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Measurement of intravesicular volumes by salt entrapment

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Abstract

Internal volume is a very sensitive parameter of vesicle morphology. Measurement of captured volumes by solute entrapment is legitimate for most types of vesicles (Perkin, W.R. et al. (1993) Chem. Phys. Lipids 64, 197–217). In this study chloride was selected as the most convenient marker ion because the ubiquity of Cl^- in physiological buffers eliminates prelabeling with exogeneous markers and because minute concentrations of trapped chloride are well detectable in the presence of large extravesicular nitrate concentrations. Perfect exchange of external chloride for nitrate was shown to be accomplished by gel filtration, dialysis, or sucrose gradient flotation – but only after significant technical improvements and/or elimination of experimental pitfalls. Reliability was cross-checked by simultaneous entrapment of Cl^- and K^+. Diafiltration and ion exchange chromatography appeared inapplicable for exchange of extravesicular salt. When a representative variety of vesicle preparations was analyzed for internal volume (as well as for external surface and size) unexpected features of vesicle morphology were discovered. This emphasizes the genuine role of macroscopic vesicle characterization in complementing information from electron microscopy.

Keywords: Captured volume; Lipid vesicle; Vesicle morphology; Chloride electrode

1. Introduction

Liposome morphology can be characterized by direct visualization in the electron microscope (Mui et al., 1993) or by measurement of macroscopic parameters, such as external surface (Gruber and Schindler, 1994; Thurnhofer et al., 1989), size distribution (Lesieur et al., 1993; Ostrowsky, 1993; Reynolds et al., 1983), and internal volume (Perkin et al., 1993). Large captured volumes are desired in numerous medicinal applications (Ahl et al., 1992) or when studying the functions of transport proteins (Kasahara and Hinkle, 1977).

As reviewed recently (Perkin et al., 1993), the internal volume can be assayed for by solute exclusion ('VIVO', meaning V_{in} by V_{out}), by solvent distribution plus solute exclusion, or by solute entrapment. The first two strategies involve the use of radioisotopes or of EPR instrumentation, and pelleting of vesicles is frequently required to maximize the low sensitivity of these methods. No such restrictions are encountered with solute entrapment but admittedly it rests on the assumption that marker ion concentrations inside the vesicles reflect those of the buffer used for vesicle preparation. This assumption is known to fail in a few cases but to be applicable to the majority of vesicle types (Gruner et al., 1985; Perkin et al., 1988; Perkin et al., 1993).

Cl^- appears to be the ideal marker ion for the internal volume because its ubiquity in physiological buffers eliminates the need for special prelabeling. Chloride has already been used for this purpose (Mimms et al., 1981) but no evidence for general applicability has been given, and our initial attempts to use existing methods were not at all satisfying, especially in the case of heterogeneous or large vesicles.

Abbreviations: BBVES, vesicles from Bio-Bead treatment of detergent/lipid mixture; CHAPS, (3-((3-cholamidopropyl)dimethylammonio)-1-propanesulfonate; DMPE, 1,2-dimyristoylphosphatidylethanolamine; eggPC, egg yolk phosphatidylcholine; FATMLV, multilamellar vesicles produced by freeze-and-thaw cycles; FTSVES, vesicles produced by freezing, thawing, and sonication; HF bundle, hollow fibre bundle; HFD, hollow fibre dialysis; LUV, large unilamellar vesicles; OLV, oligolamellar vesicles; PEG, poly(ethylene glycol) with $M_r \sim 5000$; SBL, soy bean lipids; SUV, sonicated unilamellar vesicles; TLD, thin-layer dialysis; TNBS, 2,4,6-trinitrobenzenesulfonic acid; VET, vesicles produced by extrusion technique.

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How can the internal volume of vesicles be measured correctly via salt entrapment? First, a procedure for exchange of extravesicular salt ('washing of vesicles') is needed which fulfills the following criteria: (i) external marker ion concentrations typically have to be lowered by a factor of $> 10^4$ in order to enable measurement of trapped ions; (ii) marker ion escape from vesicle interior must be slow on the time scale of external salt exchange; (iii) no loss (or at least no size-selective loss) of lipid vesicles should occur during salt exchange; (iv) vesicle integrity must be preserved. Moreover, (v) the method used for marker ion detection must be sensitive and selective enough to measure micromolar concentrations of marker ion in the presence of several 100 mM replacement ion; (vi) marker ion detection must be insensitive to the presence of lipid, as well as to the detergent which is used to release the marker ions from washed vesicles; and (vii) the lipid concentration in the washed vesicles must be determined correctly, with acceptable efforts and minimal hazards.

In this study all common methods for salt exchange were tested along these criteria. The best results in terms of reliability and general applicability were obtained with rapid dialysis. Subsequently, vesicle morphology could be characterized for a large variety of vesicle types by simultaneous measurement of internal volume, size, and lamellarity, yielding distinct information about correlation between lipid charge and vesicle morphology. Captured volume data were also found useful for the monitoring of vesicle integrity during potentially harmful treatment, such as pelleting or osmotic stress.

2. Materials and methods

2.1. Materials

EggPC and SBL were prepared chromatographically as described previously (Gruber and Schindler, 1994). DMPE, as well as methoxypolyoxyethylene amine (Sigma M-4147, $M_r \sim 5000$ Da), fluorescamine, Triton X-100, TNBS (5% solution), chlorhexidine gluconate (20% solution), dialysis tubing with 10 mm flat width (D-9277), Dowex 50X8 (50–100 mesh), and blue latex beads (55 nm mean diameter) were from Sigma. All color-less latex beads, as well as Dowex 1X8 (50–100 mesh), dialysis tubing with 25 mm flat width (Visking No. 44110), and CHAPS were obtained from Serva. Solvents, SDS, and NaCl were p.a. grade and purchased from Riedel de Haen. All other aqueous buffer components (p.a. grade) were obtained from Merck. Bio-Beads SM-2 and prepacked Econo Pac 10DG desalting columns (filled with Bio-Gel P6) were obtained from Bio-Rad. Prepacked PD-10 desalting columns (filled with Sephadex G-25 M), as well as Sephacryl S-1000, Sepharose CL-6B, and CNBr-activated Sepharose 4B were purchased from Pharmacia. A test sample of NHS-activated Sepharose 4 Fast Flow (Code No. 17-0906-02) was the kind gift of Dr. Bengt Törnblad from Pharmacia, Sweden.

