from fast reaction between reagent and readily accessible marker lipids. Furthermore, suitable stategies were found to obtain accurate 100% values (reaction of all marker lipids present), required to relate external vesicle surface to total surface. This included corrections for light scattering (NBD assay) and for turbidity (TNBS assay). These three methods appear to close a gap in the methodology to determine external surface of vesicles for typical practical needs. In particular, the reliability range of the NBD assay could be extended to marker lipid densities as low as 1 marker lipid per 3000 lipids.

Key words: Ascorbate; Lipid vesicle; External surface; CAT-16; NBD-PE; TNBS; EPR

1. Introduction

Different kinds of morphologically well-defined vesicles are required for different purposes: For instance, MLV and/or LUV are used for drug encapsulation (Mayer et al. [1]) and for physico-chemical studies (e.g., Sen et al. [2]). Most frequently, however, LUV with a narrow size distribution are desired, such as for studying transport function.

There is no general method available that allows to produce large vesicles (LUV or MLV) with predictable morphology under all circumstances. Moreover, appropriate methods for particular purposes depend on the lipid used [3,4] or, if proteins are included, on conditions for their viability (e.g., see Refs. 5 and 6). Finding

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Abbreviations: BHT, butylated hydroxytoluene; CAT-16, 4-(*N,N*-dimethyl-*N*-hexadecyl)ammonium-2,2,6,6-tetramethylpiperidine-1-oxyl iodide; DMPE, dimyristoylphosphatidylethanolamine; eggPC, egg phosphatidylcholine; EM, electron microscopy; FATMLV, multilamellar vesicles by freeze-and-thaw procedure; LUV, large unilamellar vesicles; LUVET, large unilamellar vesicles by extrusion technique; MLV, multilamellar vesicles; NBD chloride, *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) chloride; NBD-DMPE, *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)dimyristoylphosphatidylethanolamine; NBD-DPPE, *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)dipalmitoylphosphatidylethanolamine; NBD-PE, *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)phosphatidylethanolamine;PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PS, phosphatidylserine; SBL, soybean lipids; SM, sphingomyelin; SUV, small unilamellar vesicles; TNBS, 2,4,6-trinitrobenzenesulfonic acid.

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the appropriate method often requires a laborious 'trial and error' search among different methods and conditions. During this, reliable and fast assays are desired for vesicle topology/lamellarity in order to judge suitability of vesicles obtained for the particular purpose.

External, or accessible surface determination is one out of several ways to estimate lamellarity of lipid vesicles [7]. Various methods have been proposed for the measurement of accessible vesicle surface. There are some methods available, particularly devised for this purpose [3,8–11]. Other methods, originally devised to monitor asymmetric lipid distribution (e.g., see Refs. 12–15), are readily adaptable to external surface determination.

Table 1 summarizes all assays which may be considered. The basic design is the same for all assays. All methods require particular marker lipids providing a signal which changes upon addition of particular external reagents (see Table 1). Briefly, the EPR and NBD signals of marker lipids are abolished by reducing agents, the NMR signal is quenched or shifted by paramagnetic ions, the UV absorbance of TNBS is increased when alkylating aminolipids, and periodate oxidizes the listed marker lipids which is subsequently detected from formaldehyde production. EPR and NBD methods require artificial markers lipids while the other methods rely on the presence of native markers lipids of method-specific type.

In all assays the external vesicle surface is estimated from the signal change upon reaction of outer marker lipids with impermeant reagent, normalized to the signal change for reaction of all marker lipid present. Such estimates reflect true external surface only if: (i) marker lipids are randomly distributed over all lipid layers in MLV or LUV, (ii) redistribution of marker lipids (flip-flop) during reaction is negligible, (iii) membrane permeation of reagent is negligible for time of analysis, and (iv) the 100% signal (reaction of all marker lipids) is determined to sufficient accuracy, normally after treatment of the vesicles with detergent.

The assays presented in this study are based on published procedures. When testing for the four criteria we found that (i) in LUV and MLV marker lipids are generally randomly distributed while this did not apply to SUV. We, therefore, restricted the present study to large vesicles only. (ii) None of the marker lipids in Table 1 showed flip-flop except for CAT-16. (iii) The reagents TNBS, Mn^{2+}, and to a certain extent dithionite were found to permeate vesicle membranes during typical times for analysis, and (iv) determination of 100% signals needed improvements of variable degrees, dependent on the assay used. On the basis of these findings we suggest experimental protocols for accurate determination of external surface by a set of methods covering a wide range of uses.

2. Materials and methods

DMPE, NBD-DPPE, NBD chloride, and 5% aqueous TNBS solution were purchased from Sigma (Munich), CAT-16 was obtained from Molecular Probes (Eugene, OR).

2.1. Preparation of NBD-DMPE

32 mg DMPE and 20 mg NBD chloride were dissolved in 3 ml chloroform, followed by addition of 10 μl triethylamine. After 1 h the mixture was applied to two 20×20 cm preparative TLC plates (silica 60, Merck) and developed with chloroform/methanol/water (65:25:4, v/v/v). The lower 3/4 of the product band were harvested, tightly packed in a glass column, extracted with chloroform/methanol/water (5:5:1, v/v/v), taken to dryness and found to be completely pure by TLC.

2.2. Preparation of pure SBL

Crude soybean lecithin (Sigma type II-S) was freed from neutral lipids, free fatty acids, lyso-PC, and from all protein content by chromatography on silica gel 60 (Merck). 250 ml of chloroform/ methanol/ NH_3 (65:25:4, v/v/v) and 1000 ml of chloroform/ methanol/water (65:25:4, v/v/v) were prepared as eluent 1 and eluent 2, respectively. 140 ml of dry silica gel were suspended in eluent 1, packed into a 45 mm glass column, and excess solvent was recovered. Twenty five 50-ml flasks, each containing 0.3 ml of 1 mg/ml BHT in n-hexane were placed on ice. 3 g crude lecithin were dissolved in 15 ml of eluent 1, 1 ml of the BHT solution was added, and after sample loading all of eluent 1 was used for elution while collecting 30-ml fractions. Elution was continued with eluent 2 but now 50-ml fractions were collected. Fractions were analyzed by TLC in chloroform/methanol/water (65:25:4, v/v/v). All fractions containing only the four major phospholipids of SBL were combined and taken to dryness, redissolved in chloroform, filtered from silica particles through filter paper and subjected to high vacuum for 2 h. Dry material was redissolved in chloroform and dried in portions which were stored at −70°C.

2.3. Preparation of pure eggPC

Ovothin 200 was obtained in bulk quantities from Lucas Meyer (Hamburg, Germany). Its degree of peroxidation was checked by the thiobarbituric acid method [16] and found to be comparable to the least oxidized eggPC commercially available. Impurities (free fatty acid, lyso-PC, sphingomyelin, and yellow pigment,

alltogether 1–2%) were removed by the same type of silica chromatography as described for SBL, except that 150 ml dry gel and only eluent 2 was used. Fifteen 50 ml fractions were collected after loading. Fractions with pure eggPC devoid of yellow pigment were combined and processed as described for SBL.

2.4. Prelabeling of lipid mixtures with fluorescent lipids or with the spin label CAT-16

Dry components and/or freshly prepared chloroform stock solutions thereof were mixed, completely dissolved in chloroform, taken to dryness and subjected to high vacuum for at least 1 h. Definition of 100% always refers to the content of unlabeled lipids, excluding fluorescent or spin-labeled lipids.

2.5. Preparation of defined vesicles

FATMLV were prepared as described by Mayer et al. [1], with the following modifications: Dry lipid films were always rehydrated with 'Na buffer' (100 mM NaCl, 10 mM Hepes-NaOH, pH 7.4 at 20°C) unless specified otherwise. EggPC/DMPE mixtures were freeze-thawed at 200 mg/ml, SBL at 100 mg/ml. Nitrogen gas was perfused through the septum-covered flask in each freezing step (not argon, danger of explosion during warming!) while furtheron vesicles were handled under argon atmosphere whenever possible. Finally vesicle aggregates were dispersed by bath-sonication for 15 s under argon in a test tube that had never been subjected to freezing in order to avoid breaking (always at 50 mg/ml lipid concentration). LUVET were prepared exactly as described by Hope et al. [9], with one very helpful modification: Vesicles were first extruded through a single 0.1 μm Nuclepore membrane, followed by the usual eight cycles of extrusion through a double layer of the same membranes. Without the initial extrusion through a single membrane the flow in subsequent double membrane extrusions was frequently unsatisfactory, propably due to residual silica particles which had not been removed by paper filtration (see above). SUV were prepared by bath sonication of FATMLV or LUVET to near clarity for about 5–10 min under argon.

2.6. EPR stopped-flow assay

EPR signals were measured at room temperature on a Bruker X-band spectrometer (model ER-200-D) operating at 9.6 GHz. A normal aqueous flat cell was adapted for stopped flow experiments in order to minimize the dead volume and save precious sample. Teflon tubing was inserted into the lower end and connected to a 100 μl mixing chamber which was covered by a rubber septum (Aldrich Z10,075-7). Two 1 ml tuberculin syringes were simultaneously pushed by a hand-driven piston, with two 22 gauge needles serving as reversible connection to the mixing chamber. The degree of measuring cell equilibration after injection with two filled syringes was 85% only, but it was very reproducible. Before each vesicle injection the system was rinsed by three cycles of injecting 10 ml of air plus 5 ml of buffer. Air bubbles had to be avoided both before and after sample injection since they changed both the vertical position of the baseline and the horizontal position of the spectrum. Bubbles were detected unequivocally by dramatic changes in the microwave diode current (at untouched iris setting) and they could be removed by an additional cycle of rinsing. If bubbles were generated during sample injection the data were discarded. Vesicle concentration in syringe 1 was always 10 mg/ml and the spin label content 0.1 mg/ml. Syringe 2 either contained Na buffer (controls) or Na ascorbate. Ascorbate reagent (110 mM ascorbic acid, 10 mM Hepes) was prepared by dissolving ascorbic acid, titrating to pH 7.4 with NaOH under argon, and storing in the dark under argon at 4°C for no more than one week.

2.7. TNBS assay

A modification of the published protocol [7] was used during the optimization of the procedure (see legends to Figs. 3, 4, and 6). In the following, the final standard assay is described (pipetting volumes for the two down-scaled versions are given in parentheses). Eppendorf Multipettes were used to achieve sufficient precision in the pipetting steps. 200 μl (100 μl or 50 μl) of vesicles were adjusted to high pH by the addition of 100 μl (50 μl or 25 μl) of borate/sucrose reagent (with or without 4% (w/v) Triton X-100 for 'total values' and all other samples, respectively). First 12 'totals' were quickly started by the addition of 100 μl (50 μl or 25 μl) of TNBS reagent and the initial time was taken to be zero. Triplicates of these 'totals' were stopped with 200 μl (100 μl or 50 μl) of 1.5 M HCl at intervals of 15, 30, 45, and 60 min from the start. Shortly after the 'totals' 12 intact vesicle 'samples' were started in the same way and triplicates were stopped by 200 μl (100 μl or 50 μl) of stop solution at intervals of 15, 30, 45, and 60 min from start. Five blank samples were 'stopped before starting' i.e., 200 μl (100 μl or 50 μl) of stop solution were added before 'starting' with 100 μl (50 μl or 25 μl) of TNBS reagent. When the last regular 'samples' and 'totals' had been stopped, all samples were diluted to fill a standard UV cuvette by the addition of 500 μl (1000 μl or 1000 μl) of water (thus the final volume is not identical in the three versions!) and A_{410}–A_{510} was immediately determined against a water blank (the subtraction of A_{510} is purely optional). The vesicle samples were to contain

no more than 0.3 mM and not much less than 0.1 mM aminolipid, and they were adjusted to 4 mM $CaCl_2$ in the case of SBL which contains over 20% of anionic lipids. The borate/sucrose solution in our case was 0.2 M in boric acid and 1 M in sucrose. It was critical to keep to the following procedure in order to obtain the correct pH: The components were dissolved in 95% of the final volume, the pH was kept near 9.0 with NaOH while the solids were dissolving, then the pH electrode was quickly recalibrated at pH 9.00 and the pH of borate/sucrose was fine-adjusted to 9.00 by NaOH. Aliquots were stored frozen at −20°C. The TNBS reagent (0.1%) was always freshly prepared by diluting the commercially available 5% solution with vesicle buffer. The stop solution for 'samples' and 'blanks' contained 1.5 M HCl and 2% (w/v) Triton. TNBS-containing samples were protected from light as much as possible.

2.8. Dithionite reduction of external NBD-PE

The basic procedure is a very slight modification of the described one [12]. 1.9 ml of Na buffer were mixed with 100 μl of vesicles (10 mg/ml of lipid, below 1% of NBD-PE with respect to unlabeled lipid) irradiated at 470 nm (5 nm slit) and fluorescence was followed in time at 540 nm (5 nm slit). Subsequently 20 μl of dithionite reagent were added under stirring, followed by 100 μl of 20% (w/v) Triton X-100 (see Fig. 8). The 1 M dithionite reagent was prepared in the hood by dissolving 3.48 g $Na_2S_2O_4$ and 2.43 g Tris base with water to give a final total weight of 21.4 g. The pH value was automatically around 10 as required. Since the reagent strongly absorbs oxygen it was opened for as short as possible and could only be used for one day. The extension of the method for turbid samples is described in Results.

2.9. ^{31}P-NMR method of external surface determination

NMR spectra were recorded on a Bruker WM-360 NMR spectrometer exactly as described by Hope et al. [9].

3. Results

Our strategy was to compare results from independent methods using the identical vesicle sample. Reliability is then judged from the equivalence of results at highest resolution possible for each method. However, not all methods can be interrelated, due to constraints on the choice of lipids and marker lipids. For this study we have selected the following set of methods the results of which could be compared and which, together with the periodate method and the ^{31}P-NMR method (see Table 1), appear to cover the needs of any application.