2.2. Buffers

Usually vesicles were prepared in buffer A (100 mM NaCl, 10 mM Hepes, pH 7.4 adjusted with NaOH). For exchange of extravesicular salt buffer B was used (110 mM $NaNO_3$, 5 mM Hepes, pH 7.4 adjusted with NaOH). In this buffer 0.002% chlorhexidine gluconate was included for short term preservation. In dim light and at 3–4°C this preserving agent was completely stable for up to 1 week, whereas at room temperature release of chloride was detectable after 1 day, and after 1 week under daylight the preservation effect was completely lost, as judged from destruction of Sephacryl S-1000 gel by microbial growth. Buffer C was 3-fold more concentrated in all components than buffer B, except that the chlorhexidine concentration was also 0.002%. If the intravesicular volume was to be assayed both by K^+- and by Cl^- entrapment, vesicles were prepared in buffer D (100 mM KCl, 5 mM Hepes, pH 7.4 adjusted with KOH). Buffer E (100 mM NaCl, 5 mM NaN_3, 10 mM Tris, pH 7.4 adjusted with HCl) was used for the Sephacryl S-1000 column, and for long term storage of dialysis equipment, of Bio-Beads SM-2, and of desalting columns.

2.3. Formation of defined vesicles

Final lipid concentrations were always close to 10 mg/ml unless stated otherwise. FATMLV and VET were prepared according to published procedures (Mayer et al., 1985; Mayer et al., 1986), with slight modification: VET_{800} and VET_{400} were prepared from FATMLV by the usual 8 extrusions through a double layer of 0.8 or 0.4 μm Nuclepore membranes, respectively: For VET_{200} and VET_{100}, however, FATMLV were first passed through a single layer of a 0.2 or 0.1 μm membrane, respectively, before performing 8 passages through double layers of Nuclepore membranes (Gruber and Schindler, 1994). VET_{50} were prepared from VET_{100} by 8 passages through stacked 0.05 μm membranes and, in turn, VET_{30} were prepared from VET_{50}. OLV were prepared by a single passage of FATMLV through a double layer of 0.8 μm membranes.

FTSVES were prepared by a modification (Gruber et al., 1994) of the classic procedure (Kasahara and Hinkle, 1977) which takes into account that fusion of SUV is enhanced by K^+ and suppressed by Na^+ (MacDonald and MacDonald, 1983). 3 ml of water with 40 mg/ml SBL were sonicated for 5 min under argon in a bath type sonicator, resulting in a completely clear SUV suspension (salt was omitted to ease sonication and to enhance vesicle fusion in the subsequent step). To this, 3 ml of 600 mM KCl, 15 mM Hepes, pH 7.4 adjusted with KOH, were added, and after mixing the suspension was frozen in

liquid N_2 and thawed in a 20°C water bath. The vesicles were transferred to a thin-walled test tube that had never been subjected to freezing before (to avoid rupture during sonication) and sonicated under argon for 15 s in the focus of a bath type sonicator. Subsequently vesicles were diluted by dropwise addition of 2 volumes of water under stirring.

BBVES were prepared by Bio-Bead treatment of detergent/lipid mixtures at 3–4°C. Bio-Beads SM-2 were pretreated as described (Holloway, 1973) and stored in buffer E. Before use, 8 ml portions of packed bead volumes were drained by aspiration with the blue tip of a digital pipette, the end of which had been squeezed flat to prevent entering of Bio-Beads and the beads were washed by 3 cycles of resuspension in buffer A and subsequent drainage. After the second resuspension the beads were degassed by evacuation. To 8 ml of these pre-equilibrated, freshly drained beads 10 ml of detergent/lipid mixture were added and rotated slowly for 1 h. The supernatant was removed with a digital pipette (as above) and transferred to another 8 ml of Bio-Beads, and after 1 h rotation a third cycle was performed in the same way. For the formation of SBL vesicles, 150 mg of CHAPS were first dissolved in 1.87 ml of water containing 40 mg/ml SBL, and only after clarification 0.333 ml 3 M NaCl and 1.0 ml 100 mM Hepes (pH 7.4 adjusted with NaOH) were added and complemented with water to give 10 ml of detergent/lipid mixture which was cooled on ice for at least 30 min before Bio-Bead treatment. For the formation of eggPC vesicles, 1.0 ml of water containing 100 mg/ml eggPC were used in place of the aqueous SBL stock solution, all other details were the same.

2.4. Exchange of extravesicular salt

In a typical gel filtration experiment 0.5 ml of vesicles were loaded on a 1.5 cm × 10.5 cm column and eluted at 1.5 ml/min with buffer B. Prepacked columns were also loaded with 0.5 ml of vesicles and eluted at gravity flow. PEG-Sepharose 4B was produced by coupling methoxypolyoxyethylene amine to CNBr-activated Sepharose 4B according to the manufacturer's instructions. 5 g dry gel were swollen (16 ml bed volume) and reacted with 1 g of PEG derivative, 0.4 g of which were irreversibly bound to the gel as determined by the fluorescamine method (see below). PEG Sepharose 4 Fast Flow was prepared in an analogous way from NHS-activated Sepharose 4 Fast Flow (25 ml bed volume in isopropanol). 0.44 g out of 2 g methoxypolyoxyethylene amine were irreversibly coupled and the final bed volume was 36 ml in aqueous buffer.

For diafiltration, Immersible CX-10 concentrator fingers (Millipore) were mounted on the corresponding vibration unit (Millipore, this device created the tangential flow) and immersed into mixtures prepared from 0.5 ml of vesicles plus 4.5 ml of buffer B in a centrifuge tube which just fitted the concentrator finger (Kontron TST 28.17, thick wall). During each cycle the vesicles were concentrated down to 0.5 ml total volume by applying aspirator vacuum to the concentrator finger, and the original suspension volume of 5.0 ml was restored by the addition of buffer B.

For sack dialysis, dialysis sacks with 10 cm length were formed from dialysis tubing (10 mm flat width) by knot tying, and loaded with 0.7 ml of vesicles each. Filled sacks were mounted on a home-built multiple dialyzer and dialyzed against 250 ml of buffer B. At indicated time intervals one of the sacks was cut from the multiple dialyzer with a clean blade, sack contents were harvested by rinsing with additional buffer, resulting in 3–5 ml of dialyzed vesicles. The multiple dialyzer with the remaining dialysis sacks was placed into fresh buffer B after each removal of one sack.

For hollow fibre dialysis, a HF bundle with 2 ml sample volume was primed according to instructions and stored in buffer E. Storage and all operations were at 3–4°C. Before each use the HF bundle was equilibrated by careful injections of air and buffer B, and the outside was rinsed with buffer B from a squeeze bottle. This cleaning cycle was repeated three more times. 12 ml of VET_{100} (prepared from eggPC in buffer D) were loaded and recycled at a rate of 8 ml/min, and after rinsing the outside of the HF bundle with buffer B the HF bundle was immersed in 450 ml of buffer B at time zero. Both the outer buffer and the vesicle suspension were stirred continuously. At 5, 10, 15, 20, and 25 min the HF bundle was removed from the old dialysis buffer, rinsed with buffer B on the outside, and placed in a new 450 ml portion of buffer B. The first of the used buffers was discarded, the others were re-used in a subsequent experiment in proper order, and the last portion of buffer B was always fresh. The recirculation rate in the HF bundle was reduced to 4 ml/min after 2 min, and to 1–2 ml/min if dialysis times were > 1 h. Flow-induced backpressure in the HF bundle resulted in volume loss of the vesicle suspension which was measured by using a measuring cylinder as vesicle reservoir. The volume losses were compensated for by frequent additions of buffer B while the volumes of the 1 ml aliquots which were removed at 4, 9, 14,....59 min were not compensated for by buffer additions. In this way the lipid concentration within the HF bundle remained approximately constant.

Thin layer dialysis was performed in close analogy to the HFD method (also at 3–4°C). The TLD setup was constructed by inserting a tightly fitting teflon rod (18 mm diameter) into prewashed dialysis tubing (25 mm flat width in dry form) and sealing of both ends with O-rings. Vesicles were perfused through the narrow space between teflon rod and dialysis tubing via bore holes on both ends of the rod (flow rate 8 ml/min) and turbulent mixing was achieved by a thread which had been cut into the surface of the teflon rod. The area of dialysis was 160 cm^2, and the dead volumes of the TLD dialyzer and of external tubing were 4 ml and 1 ml, respectively. This rod dialyzer

was immersed in an open chromatography column filled with 130 ml of buffer B (nitrate) which was also recirculated (≤ 200 ml/min). The external buffer B was exchanged batchwise every 5 min up to a total dialysis time of 0.5 h, every 10 min up to 1.5 h, and every 20 min up to 4 h. During dialysis, vesicles were also recirculated through an open reservoir (~ 1 ml filling volume). At indicated time intervals a vesicle sample corresponding to about 0.4 mg of lipid was withdrawn, and the reservoir was replenished with buffer B. (Correspondence about further details of TLD is welcome.)

2.5. Determination of vesicle lamellarity by external surface labeling with TNBS

Trinitrophenylation of aminolipids with TNBS results in a 6.4-fold absorbance increase (Gruber and Schindler, 1994). Due to the low membrane permeance of TNBS, the absorbance increase in the absence and in the presence of detergent can be related to the extravesicular and to the total contents of aminolipids, respectively (Barenholz et al., 1977). In a previous study the conventional method had been corrected by explicitly separating the fast kinetics of external aminolipid labeling from the relatively slow TNBS influx into the vesicles (Gruber and Schindler, 1994; see there for details).

2.6. Determination of lipid concentration

Lipid concentrations were routinely measured in quadruplicates by a published extraction assay (Stewart, 1980). Modifications were introduced to reduce chloroform consumption and to minimize efforts for reproducibility of the data. 20–200 μl of vesicle suspension containing < 100 μg of any type of lipid were carefully pipetted (and rinsed) into 1 ml portions of $Fe(SCN)_3$ reagent (prepared by dissolving 27.03 g $FeCl_3$ and 30.4 g NH_4SCN in water, final volume 1 l) in glass test tubes. 2 ml portions of chloroform were added from a reproducible dispenser. Quantitative extraction of the colored lipid-$Fe(SCN)_3$ complex into chloroform was best achieved by 5 cycles with 2 s of vortexing and 2 s of waiting, always processing two test tubes intermittingly at a time. After 5 min centrifugation at $1000 \times g_{max}$ the aqueous top layer was removed by aspiration with a Pasteur pipette, with circular motion along the wall of the test tube. The chloroform layer was transferred into a UV-VIS cuvette with a Pasteur pipette, and A_{488} was read against a chloroform blank. Calibration was by use of standard samples containing the same lipid mixture because different types of phospholipids bind to Fe^{3+} with different stoichiometry (Stewart, 1980).

For the determination of lipid concentrations in chromatographic fractions from the Sephacryl S-1000 column a much less time-consuming method had to be used. Taking advantage of the aminolipid contents of SBL and of the eggPC/DMPE mixture a fluorescamine assay was devised on the basis of a similar peptide/protein assay (Udenfriend et al., 1972). The dose response curves with the largest linear range were achieved if 0.9 ml of aqueous samples containing < 10 μg/ml SBL (or < 50 μg/ml eggPC/DMPE = 95:5) were mixed with 0.1 ml portions of borate buffer (200 mM H_3BO_3, pH 9.0 adjusted with NaOH, containing 4% of Triton X-100), and 25 μl portions of DMSO containing 10 mM fluorescamine were added from an Eppendorf Multipette while vortexing. Within 2 min fluorescence was determined (excitation at 390 nm, emission at 475 nm, slits 5 nm on both sides). The fluorescamine assay was used only to immediately compare chromatographic fractions with the stock solution from which the column was loaded because NH_2 group oxidation was well detectable on a timescale of hours. The absolute lipid concentrations in vesicle stocks were determined by the extraction assay (see above).

After salt exchange by gel filtration, by HFD and TLD, and by flotation, lipid concentrations were measured with the Menagent Phospholipids assay (Menarini Diagnostics, Florence, Italy) which is based on the Trinder method (Trinder, 1969) and quantitates choline groups after enzymatic release. 50 μl of vesicle suspension containing < 1 mg/ml of eggPC (or < 3 mg/ml of SBL) were pipetted and rinsed into 500 μl of commercial enzyme reagent, samples were incubated at 37°C for 15 min, cooled to 20°C, diluted with 750 μl of water, and A_{500} was read against a water blank. Eppendorf Multipettes were used for pipetting of reagent and water addition. The strategy for calibration was the same as for the Stewart method.

2.7. Definition and calculation of intravesicular volumes

The basic assumption was that the ion concentrations inside the liposomes were the same as in the buffer used for their preparation. After exchange of extravesicular Cl^- (and K^+) for NO_3^- (and Na^+) the intravesicular ions were released by the addition of Triton X-100 and the concentration of Cl^- (or K^+) was measured with an ion-selective electrode. The so-called absolute internal volume (AIV, in μl trapped volume per ml of vesicle suspension) was calculated as AIV = (1000 μl/ml) · [Cl^- in nitrate-washed vesicles]/[Cl^- in vesicle buffer]. It is important to note that [Cl^- in nitrate-washed vesicles] was always corrected by subtraction of residual [Cl^-] in the last batch of dialysis buffer. After [lipid] determination for an aliquot of the nitrate-washed vesicles the so-called relative internal volume (RIV, in μl of trapped volume per mg of lipid material) was obtained as RIV = AIV/[lipid in mg/ml]. All 'internal volumes' reported in Section 3 are given in terms of relative internal volumes. 'Apparent internal volumes' are calculated under the assumption that all Cl^- (and K^+) were trapped inside the vesicles even though exchange of Cl^- for NO_3^- (and of K^+ for Na^+) may still have been incomplete in reality.