Table 1
Available assays for the external surface of lipid vesicles

Assay method	Marker lipid		External reagent	References
	native	artificial		
EPR		PESL	$FADH_2$	Thurnhofer et al. [17]
EPR		CAT-16	ascorbate	this study
NBD		NBD-PE	dithionite	this study [a]
TNBS	PE or PS		TNBS	Barenholz et al. [8] New [7] Hope and Cullis [15] [b] this study
Periodate	PG, PI, or glycolipids		periodate	Hope et al. [12] [c] Lentz et al. [10] Szoka et al. [3]
^{31}P-NMR	phospholipids [d]		Mn^{2+}	Hope et al. [9] Mayer et al. [18] Berden et al. [23] this study
^{1}H-NMR	PC or SM		lanthanides or Mn^{2+}	Bystrov et al. [24] Berden et al. [23] Strauss and Hauser [11]

[a] Adapted from McIntyre and Sleight [13], who used dithionite to prove asymmmetric incorporation of NBD-PE into cell membranes.
[b] Used for assaying transbilayer distribution of aminolipids in LUVET.
[c] This newer method with arsenite in place of bisulfite is much less dangerous!
[d] Positively shown for PC and PC/PS mixture only.

3.1. EPR stopped-flow assay

In a previous study of CAT-16 reduction by ascorbate [17] it had been concluded that this assay is unsuitable for external surface determination of vesicles. We intend to evidence that this simple method can yield very reliable results provided that (i) the EPR signal of only membrane-bound marker lipids is monitored and (ii) the time of ascorbate addition is known precisely. The latter requirement was approached by stopped-flow experiments and the former by appropriate signal analysis at a position of maximal selectivity in the EPR spectra.

The EPR spectrum of CAT-16 in the presence of lipid membranes (see Fig. 1, lower spectrum) is composed of two components, due to the coexistence of membrane-bound CAT-16 molecules with CAT-16 molecules in micelles. The narrow spectral component in the presence of membranes matched in peak position and linewidth the spectrum of CAT-16 in micelles (upper spectrum). The broad spectrum of membrane-bound CAT-16 molecules appeared not to be significantly dependent on the choice of lipid since virtually the same spectra were obtained for two different types of lipid mixtures (SBL or eggPC/DMPE, comparison not shown). In order to exclusively monitor the concentration of the membrane-bound spin labels with time the magnetic field was locked in at the position shown by the vertical line in Fig. 1.

In a routine protocol (see Fig. 2) three successive 'ascorbate injections' (upper part of Fig. 2) and three

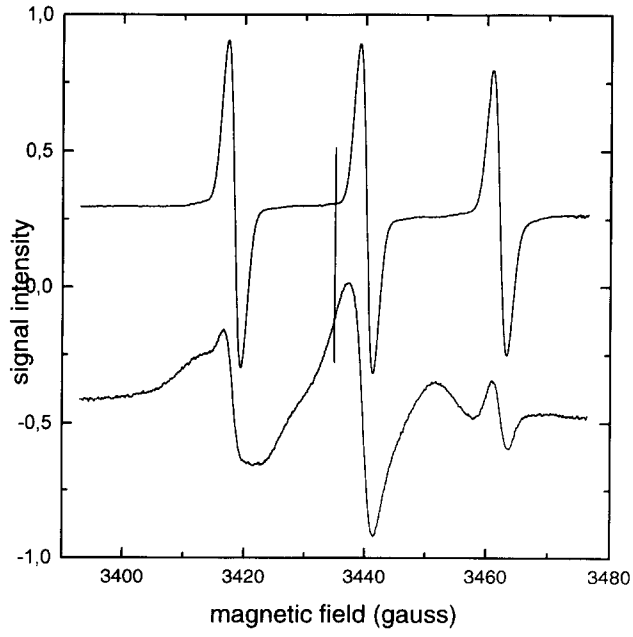

Fig. 1. Spectrum of CAT-16 in the absence and in the presence of lipid vesicles. Upper spectrum: 0.1 mg/ml of CAT-16 in Na buffer. Lower spectrum: 10 mg/ml of eggPC/DMPE-LUVET, prelabeled with 0.1 mg/ml of CAT-16.

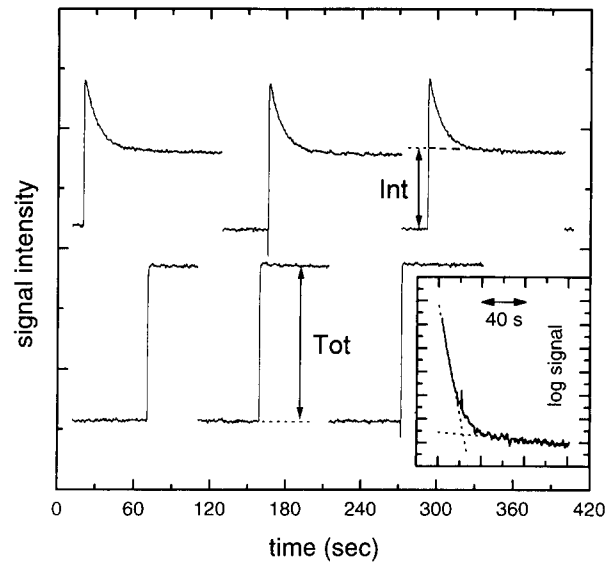

Fig. 2. Demonstration of the EPR stopped flow assay with 10 mg/ml SBL-LUVET (prelabeled with 0.1 mg/ml CAT-16) in syringe 1. Lower triplicates: control injections with Na buffer in syringe 2. Upper triplicates: ascorbate injections with ascorbate reagent in syringe 2. The dashed line indicates the graphical extrapolation of the signal of internal spin labels. (Inset) Semilogarithmic plot of the third ascorbate injection, the dotted lines indicate the biexponential time-course.

control injections (lower part of Fig. 2, buffer in place of ascorbate) were performed. The fraction of external spin labels (E) was determined graphically from the 100% signal (Tot) and the signal of spin labels not accessible to ascorbate reduction (Int) using the relation $E = 1 - (Int/Tot)$ as shown in Fig. 2. For a formally more correct data analysis semilogarithmic plots were used (see inset in Fig. 2). They revealed that the signal decrease followed a biexponential time-course. Extrapolation of the slow phase (due to reduction of internal spin labels) to time zero allowed then to determine the fraction of internal spin labels (Int) at time zero. It turned out that results from such numerical analysis (see Table 2) are not significantly different from those obtained from the simple graphical extrapolation of Int (see values in parentheses in Table 2).

Why was it necessary to set the field in the stopped-flow experiments (Fig. 2) to a position where only membrane-bound spin lables were monitored (Fig. 1), rather than at the position of maximum intensity? The answer is that CAT-16 was used as a marker for outer and inner membrane surfaces in the present study, therefore suppression of the signal from *not* membrane-bound spin labels was needed to obtain the desired information on surface accessibility.

The EPR stopped flow assay was applied to two morphologically distinct preparations of model vesicles which have been very well characterized with respect to lamellarity in the literature [9,18]. The one type

were multilamellar vesicles (FATMLV), where only a minor fraction of the CAT-16 molecules is expected to reside on the external surface while the other were large unilamellar vesicles (LUVET) which are expected to give values of approx. 1/2 for the relative fraction of internal labels (*Int/Tot*). For both types of vesicles two quite different lipid compositions were used, eggPC/DMPE (95:5, w/w) and SBL.

Table 2 shows external surface values obtained: LUVET from SBL indeed appeared to be perfectly unilamellar, exhibiting 50% external surface. LUVET from eggPC/DMPE (95:5, w/w) may still have contained a few oligolamellar vesicles since their accessible surface was 45%, rather than 50%. The degree of multilamellarity of FATMLV from SBL is quite low, judged from 33% external surface. We consistently obtained similar results when trying to produce very multilamellar vesicles from SBL, by any method proposed in the literature [7]. In contrast to SBL, FATMLV from PC/PE revealed high lamellarity in accord with literature data for eggPC vesicles [18]. This agreement with expectations does not prove, however, that this method gives accurate data for any particular condition. To establish reliability at a safer level we chose to compare the EPR assay with another assay (TNBS). Both assays differ in the marker lipids used, native lipids in the TNBS while artificial in the EPR assay (cf. Table 1).

3.2. TNBS assay

A glance at Table 2 shows that the TNBS method can give results which match those obtained from the EPR stopped flow assay, using the same vesicle preparation. This goal was achieved, however, only after a thorough investigation on all essential kinetic parameters, with respect both to the trinitrophenylation reaction itself and to the permeation of TNBS through the lipid bilayer.

Initially, when attempting to use a recent version [7] of the conventional TNBS method of Barenholz et al. [8] we regularly found the fraction of external aminolipids to be overestimated by 10–30%, as judged by parallel EPR assays on identical batches of vesicles. According to the conventional procedure the extent of external lipid labeling (*Ext*) and of total lipid labeling in the presence of Triton (*Tot*) are assumed to be proportional to the absorbance increases at 410 nm measured 30 min after exposure to TNBS. The relative external surface E is then calculated from $E = Ext/Tot$. This definition of outer lipid labeling would require TNBS to be perfectly impermeant and outer lipid labeling to be complete after 30 min. We, therefore, examined the whole time-course of vesicle labeling under the conditions of the conventional procedure. This is shown in Figs. 3 and 4 (open squares) for LUVET prepared from eggPC/DMPE (95:5, w/w)

Table 2
Comparison of external surface determination by the EPR method, the TNBS method, as well as the uncorrected and the corrected NBD method

Lipid	Vesicle type	Marker lipid	External marker lipid fraction ± S.D. (%)			
			ESR [a]	TNBS	NBD_{uncorr}	NBD_{corr}
SBL	LUVET	1% CAT-16	49.8 ± 1.9 *(50.3 ± 1.4)*	47.4 ± 1.2	–	–
PC/PE	LUVET	1% CAT-16	44.6 ± 1.6 *(44.7 ± 1.6)*	46.1 ± 1.7	–	–
SBL	FATMLV	1% CAT-16	32.6 ± 0.9 *(32.7 ± 1.0)*	33.9 ± 1.9	–	–
PC/PE	FATMLV	1% CAT-16	15.8 ± 4.2 *(15.7 ± 4.2)*	20.8 ± 0.5	–	–
PC/PE	LUVET	0.5% NBD-PE	–	40.5 ± 1.1	42.2 ± 1.8	–
PC/PE	FATMLV	0.5% NBD-PE	–	18.9 ± 1.0	16.6 ± 0.2	–
PC/PE	FATMLV	1.0% NBD-PE	–	20.9 ± 1.2	16.3 ± 1.1	19.0 ± 1.2
PC/PE	FATMLV	0.33% NBD-PE	–	24.8 ± 0.8	17.9 ± 2.1	21.3 ± 2.8
PC/PE	FATMLV	0.10% NBD-PE	–	18.9 ± 0.8	13.2 ± 2.3	18.0 ± 2.5
PC/PE	FATMLV	0.033% NBD-PE	–	22.7 ± 1.5	15.2 ± 1.4	23.0 ± 1.5
PC/PE	FATMLV	0.010% NBD-PE	–	20.4 ± 0.8	12.1 ± 1.4	30 ± 2
PC/PE	FATMLV	0.000% NBD-PE	–	21.7 ± 1.1	4.2 ± 0.4 [b]	0.0 [c]

[a] Values in parentheses were determined graphically from the original EPR time scans (compare Fig. 2, third ascorbate injection), the other values were calculated numerically as shown in the inset of Fig. 2.
[b] This value corresponds to the negligible reduction of light scattering upon dithionite addition.
[c] No markers were present.

and from SBL, respectively. The fast absorbance increase reflects external lipid labeling. The slow further increase is assigned to internal lipid labeling (evidenced later in Fig. 6). It is obvious that at 30 min a considerable contribution of internal lipid labeling obscures external surface analysis. However, extrapolation of the slow phase to time zero yields an improved estimate of external labeling.

Replacement of bicarbonate buffer by borate or borate/sucrose in presence and absence of Ca^{2+} (Figs. 3 and 4) revealed that a high osmolarity is essential for a fast initial reaction and for a clear separation of fast and slow phase. Additional sucrose accelerated external lipid labeling considerably over using only isotonic borate buffer (cf. insets in Figs. 3 and 4) for both types of lipid mixture. Additional Ca^{2+} always accelerated external lipid labeling, both for eggPC/DMPE (Fig. 3) and for SBL vesicles (Fig. 4). However, for eggPC/DMPE internal lipid labeling (slow phase) was even more accelerated by Ca^{2+} so that Ca^{2+} was disadvantageous. In contrast, using SBL vesicles, Ca^{2+} was of advantage since it had little effect on the slow phase, even decelerating internal lipid labeling slightly.

In the course of these experiments it became clear that the choice of borate/sucrose ± Ca^{2+} condition allowed to avoid disadvantages of bicarbonate buffer which are: (i) dependences of activity coefficients of bicarbonate buffer on final salt concentrations made pH adjustments inconvenient, (ii) 'aging' of trinitrophenylated lipids, leading to additional absorbance increase (see Fig. 6) was more pronounced in bicarbon-