3. Results

In this study Cl^- (and occasionally K^+) was used as the marker ion for the intravesicular volume. Because of its ubiquity in physiological salt solutions, chloride ion was the most preferable candidate. Fortunately, chloride also proved to be the most suitable physiological ion for this purpose, due to the unsurpassed selectivity and stability of chloride-selective electrodes. Even at 100 mM or 300 mM NO_3^-, chloride concentrations down to a few micromolar could be measured (Fig. 1, solid and dotted line, respectively), and the Cl^- electrode was insensitive to the presence of lipid and of Triton X-100 (used for release of intravesicular Cl^-). Selectivity and sensitivity of the K^+ electrode for K^+ was comparable to that of the Cl^- electrode when Na^+ was used as replacement ion for K^+. However, the K^+ electrode slightly 'remembered' whether the previous samples had been high or low in K^+, and therefore the measurement of a new unknown sample with a dramatically different $[K^+]$ than before had to be preceded by measurement of several calibration standards with $[K^+]$ near the expected new value. Thus, in principle the K^+ electrode is equally suited as the Cl^- electrode but in practice the Cl^- electrode is far more convenient. The most important message from Fig. 1 is that with a suitable protocol (see legend to Fig. 1) the Cl^- electrode allows to detect trapped chloride even from small vesicles and even at lipid concentrations as low as a few mg/ml (exemplified in Fig. 3).

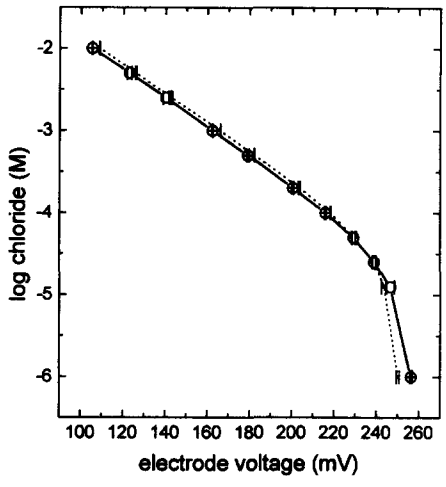

Fig. 1. Detection of Cl^- by a Cl^--selective electrode. A Cl^- electrode (Orion 9417SC) with a separate reference electrode (Orion D/junct.ref.) was used. Standard solutions with the indicated Cl^- contents were prepared by diluting buffer A with either buffer B (110 mM $NaNO_3$ besides Hepes, circles, —) or buffer C (330 mM $NaNO_3$, · · ·, standard deviations only, without symbols). To 800 μl samples 50 μl portions of 20% Triton X-100 (w/w in water) were added from an Eppendorf Multipette before the mixed samples were transferred to plastic trays (Orion) suitable for micro-scale assay. A similar calibration curve was obtained for the K^+ electrode (Orion 9319BN) for standards prepared from buffer D and buffer B.

Table 1
Recoveries of FATMLV after gel filtration on different chromatographic gel media

Gel type	eggPC vesicles	SBL vesicles
Sephadex G-25 M	66% (74%)	14% [a]
(PD-10 column)	70% (73%)	9%
	71% (75%)	9%
Bio-Gel P6	66% (71%)	–
(desalting column)	64% (64%)	–
Sepharose CL-6B	93% (91%)	49% (82%) [b]
	95% (85%)	–
	90% (82%)	–
PEG-Sepharose 4B	81% (65%)	–
	82% (61%)	–
	75% (57%)	–
PEG-Sepharose 4	79% (53%)	93% (87%)
Fast Flow [c]	–	92% (86%)
	92% (92%) [e]	92% (93%) [d]

Lipid recoveries were determined by the enzymatic method or by turbidity (values in parentheses). Accuracy was within 1% in the former and within 4% in the latter method, respectively. Multiple numbers indicate consecutive experiments on the identical gel filtration column.

[a] Before these three elutions of SBL-FATMLV, six consecutive gel filtration experiments had been performed with SBL-VET_{100}, the recoveries being 74, 70, 70, 60, 58, and 58%, respectively. During the three column runs with FATMLV the gravity flow rate was reduced by an order of magnitude. Resolution between the lipid and the salt peak, however, was fully retained. After the last column cycle the adsorbed lipid was instantaneously desorbed from the gel by washing with distilled water.

[b] Only half of the recovered lipid eluted as a sharp void peak, the rest of the recovered lipid eluted as a broad peak near the included volume.

[c] The order of column runs on the new column was: SBL-FATMLV, eggPC-FATMLV, SBL-VET_{200}, and eggPC-VET_{200}.

[d] These vesicles were VET_{200} and not FATMLV.

In the following, we tested common methods of salt exchange for their applicability to lipid vesicles: gel filtration, ion exchange chromatography, ultrafiltration, different types of dialysis, and centrifugation.

3.1. Exchange of extravesicular salt by gel filtration

Gel filtration has been the most widely used method for exchange of extravesicular solute (e.g., Brunner et al., 1980; Mimms et al., 1981; Penefsky, 1979) but to our knowledge lipid recoveries have never been reported, except for the 70% recovery of VET_{100} (eggPC/cholesterol) from spin columns filled with Bio-Gel A15m (Chonn et al., 1991). We also observed such moderately satisfying yields around 70% with all typical desalting gels when applying small vesicles with 100 nm diameter from any type of lipid (data not shown), and presaturation often aggravated, rather than alleviated the problem (see footnote a in Table 1).

Not unexpectedly, the recovery of large, heterogeneous

vesicles (FATMLV, see Table 1) was generally lower than of small vesicles, thus FATMLV seemed ideal to test different gel types under worst case conditions. Table 1 shows that unmodified Sepharose CL-6B is uniquely suited for washing of large eggPC vesicles while for large SBL vesicles (containing ~ 10% each of the anionic lipids phosphatidic acid and phosphatidylinositol) the replacement of normal desalting gels by Sepharose CL-6B only meant a step in the right direction but not a solution to the problem of insufficient lipid recovery. The perfect gel for large, partially anionic vesicles was created by covalent attachment of PEG chains (M_r ~ 5000) to NHS-activated Sepharose 4 Fast Flow (very coarse and rigid, like Sepharose CL-6B), as evidenced by lipid recoveries of > 90% (Table 1). The good agreement between the biochemically and the turbidimetrically determined lipid concentrations must be interpreted in the sense that vesicle adsorption to the gel media was not size-selective because otherwise the ratio of turbidity over lipid concentration should have changed (Table 1).

It is very important to note that PEG-coating of gels was even disadvantageous in the case of purely zwitterionic lipid vesicles (Table 1), thereby emphasizing the main message of Table 1 that there is a suitable gel for any type of lipid composition but appropriate tests are necessary in every specific case.