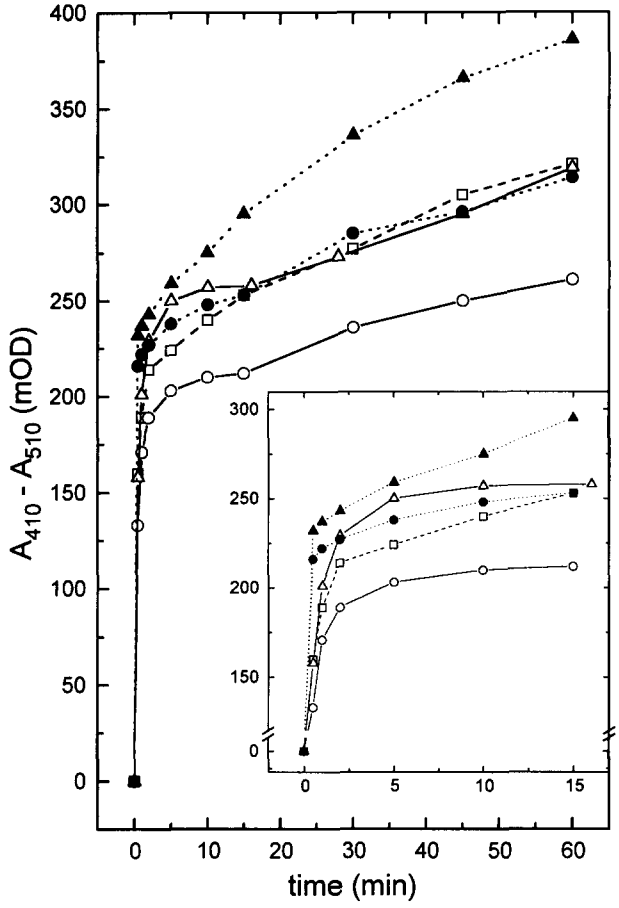

Fig. 3. Time-course of TNBS labeling of LUVET from eggPC/DMPE (95:5, w/w) at 19.5 ± 0.5°C. 600 μl of vesicles with 2.5 mg/ml lipid concentration and with 0 mM $CaCl_2$ (open symbols) or with 2.83 mM $CaCl_2$ (filled symbols) were adjusted to high pH by the addition of 200 μl of 0.8 M bicarbonate (pH 8.5) (□, - - - - - -) or 200 μl of 0.2 M borate buffer (pH 9.0 with NaOH) (○, ●) or 200 μl of the same borate buffer with additional inclusion of 1 M sucrose (△, ▲). Trinitrophenylation was started with 50 μl of 0.6% TNBS containing 120 mM NaCl (freshly prepared from the commercial 5% TNBS stock solution). The reaction was terminated at indicated time intervals by the addition of 400 μl stop solution (1.5 M HCl and 1.2% Triton). Experiments were done in triplicates but error bars were omitted for clarity. Standard deviations ranged from 5 to 10 mOD. The final pH was always 8.9 in every experiment. The high background absorbance from unreacted TNBS (about 400 mOD) has been subtracted from every time-course in order to allow for an accurate comparison between different kinetics because the background was slightly dependent on the different buffer systems.

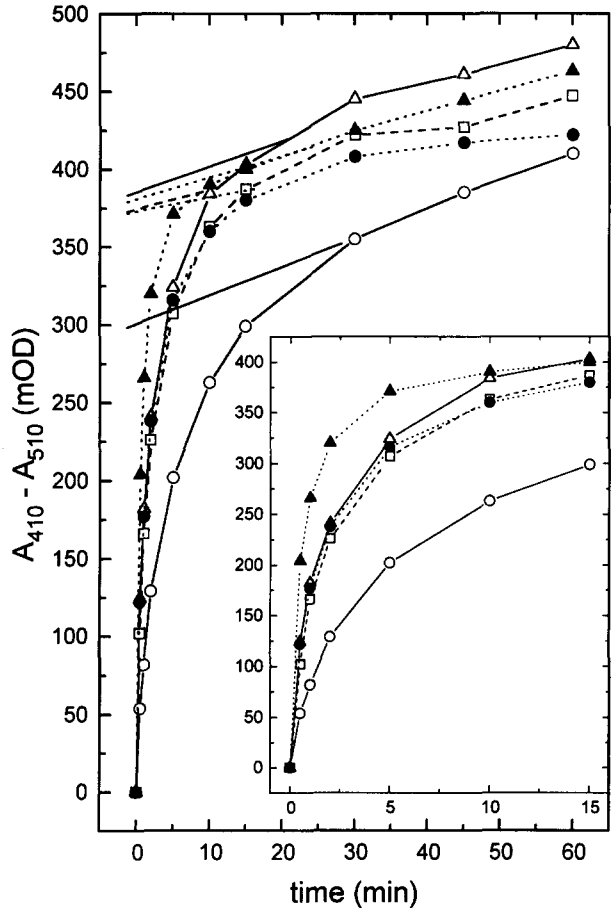

Fig. 4. Time-course of TNBS labeling of LUVET from SBL at 19.5 ± 0.5°C. The lipid concentration in the 600 μl samples was 0.5 mg/ml. Straight lines represent linear fits to the time points from 15 to 60 min, except for pure borate buffer where the fit was for the interval from 30 to 60 min. All other details are the same as in Fig. 3.

ate than in borate buffer, and (iii) with bicarbonate buffer CO_2 release after reaction stop by HCl frequently occurred delayed, hampering optical absorbance measurements.

For a reliable and accurate determination of external surface using borate/sucrose ± Ca^{2+} buffer, four determinations in 15 min intervals turned out to be sufficient as shown in Fig. 5. Results in Table 2 were obtained in this way which agree with results from the EPR assay within narrow limits. It should be added that the slow, but significant absorbance increase found for Triton-solubilized vesicles (upper data in Fig. 5) was unexpected since the fast reaction was found to be completed after 1 to 2 min (not shown). It may reflect slow chemical changes of the reactions products ('aging').

Justification for the linear extrapolation in the standard assay (Fig. 5) was obtained from data as shown in Figs. 6A and 6B for the two types of lipid mixture used throughout this study, respectively. At 20°C the overall time-course of labeling showed that linear extrapolation is reasonable for the 15 to 60 min time range of the standard assay (see dashed lines in Fig. 6). The figure also shows that the slow phase is selectively decelerated by lowering temperature to 2°C (see solid lines in Fig. 6). The linear phase now extends to several hours. Extrapolation to zero time for 20°C and for 2°C leads to the same value with a comparable S.D. so that cooling brought no advantage in practical uses. We have included these data mainly to show that external surface data are independent of temperature as they ought to be.

One determination of external surface by this TNBS method requires 0.5 to 1.5 mg of aminolipid, a serious limitation in practical uses. Therefore, we have devised an alternative TNBS assay, described in Fig. 7, which consumes 20-fold less material at the expense of accuracy (adapted from Hope and Cullis [15], see Table 1).

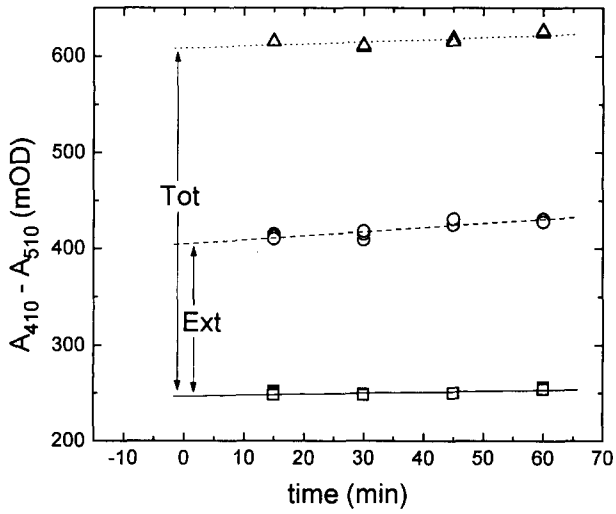

Fig. 5. New standard version of external surface determination by TNBS labeling: 200 μl aliquots of 1 mg/ml SBL-SUV with 4 mM $CaCl_2$ were processed in triplicates (mostly concealed by overlap) at 20°C as described in Materials and Methods. Blank values (□, ———) were determined by including lipid but stopping with HCl/Triton before 'starting' with TNBS. This blank definition is formally more correct than the conventional strategy of omitting the lipid alltogether in the blank samples but both methods failed when Triton batches contained TNBS-reactive contaminants. In this case it was necessary to mix both strategies, i.e., to perform the complete TNBS standard assay shown above once in the presence of lipid (yielding *Tot* and *Ext*) and once in the absence of lipid (yielding Tot_o and Ext_o) and to calculate the corrected values $Tot_{corr} = Tot - Tot_o$ and $Ext_{corr} = Ext - Ext_o$,respectively. External lipid labeling (○, - - - - - -) was followed with intact vesicles, while total labeling (△, · · · · · ·) was measured with Triton-solubilized vesicles. Lines are linear least-squares fits.

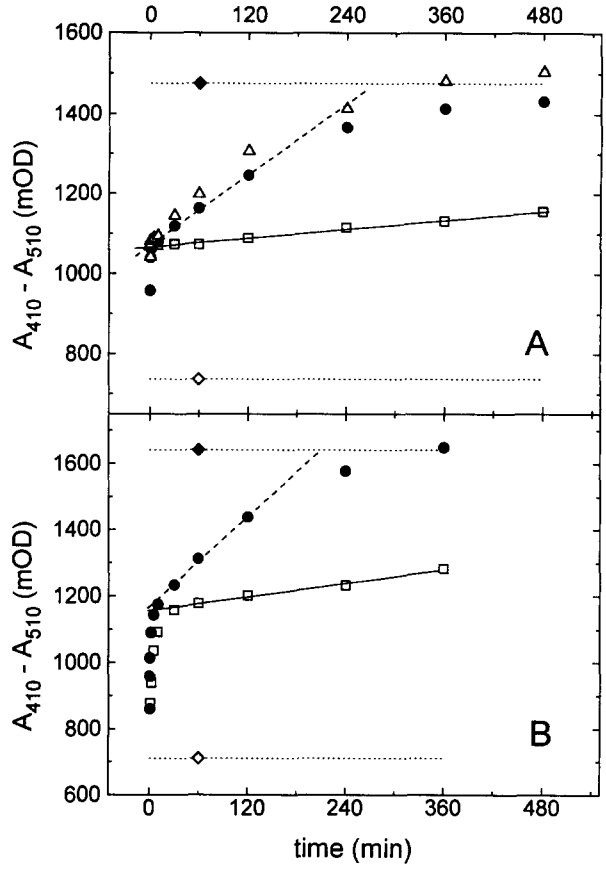

Fig. 6. Temperature dependence of vesicle labeling by TNBS. 600 μl of vesicles in Na buffer were adjusted to pH 8.9 by the addition of 200 μl of 0.2 M borate (pH 9.0) with 1 M sucrose included. The reaction was performed on ice (□, ———) or at 20°C (●, - - - - - -). The same protocol as in Fig. 3 was used. (A) 5 mg/ml of eggPC/DMPE-LUVET in the vesicle sample, – for comparison one series was run by adding 200 μl of 0.8 M bicarbonate (pH 8.5) (△, ·–·–·) instead of borate/sucrose at 20°C. (B) 1 mg/ml SBL-SUV and 2.83 mM $CaCl_2$ in the vesicle sample. All experiments were done in triplicates but the error bars were omitted for clarity. Blank values (◇, · · · · · ·) were produced by 'stopping' with stop solution before starting with TNBS reagent, and total values (♦, · · · · · ·) were defined by including 1.6% (w/w) Triton in the borate/sucrose buffer while using 0.4% Triton only in the stop solution.

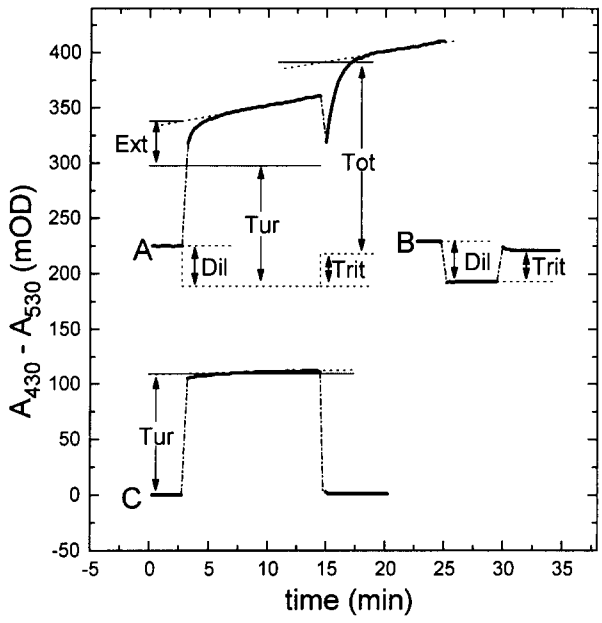

Fig. 7. Direct observation of TNBS labeling of intact vesicles. The method of Hope and Cullis [15] was modified to separate the contributions of vesicle turbidity from the absorbance effect of aminolipid labeling by TNBS. Three successive experiments were performed (in triplicates) in a standard 1 ml cuvette. In experiment A 500 μl Na buffer and 300 μl borate/sucrose were added, followed by 200 μl of 0.05% TNBS in Na buffer. Mixing was achieved by withdrawing from and redelivering the mixture into the cuvette using the yellow tip of a digital pipette, and the tip with residual droplets was saved in the dark. $A_{430} - A_{530}$ was measured at 15 s intervals (430 nm, rather than 410 nm were chosen because of the spectral shift at basic pH). After establishment of a stable baseline 200 μl of vesicles in Na buffer, containing 1.5 mg/ml of eggPC/DMPE (95:5, w/w) were added. Subsequently, 25 μl of 20% (w/w) Triton X-100 were added. In experiment B 200 μl of Na buffer were added in place of 200 μl of vesicles. In experiment C 200 μl of Na buffer were added in place of the TNBS reagent. Because the signal of interest was small in comparison to the high blank level of unreacted TNBS and in comparison to turbidity of MLV it was essential to use Eppendorf Multipettes, and to use the identical yellow tip for the three mixing steps of each single experiment.

Essentially, vesicles are added to borate/sucrose buffer containing TNBS (at $t = 3$ min in Fig. 7A) and absorbance increase is monitored in time. At $t = 15$ min Triton is added to assay the 100% value (*Tot*). Both additions cause discrete changes of baseline, due to the dilution when adding vesicle sample (*Dil* in Fig. 7A) and due to TNBS-reactive contaminants in the batch of Triton used in this experiment (*Trit*). These changes were determined by using buffer instead of vesicle sample (see part B of Fig. 7). Finally, turbidity of vesicles (*Tur*) contributing to the signal was determined from the same experiment as in part A of Fig. 7 but omitting TNBS (see part C). Moreover, turbidity was greatly suppressed by using $A_{430} - A_{530}$ differences (as shown in Fig. 7), rather than A_{430} values directly (in which case no significant result could be obtained, not shown). Linear extrapolations as shown in Fig. 7A lead to a value for the external surface according to $E = Ext/Tot$. All details of practical importance are given in the legend to Fig. 7. This method gave 26.9 ± 3.6% external surface for the data shown in Fig. 7 which compares to 29.8 ± 0.5% determined from the standard TNBS assay for the same batch of vesicles.