3.2. Test for salt exchange by ion exchange chromatography

In contrast to gel filtration media the cation exchange resin Dowex 50X8 is known to yield 100% lipid recovery (Garty et al., 1983; Talvenheimo et al., 1982). In this study we tested the anion exchange resin Dowex 1X8 (50–100 mesh, 9 ml bed volume for washing of 0.5 ml vesicle portions) and we found 95.6 ± 2.4% lipid recovery even for the partially anionic SBL vesicles. Exchange of extravesicular Cl^- for NO_3^- was also achieved but after every 1–2 column cycles the resin had to be regenerated by cumbersome elution with 100 ml 1 M $NaNO_3$ plus 20 ml buffer B in order to sufficiently lower the baseline value of Cl^-. Thus, the ion exchange resin is not suitable for practical use.

3.3. Test for exchange of external salt by diafiltration

The mildest, and most efficient version of diafiltration was chosen, i.e., where polarization on the ultrafiltration membrane is minimized by tangential flow. As seen from Fig. 2A (open diamonds) external salt was removed by about 3–4 cycles of concentration but with successive cycles no stable value of Cl^- entrapment was obtained. From the progressive loss of Cl^- it had to be concluded that the ultrafiltration process destroyed the vesicles and that diafiltration was unsuitable for the purpose of this study.

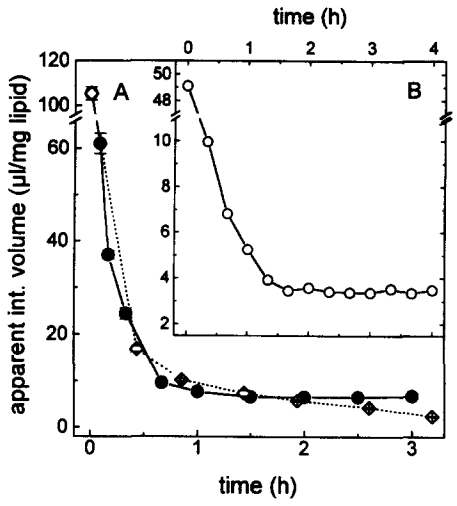

Fig. 2. Comparison of sack dialysis and tangential flow diafiltration with respect to external salt exchange. SBL-OLV (panel A, experiments at room temperature) eggPC-OLV (panel B, experiments at 2–4°C) were prepared in buffer A and outer chloride was exchanged for nitrate by repeated sack dialysis (solid circles in panel A for SBL, open circles in panel B for eggPC) or by diafiltration (open diamonds in panel A). As for diafiltration, successive data points on the time axis indicate increasing numbers of operating cycles to which parallel samples were subjected, the time indicates total operation time up to the moment of sample recovery. The dialyzed and the diafiltered vesicles were analyzed for lipid (by the extraction method, see Section 2) and for Cl^- concentrations (see legend to Fig. 1) in order to calculate the apparent trapped volumes (see Section 2).

3.4. Exchange of external salt by sack dialysis

A priori, sack dialysis offers two advantages: no loss (or at least no size-selective loss) of vesicles can occur, and second, vesicles are not exposed to large surfaces or other sources of stress, rendering sack dialysis the least invasive of all methods. Yet two questions were to be answered by proper tests: (i) whether sack dialysis was fast enough to exchange extravesicular Cl^- for NO_3^- before a significant extent of the intravesicular marker ions had been lost, and (ii) whether in practice dialysis was efficient enough to achieve low and reproducible background levels of residual extravesicular Cl^- which were small in comparison to the typical concentrations of trapped Cl^- (for instance, incomplete removal of extravesicular Cl^- was to be expected in 'blind regions' near knots of dialysis tubing or near tubing closures).

Requirement (i) appears to be fulfilled in the experiments shown in Fig. 2: After 2 h dialysis of SBL-OLV (solid circles in panel A) or eggPC-OLV (open circles in panel B) a stable level of chloride entrapment was obtained from which the internal volume was calculated.

As a control for requirement (ii), pure buffer A (containing chloride) was dialyzed against nitrate buffer (Fig. 3, open circles) and the results were compared with those for vesicles with a very small trapped volume (SUV, Fig. 3, solid squares) in order to assess the disturbance by

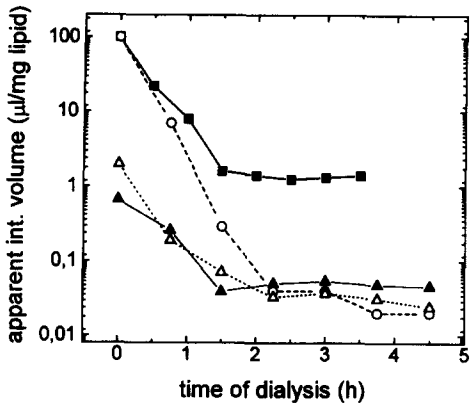

Fig. 3. Critical controls for the sack dialysis method. SUV with minimal trapped volume were prepared by hydration and bath sonication of SBL in buffer A, and dialysis against buffer B was monitored as described in Fig. 2, except that dialysis temperature was 3–4°C (solid squares, —). In order to control for possible background values of chloride-free vesicles, SUV from SBL (solid triangles, —) and from eggPC (open triangles, · · ·) were prepared in buffer B (nitrate) and analyzed in the same way. Finally, the limits of chloride removal by sack dialysis were tested by a series in which sacks with lipid-free buffer A (chloride) were subjected to dialysis (open circles, - - -).

background levels of Cl^- in the dialysis sack under worst case conditions. It can be seen that the error due to background levels of Cl^- was little over 1%, and the same applied to the residual 'chloride-like' contaminant of chloride-free vesicles after extensive dialysis (Fig. 3, solid and open triangles for SBL- and eggPC-SUV, respectively). The very same chloride-like contaminant was also well removed by gel filtration, in which case the residual intravesicular fraction of this contaminant was also negligible in comparison to normal trapped Cl^- (data not shown).

3.5. Rapid exchange of external K^+ and Cl^- for Na^+ and NO_3^-, respectively, by hollow fibre dialysis

In the sack dialysis experiments depicted in Figs. 2 and 3 the rates of intravesicular chloride escape were almost negligible for large vesicles from SBL or eggPC and for small SBL vesicles. However, on the basis of published permeability coefficients of eggPC membranes for Cl^- and K^+ (Hauser et al., 1973; Mimms et al., 1981) the escape rate of Cl^- (but not of the less permeant K^+ ion) from small eggPC vesicles was predicted to be uneasily close to the rate of salt exchange by sack dialysis. For rigorous analysis of this potential problem, eggPC vesicles with an unfavorable volume/surface ratio (VET_{100}, see Mui et al., 1993) were double-labeled with Cl^- and K^+, and rapid dialysis (HFD) was applied in order to accurately monitor escape of intravesicular marker ions. Fortunately, both K^+ and Cl^- efflux even from this type of eggPC vesicles was found to be much slower than extravesicular salt exchange by HFD (Fig. 4A and B), and quite obviously slow enough to allow for use of sack dialysis (and calculation of trapped volumes by extrapolation to time zero) in the absence of a HFD setup. The perfect agreement between Cl^- and K^+ entrapment for different vesicle preparations (Table 2) proved that chloride is a valid marker for captured volume.