3.3. NBD assay

We have adapted the recently published strategy to abolish NBD-PE fluorescence by impermeant dithionite [13] for external surface determination. Fig. 8 shows the time-course of NBD fluorescence of intact vesicles and reductions caused by addition of dithionite (arrow 1) and of Triton (arrow 2). The continuous linear decrease of the signal is due to slow reduction of internal NBD fluorescence by dithionite influx (see squares) under avoidance of fluorescence bleaching (see legend to Fig. 8) while in the second experiment (see circles) bleaching also contributed to signal decrease. In any case, extrapolations of signal decrease gave accurate values for dithionite-induced drop (*Ext*) and Triton-induced drop (*Tot*), yielding external surface value $E = Ext/Tot$. For practical use in routine assays only a few minutes fluorescence monitoring was sufficient for accurate analysis which may be seen from Fig. 9A. Results using this assay (NBD_{uncorr}) are compared in Table 2 with results from the TNBS assay for PC/PE vesicles. Agreement is found within S.D. for

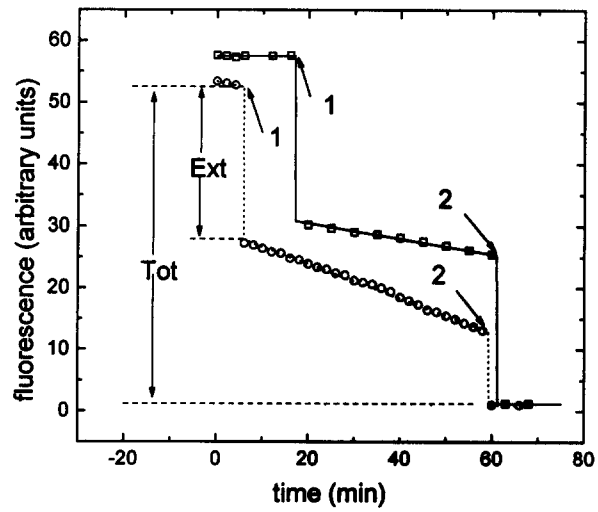

Fig. 8. Effects of bleaching, of dithionite addition, and of Triton addition on the fluorescence signal of SBL-SUV with 0.25% NBD-DMPE label density. Scans were started after mixing 2 ml of Na buffer with 100 μl of 10 mg/ml lipid vesicles in the same buffer. The first drop (arrow 1) in intensity was produced by addition of 20 μl dithionite reagent and the second drop (to nearly zero, arrow 2) by 100 μl of 1.6% Triton (○, ·····). In a parallel experiment the cuvette was protected from illumination except for the moment of measurement (□, ———).

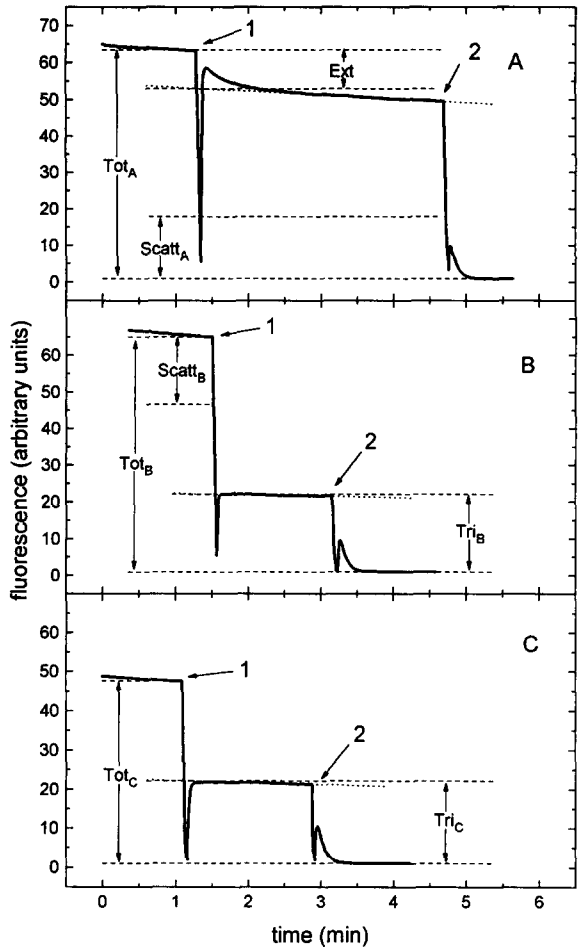

Fig. 9. Determination of the relative external surface of FATMLV from eggPC/DMPE/NBD-DPPE (95:5:0.033, w/w/w). (A) 1.9 ml of Na buffer and 100 μl of vesicles (20 mg/ml) were mixed before scanning, followed by 20 μl of dithionite reagent (arrow 1, spike due to cover opening) and by 100 μl of 20% Triton (arrow 2). (B) Same experiment as in A but Triton (arrow 1) was added before dithionite (arrow 2). (C) Same experiment as in B but the 20 mg/ml vesicle suspension was bath-sonicated for 10 min under argon before use.

vesicles prepared by extrusion (LUVET); the value of about 41% indicates that in this preparation not all vesicles had been unilamellar. Agreement for vesicles prepared by freeze-thaw cycles (FATMLV) was not within S.D. With decreasing NBD-PE content deviation from TNBS data increased as shown in the lower part of Table 2. This difference could be shown to originate from light scattering when analyzing multilamellar vesicles by the NBD assay. Determination of the contribution of scattering to the measured signal (*Scatt* in Fig. 9) required two further experiments shown in parts B and C of Fig. 9. Both experiments follow the same protocol as in Fig. 9A except that Triton was added first, followed by dithionite addition. In experiment B the same vesicles were used as in A whereas in experiment C these vesicles had been sonicated to virtually abolish scattering, leaving NBD-PE content, however, constant. The ratio of signals after Triton application (Tri_B/Tri_C) is known to be equal to the ratio of NBD-PE contents [19,20] which we confirmed for our conditions (not shown). The scattering contribution ($Scatt_B$) is then obtained from the difference of signals Tot_B and Tot_C, normalized to Tri_B and Tri_C, respectively, i.e., $Scatt_B/Tri_B = (Tot_B/Tri_B) - (Tot_C/Tri_C)$. In A and B of Fig. 9 the same vesicles were used so that $Scatt_A = Scatt_B$ (Tot_A/Tot_B). External surface values, corrected for scattering, are thus calculated from

$$E = Ext/(Tot_A - Scatt_A)$$
$$= (Ext/Tot_A)\cdot(Tot_B/Tot_C)\cdot(Tri_C/Tri_B)$$

The last column in Table 2 shows scattering-corrected data (see NBD_{corr}). They now agree within S.D. with the TNBS data, down to a weight ratio NBD-PE/lipid of 3×10^{-4}, which is equivalent to only 30 NBD-PE molecules per vesicle of 100 nm diameter. This corresponds to the conditions in Fig. 9. At the lowest NBD-PE concentration used (0.01%), where analysis starts to fail, NBD fluorescence and light scattering by multilamellar vesicles (FATMLV) reached equal values (see Fig. 10, squares). At the same time the basic assumption of the scattering correction procedure is no longer valid, i.e., scattering by SUV becomes non-negligible (Fig. 10, circles). At conditions of Fig. 9 (0.033% NBD-PE), the apparent limit of applicability of the NBD assay (at least to multilamellar vesicles),

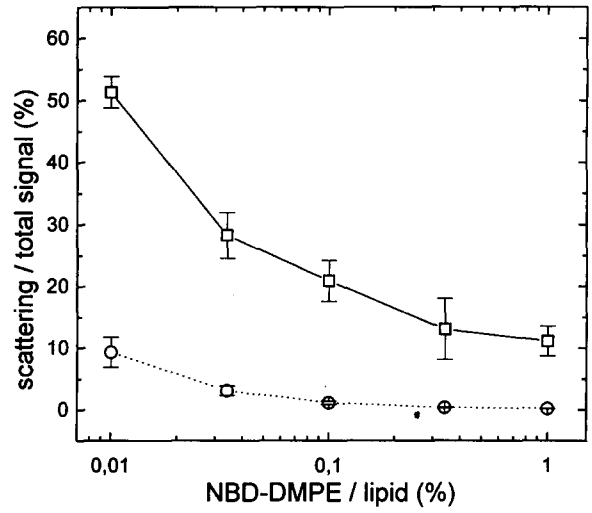

Fig. 10. The increase of the contribution of light scattering to the total fluorescence signal by NBD-PE-labeled vesicles at decreasing percentages of NBD-PE. The relative contribution of scattering by FATMLV from eggPC/DMPE (□, ——) was determined in the course of the external surface determination by the corrected NBD method (compare the lowest five entries in Table 2). Values for SUV from eggPC/DMPE (○, ·····) could only be determined by use of unlabeled vesicles.

light scattering contributed by about 30% to the total signal. Even at 1% NBD-PE scattering cannot be ignored when studying MLV. For the uncorrected external surface value found at 0.5% NBD-PE (see Table 2) the data in Fig. 10 predict a correction by +2.0% which now gives match with the TNBS value within S.D.