From the slow decreases in K^+ and Cl^- entrapment the permeability coefficients of eggPC membranes for these ions could be calculated (see Table 2), taking into account geometric parameters of VET_{100} (see footnote b to Table 2). The Cl^- permeability coefficient is smaller than of corresponding SUV ($P_{Cl} = 6.5 \cdot 10^{-12}$ cm/s at 3°C, Hauser et al., 1973) or of comparable LUV produced by β-octyl glucoside dialysis ($P_{Cl} = 76 \cdot 10^{-12}$ cm/s at 24°C, Mimms et al., 1981). Apart from elevated temperature, the chloride permeability of the latter vesicles may additionally have been increased by residual detergent since in this example chloride permeability was about 10-fold higher than permeabilities for alkaline ions, whereas a ratio of only 2.3 was measured in this study for P_{Cl}/P_K.

3.6. Thin-layer dialysis as a substitute for hollow fibre dialysis

Unfortunately, the manufacturing of small scale HF bundles has been discontinued, and no equivalent product

Table 2
Simultaneous determination of Cl^- and K^+ entrapment by the HFD method

Lipid mixture	Measured parameter	By K^+ entrapment	By Cl^- entrapment
eggPC/DMPE	int. volume (µl/mg)	1.55 ± 0.02	1.57 ± 0.03
eggPC	int. volume (µl/mg)	1.30 ± 0.08	1.34 ± 0.04
eggPC [a]	int. volume (µl/mg)	1.5 ± 0.1	1.43 ± 0.02
	$\tau_{1/2}^{fast}$ (min)	3.2	3.1
	$\tau_{1/2}^{slow}$ (h)	129	55
	P (cm/s) [b]	$0.94 \cdot 10^{-12}$	$2.2 \cdot 10^{-12}$

VET_{100} with the indicated lipid composition were prepared in buffer D, subjected to HF dialysis against buffer B, and internal volumes were calculated from trapped K^+ ions or trapped Cl^- ions (see legend to Fig. 4).

[a] Data from HFD experiment in Fig. 7B.

[b] Permeability coefficients P of VET_{100} membranes for K^+ and Cl^- were calculated according to Fick's first law of diffusion from the relation $\tau_{1/2}^{slow} = (V/A)$ (ln $2/P$), the surface A ($31 \cdot 10^3$ nm^2) was assumed for a sphere with 100 nm diameter, the lipid volume ($131 \cdot 10^3$ nm^3) was assumed from an outer shell with 4.6 nm thickness (Hauser et al., 1973), and the inner volume V ($198 \cdot 10^3$ nm^3) was calculated from the experimentally determined trapped volume (1.5 µl/mg lipid) by assuming a partial volume of 1 µl/mg of lipid.

seems to be commercially available, as we learned towards the end of this study. We, therefore, developed a TLD setup (see Section 2) which also affords rapid exchange of extravesicular salt and allows to monitor the slow further decrease of marker ion entrapment in a continuous fashion (Fig. 4C). Thus, with a proper protocol (see Section 2), the TLD method can be used as a satisfactory substitute for HFD even though the high surface/volume ratio of hollow fibres cannot fully be reached by TLD.

3.7. Application of the HFD method to monitor vesicle integrity during centrifugation

Covalent or noncovalent binding of ligands (Tortorella et al., 1993), peptides (Murata et al., 1993), or proteins (Shen et al., 1982) to liposomes is frequently assayed by

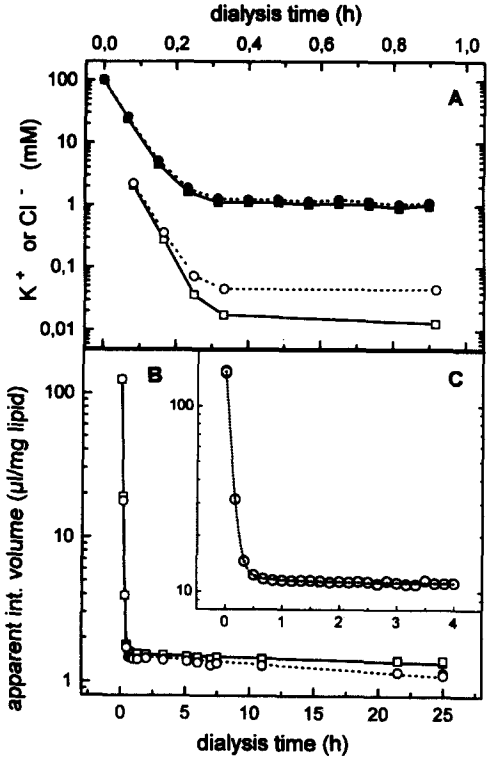

Fig. 4. Exchange of external salt by HFD and TLD. (A) Short term HFD of eggPC-VET_{100}. 12 ml of vesicles (prepared in buffer D) were dialyzed against buffer B. At indicated time intervals aliquots from dialyzed vesicles (solid symbols) as well as from the used dialysis buffers (open symbols) were analyzed for K^+ (squares, —) and Cl^- concentrations (circles, · · ·) by ion-selective electrodes (see Fig. 1). (B) Examination of long term permeability of eggPC vesicles for K^+ and Cl^- by HFD. 24 ml of VET_{100} (prepared in buffer D) were loaded and dialyzed against 450 ml portions of buffer B which were exchanged frequently enough to maintain a 10^2- to 10^3-fold concentration gradient of K^+ and Cl^- across the HFD membrane (explicitly controlled, data not shown). Lipid concentrations were determined enzymatically. Apparent internal volumes were calculated from entrapment of K^+ (squares, —) or Cl^- (circles, · · ·) as described under Section 2. (C) TLD as a substitute for HFD. 6 ml of eggPC-OLV (20 mg/ml) were processed in the TLD setup as described under Section 2 and apparent internal volumes were determined from Cl^- entrapment. The meaning of x and y is the same as in panel B.

Table 3
Persistence of vesicle morphology upon pelleting or flotation in sucrose gradients

Lipid	Treatment	External surface	Internal volume in µl/mg lipid
SBL	none	46.0%	2.5
	flotation	46.2%	2.5
	pelleting	46.3%	2.8 [a]
eggPC	none	–	2.0
	flotation	–	2.0
	pelleting	–	2.4 [a]

VET_{200} were prepared from SBL or eggPC in buffer A and either pelleted by ultracentrifugation (3 h, 95000× g_{max}), with subsequent resuspension in buffer A, or subjected to flotation in a sucrose gradient. For flotation, 18 ml of 20% sucrose (w/v, prepared by dilution of 80% w/v sucrose with buffer A) were placed in a 38 ml centrifuge tube and a mixture of 10 ml of VET_{200} with 4 ml of 80% sucrose (w/v, corresponding to 62% w/w, containing 100 mM NaCl, in addition) was layered underneath. Layers of 15, 10, and 5% sucrose were placed on top, and tubes were filled with buffer A. After 3 h centrifugation at 95000× g_{max} vesicle bands were harvested and resuspended in buffer A. External surface was measured by the TNBS assay and internal volume by the HFD method (see Fig. 4).

[a] The apparent increase in trapped volume is explained by the observation that the smallest vesicles could not be pelleted completely, as judged from slight residual turbidity of the supernatants.

pelleting or flotation of vesicles. In the context of another study in this lab, immunoliposomes (VET_{200}) were to be separated from non-immobilized antibodies (Haselgrübler et al., 1995). We therefore used measurements of external surface and internal volume to monitor liposome stability during sucrose gradient flotation or pelleting. Table 3 shows that vesicle morphology remained completely intact by both criteria, irrespective of lipid composition.

3.8. Exchange of extravesicular salt by sucrose gradient flotation

The observed integrity of vesicles in a sucrose gradient prompted an attempt to utilize flotation for exchange of external Cl^-. Sucrose gradients were formed with nitrate buffer and both large (FATMLV) and relatively small vesicles (VET_{200}) with different lipid compositions were 'washed' by flotation in these gradients. Trapped volumes obtained in this way correlated well with those obtained by the HFD method (Fig. 5). Thus, sucrose gradient flotation affords perfect exchange of extravesicular solutes except that vesicle flotation speed becomes inconveniently slow in case of $\ll$ 200 nm vesicle diameter.

3.9. Characterization of standard vesicle preparations by simultaneous determination of lamellarity and trapped volume

On the basis of the investigations presented above, several salt exchange procedures are now available for the reliable determination of intravesicular volumes. In a pre-

ceding study (Gruber and Schindler, 1994) reliability had been established for measurement of external surface (normalized to total membrane surface) of lipid vesicles. In the following, these two complementary features of vesicle morphology were combined in order to test for the usefulness of macroscopic vesicle characterization. For the sake of general interest, a wide variety of vesicle types with two different, representative lipid compositions was subjected to such analysis.

Results for two series of extruded vesicles are shown in Fig. 6. Vesicle morphology greatly differed for the two types of lipid mixture used: FATMLV from SBL (containing about 20% of anionic lipids, see above) had an externally accessible surface fraction of 34% (Fig. 6A, open circles), i.e., they were not very multilamellar. Surprisingly, extrusions caused little further increase in relative external surface (i.e., little decrease in the number of lamellae per vesicle), except when pore sizes as small as 100 nm were used. In parallel, internal volumes also showed little response to extrusion (Fig. 6B, open circles), except for the significantly reduced internal volumes of VET_{200} and VET_{100}. The apparent 'resistance' of SBL vesicles towards extrusion was also noticed in a very practical sense since much higher pressures were needed for extrusion of SBL vesicles as compared to corresponding vesicles from eggPC/DMPE and extrusion of SBL vesicles through 50 nm or 30 nm pore sizes was completely impossible for the same reason.

Unextruded vesicles from zwitterionic lipids (eggPC/DMPE = 95:5), on the other hand, were very

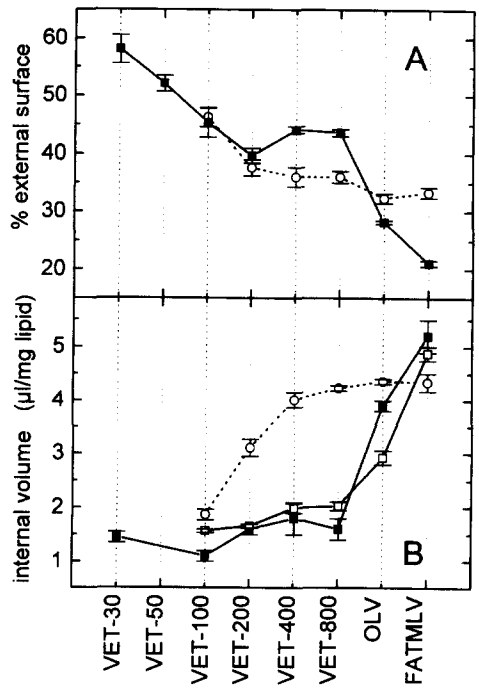

Fig. 6. Characterization of extruded vesicles by simultaneous determination of external surface and internal volume. Various types of vesicles (indicated on the x-axis) were prepared either from SBL (open circles, · · · in panels A and B) or from a 95:5 mixture of eggPC/DMPE (solid squares, — in panels A and B). Aliquots from identical batches were subjected to external surface determination (A) by the TNBS method and to internal volume assay (B) by the sack dialysis method. Aliquots from the very same batches of eggPC/DMPE vesicles were also sized on a Sephacryl S-1000 column (see text). A separate series of eggPC/DMPE vesicles was prepared for the determination of internal volume by the HFD method (open squares, — in panel B).

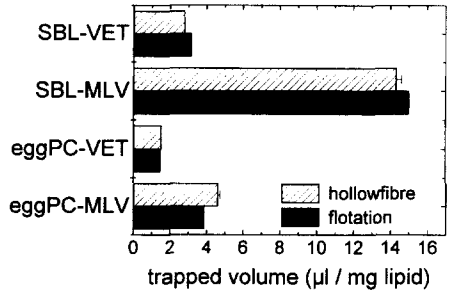

Fig. 5. Exchange of external salt by sucrose gradient flotation. FATMLV ('MLV') or VET_{200} were prepared from eggPC or SBL in buffer A. 1 ml samples of vesicles were mixed with 0.6 ml portions of 80% sucrose (w/v, corresponding to 62% w/w, containing the same salt concentrations as buffer B in addition) and placed in centrifuge tubes holding 12 ml volumes. The dense vesicles were overlayered most carefully with 0.5 ml layers of 26, 24, and 22% sucrose, followed by 1.5 ml of 20% and 0.5 ml of 10% sucrose. The tubes were filled with buffer B and spun at $95000 \times g_{max}$ for 3 h. Vesicle bands were analyzed for Cl^- with the Cl^- electrode (see Fig. 1) and for lipid by the enzymatic method. For comparison, trapped volumes were measured by the HFD method (see Fig. 4). Flotation of VET_{200} was not 100.0%, as seen from a slight haze in the region of 20% sucrose. In parallel control gradients lipid-free buffer A was used in place of vesicle samples in order to successfully demonstrate complete absence of Cl^- contaminations in those regions of the gradient from which vesicle bands were harvested in the sample gradients (data not shown).

multilamellar (FATMLV, Fig. 6A, solid square) with relatively large trapped volumes (Fig. 6B, squares). Upon extrusion, external surface values strongly increased with the number of extrusions (compare OLV and VET_{800}) and with smaller filter pore sizes (solid squares in Fig. 6A), except for the discontinuity at VET_{200} which was repeatedly found with various batches of Nuclepore filters dating from a period of well over 5 years. At present, we cannot offer an unequivocal explanation for the latter phenomenon.