3.4. Limitations found with the ^{31}P-NMR method

The standard method of quenching the ^{31}P-NMR signal of external lipids by Mn^{2+} is regarded to be the most general and the least invasive of all external surface assays since any phospholipid can serve as marker lipid [7]. Yet to our knowledge it has only been demonstrated to work for LUVET and FATMLV formed from eggPC and from PC/PS mixture (see Table 1). In this study the ^{31}P-NMR method was applied to FATMLV from SBL (formed in the same way as usual) and it was observed that the external 'quencher' Mn^{2+} was able to enter the vesicles with a half time that was shorter than the time required to collect a single spectrum. Thus, not even an extrapolation of signal intensity to time zero was possible, in contrast to all other successful methods described above. Cooling to 4°C might well have solved the problem but for practical reasons we did not pursue this option.

4. Discussion

The goal of this study was to analyze the reliability of external surface values obtained from different methods which utilize different marker lipids and different external reagents. Reliability was tested by pairwise comparison of the EPR and the TNBS assay, as well as of the TNBS and the NBD assay where vesicles contained both marker lipids required. Agreement of results within S.D. was consistently found for vesicles at both extremes of lamellarity, each at two representative but quite different lipid compositions and at various degrees of prelabeling with different marker lipids (Table 2). From this match we conclude that *each* of the three refined methods can safely be used *alone* to determine reliable external surface values. As the main result, there is now at least one external surface assay available for any type of lipid composition (see Table 1), when including the well-established periodate method and the ^{31}P-NMR method with the limitations reported above.

The strategy of testing accuracy by direct comparison of different methods necessarily led to improvements on each technique until each gave satisfactory results. The main line of improvement made in all three methods was (i) by unequivocal separation of fast reaction of outer marker lipids from slower reaction of inner marker lipids, and (ii) by more reliable ways to find the 100% value for reaction of all marker lipids. These improvements conform with fulfillment of the four criteria for any correct external surface assay (see Introduction).

The basic criterion of random marker lipid distribution between outer and inner membrane surfaces was obviously fulfilled in all three methods tested in this study (Table 2). The excellent agreement between different methods under widely differing conditions practically excludes the possibility that marker lipids may have partitioned in a non-random way between outer and inner vesicle surfaces since this would imply the same non-random distribution for the three different marker lipids at all conditions studied. Non-random partitioning of marker lipids, however, was quite reguarly found with any of the three methods for highly curved small vesicles (SUV) as resulting from sonication (data not included).

The second and third criteria addressed the nature of slow phases observed, either due to permeation of the external reagent into the vesicles or due to marker lipid redistribution during reaction. The latter effect appeared to dominate the slow phase in the EPR assay since the slow phase developed exponentially, as expected for marker lipid redistribution, rather than linear, as expected for reagent influx at the high excess of reagent used. In contrast, the linear slow phase found for the other two assays, i.e., TNBS and NBD assay, indicated that reagent influx dominated the slow phase. The periodate assay (according to references in Table 1) apparently fulfills these two criteria in a perfect way, judged from the published data. At this point we want to recommend the modification introduced by Hope et al. [21], in which bisulfite was replaced by arsenite, being more safe in experimenting, since one of us was injured by SO_2 evaporating from bisulfite-containing samples, in spite of working in a good hood. The ^{31}P-NMR assay revealed reagent influx which was uncontrollably fast, at least at room temperature and for the SBL vesicles studied. The fast Mn^{2+} influx observed was propably catalyzed by the high PA content of SBL, in analogy to Ca^{2+} transportation by PA [22]. The observed fast Mn^{2+} influx seems to be specifically associated with PA, and not with anionic lipids in general, since in the case of PS the ^{31}P-method was quite successful [23]. ^{1}H-NMR [24] instead of ^{31}P-NMR would be sufficiently sensitive, and thus fast enough to cope with the observed Mn^{2+} influx but the requirement for $^{2}H_2O$ appears unacceptable for practical applications.

With respect to the second and third criteria all of the presented, and cited (Table 1) methods are well

applicable to sealed compartments of native biomembranes because neither the marker lipids (see McIntyre and Sleight [13] for NBD-PE) nor the hydrophilic reagents can cross biomembranes to a significant extent on the time scale of the experiment. TNBS has routinely been used to assess the transbilayer distribution of aminolipids (Higgins [25]) and both dithionite (McIntyre and Sleight [13]) and periodate (Gahmberg et al. [26]) have been shown to be nearly impermeant. We have already applied the EPR method presented here to study the transbilayer distribution of CAT-16 in sealed erythrocyte ghosts (unpublished results): It took 2 h at 37°C to equilibrate CAT-16 across the biomembrane and reduction of all outer CAT-16 molecules was complete within < 1 min while reduction of inner CAT-16 by penetrating ascorbate was more than one order of magnitude slower. Thus, from the methodical point of view all described assays are applicable to biomembranes, as well, but the accessibility of marker lipids will have to be interpreted in terms of leakiness and/or transbilayer asymmetry, rather than in terms of lamellarity.

As to the fourth criterion of obtaining a reliable reference value (100% reaction of marker lipid), we found it best to resort in all three assays to the determination of the 100% value in parallel experiments. Similar strategies were used for proper baseline determination (0% reaction of marker lipid). This improved considerably the accuracy and precision of the data. During our attempts to fulfill the fourth criterion we found in particular that the reliability range of the NBD assay could be extended to marker lipid densities as low as 1 marker lipid per 3000 lipids.

The measurement of external surface may be viewed as an alternative, or as a complementation to EM, depending on the type of application and the desired information. EM provides a direct view at vesicle morphology and vesicle heterogeneity but (i) negative stain leads to many artifacts [7] and only 'nice' areas on the grid are selected for evaluation, (ii) freeze fracturing does not yield unequivocal quantitative information on lamellarity (Mayer et al. [27]) and (iii) cryo-EM is usually not available on a daily basis.

External surface assays, on the other hand, have the advantages of being less invasive and easily available. Furthermore these assays are much faster and simpler than EM if a representative average parameter is desired by which different vesicle samples are to be characterized. For these reasons external surface assays will be very helpful when optimizing vesicle formation protocols or monitoring vesicle integrity.

This work was meant to provide clear-cut protocols for external surface assays for practical use (please note that the experiments shown were at worst case conditions). They cover a wide range of applications at quite acceptable accuracy and precision.

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