Most dramatic (and unexpected in the extent observed) was the loss of trapped volume upon repeated extrusion of zwitterionic lipid vesicles (solid squares in Fig. 6B). VET_{100} from eggPC have indeed been described as ellipsoidal, with a trapped volume of ~ 1 μl/mg of lipid, rather than ~ 3 μl/mg as expected for spherical shape (Mui et al., 1993). Yet the measured (Fig. 6B, solid square) trapped volume of only 1.6 μl/mg for VET_{800} (with certified near unilamellarity!, see Fig. 6A, solid square) was really astonishing since expectation for a spherical unilamellar vesicle was about 20-times higher. Fortunately, aliquots from the identical vesicle batches of all extruded vesicles had simultaneously been sized by chromatography on Sephacryl

S-1000 as described in the literature (Hauser et al., 1973; Reynolds et al., 1983; Schurtenberger and Hauser, 1984) and the results agreed with the expected size distributions as known from electron micrographs of comparable extruded vesicles (Mayer et al., 1986).

Still refusing to believe in such small trapped volumes we suspected that a major fraction of the extruded vesicles had been too leaky for the time scale of sack dialysis while a minor fraction had given rise to the usual stable level of chloride entrapment (compare Figs. 2 and 3). Therefore, a new series of extruded vesicles was subjected to HFD and, due to small sample volumes, all extravesicular chloride was exchanged within ~ 10 min (rather than within ~ 20 min as shown in Fig. 4A). The results (Fig. 6B, open squares) closely matched those previously obtained with sack dialysis (solid squares) within the expected range for repeated vesicle preparations.

These findings suggest that extruded vesicles from zwitterionic lipids look like uninflated balloons. The apparent discrepancy between the spherical shapes seen in electron micrographs and the measured small captured volumes may relate to the effect of glycerol which is usually included as a cryoprotectant in electron microscopy and which has been shown to cause roundup of nonspherical vesicles (Perkin et al., 1993).

Similar trends for partially anionic as opposed to purely zwitterionic lipid vesicles were also observed with other vesicle preparation protocols (Table 4). The popular FTS protocol (Kasahara and Hinkle, 1977) gave very large internal volumes for SBL vesicles, albeit at some degree of multilamellarity (FTSVES in Table 4). When using selective detergent removal, SBL gave rather homogeneous, spherical 100 nm vesicles with near unilamellarity (SBL-BBVES in Table 4, see footnote b), while corresponding eggPC vesicles contained very little aqueous medium in spite of extremely high turbidity (eggPC-BBVES in Table 4). A similar effect of negatively charged lipids has been reported for a large series of vesicles produced by dialysis of decyl maltoside (Alpes et al., 1986).

Table 4
Characterization of vesicle preparations typically used for reconstitution of transport proteins

Lipid composition	Vesicle type	External surface	Internal volume in μl/mg lipid
SBL	FTSVES [a]	34.4 ± 1.1%	7.9 ± 0.3
SBL	BBVES [b]	43.9 ± 1.9%	3.5 ± 0.3
eggPC	BBVES [c]	–	1.8 ± 0.2

Vesicles were formed either by freeze-thaw-sonication or by detergent removal with Bio-Beads (see Section 2). External surface was determined by the TNBS assay and internal volume by the sack dialysis method (compare Fig. 2).

[a] Moderate turbidity.

[b] Very weak turbidity, mean diameter ~ 100 nm according to chromatography on Sephacryl S-1000.

[c] Extremely turbid.

4. Discussion

The object of the present study was to analyze possible systematic errors in the measurement of salt entrapment and to establish reliable protocols with general applicability. This goal has successfully been reached.

Chloride proved to be the best marker ion for intravesicular volume in every respect because of its ubiquity in biological buffers, and because the high selectivity and stability of the Cl^- electrode allows for simple and accurate measurement of internal chloride under all relevant conditions.

Exchange of external chloride for nitrate turned out to be non-trivial when critically examined. Reliable determination of chloride entrapment can be achieved with gel filtration, dialysis, and sucrose gradient flotation, taking into account the limits pointed out in this study. In contrast, ion exchange chromatography and diafiltration proved to be inapplicable.

Gel filtration was significantly improved by switching to 'new' gel types, thereby increasing lipid recovery from ~ 10% to > 90% under worst case conditions.

Dialysis is the most general method with the least pitfalls, provided that marker ion escape from vesicle interior is taken into account. Chloride loss from large or small vesicles with 20% anionic lipid is negligible, even at room temperature. The same applies to large zwitterionic lipid vesicles, at least in the cold. In the case of small zwitterionic lipid vesicles, however, it is necessary to explicitly monitor chloride efflux and to calculate trapped volumes by extrapolation to time zero. From a comparison of Figs. 2 and 4 it is obvious that regular sack dialysis can be used for this purpose. HFD and TLD simply have the advantage of higher convenience and lower data scattering.

Significant new insights into vesicle morphology were obtained when internal volume data were combined with the measurement of 'lamellarity' (Gruber and Schindler, 1994) and size distribution. The findings in Fig. 6 and in Table 4 consistently pointed to an essential role of negatively charged lipids for production of voluminous vesicles (with near unilamellarity and high mechanic and osmotic stability) as is desired in most biochemical, biophysical, medicinal, and technical applications of liposomes. The results also show that such macroscopic characterization of vesicle morphology yields valuable information which nicely complements electron microscopy.

Intravesicular volume is a very sensitive macroscopic parameter of vesicle morphology but all methods hitherto available suffered from poor sensitivity and/or need for very special instrumentation and/or necessity of prelabeling with a hydrophilic marker the presence of which conflicts with the intended application of the identical vesicle batch. All three limitations are overcome by the chloride entrapment method as proposed in this study: sensitivity is high, little equipment is required, and the ubiquity of Cl^- greatly simplifies practical application.

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