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Size analysis of biological membrane vesicles by gel filtration, dynamic light scattering and electron microscopy

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Biological membrane vesicles are analysed in terms of size and size distribution using gel filtration on Sephacryl S-1000, electron microscopy and quasi-elastic light scattering. The agreement between the three methods is satisfactory particularly for homogeneous dispersions. Gel filtration on Sephacryl S-1000 is a quick and convenient method for the routine size analysis of membrane vesicles up to a diameter of about 250 nm.

The routine size analysis of aqueous phospholipid and biological membrane dispersions consisting of unilamellar vesicles is becoming increasingly important in a number of research areas. Preparative methods currently used to isolate biological membranes from tissue usually yield sealed membrane vesicles. Such membrane vesicles have been the subject of extensive structural and functional studies. Often the successful interpretation of these studies requires the knowledge of the size and size distribution of the membrane vesicles. Here three different methods are being assessed as to their suitability for a routine size analysis of membrane vesicles: (I) gel filtration on Sephacryl S-1000; (II) electron microscopy and (III) quasi-elastic light scattering. The application of these three methods to the problem of the size analysis of aqueous phospholipid dispersions has been discussed previously [1–3]. Sephacryl S-1000 is used here for both preparative and analytical purposes because it has a larger exclusion limit compared to the Sepharose gels, allowing the particle size analysis to be extended up to diameters of about 250 nm [1,2]. The results reported here were obtained with brush-border membrane vesicles from rabbit small intestines and chromatophores from *Rhodospirillum rubrum* as representative examples of a plasma membrane and an intracytoplasmic membrane, respectively. We show that Sephacryl S-1000 lends itself well to both the fractionation and size analysis of biological membrane vesicles provided their size does not much exceed a diameter of about 220 nm. Consistent results are obtained with the three different methods: the agreement is best for dispersions homogeneous with respect to particle size.

The elution profile of brush-border membrane vesicles prepared from rabbit small intestines [4] consisted reproducibly of a broad, asymmetric peak with an elution volume V_e close to the void volume (Fig. 1A). All fractions were monitored for ultraviolet absorption and turbidity and those with significant absorbance at 280 nm were analyzed for protein [5] and lipid phosphorus [6], both analytical methods giving consistent results. Brush-border membrane vesicles were polydisperse, the elution volume of the asymmetric peak $V_e = 15 \pm 1.5$ ml corresponding to a Stokes' radius of 97 nm. The Sephacryl S-1000 column was calibrated with polystyrene (latex) beads and

brome mosaic virus of known diameter [3]; the inverse error function relationship of Ackers [7] was used to convert elution volumes V_e to Stokes radii. The error of the V_e measurement was large in this case so that V_0 was within the error of the V_e measurement. This produced a particularly large error in the determination of the Stokes' radius ranging from about 75 nm to practically the exclusion limit of the column of about 125 nm. Freeze-fractured samples of the same brush-border membrane preparation were examined by electron microscopy and the results are presented as the bar histogram in Fig. 2A. A non-Gaussian distribution confirming the polydispersity of the sample was obtained from which different averages of mean radii were computed (see Table I). The various average radii thus derived are smaller than the average Stokes' radius derived from gel filtration, but are still within the large error margin of the gel filtration measurement.

A representative elution profile of chromatophores prepared from *R. rubrum* [8] is shown in Fig. 1B. The column effluent was analyzed for protein, lipid phosphorus and absorbance at 880 nm as a measure of the chromophore content. All three methods of analysis gave consistent results. Reproducibly bimodal elution profiles were obtained, the first peak (I) eluting at the void volume V_0, the second major peak at $V_e = 21.3 \pm 0.9$ ml ($n = 9$) corresponding to a Stokes' radius of 42 ± 4 nm. Fractions 36–39 (Fig. 1B) were pooled, concentrated and rechromatographed (Fig. 1C). Simulation of the elution profiles in Figs. 1B and C in terms of Gaussian peak distribution shows that the elution peaks are nearly Gaussian. Electron micrographs of freeze-dried samples of the same chromatophore preparation (Fig. 2B) were analyzed in terms of size distribution and the results are presented as the bar histogram in Fig. 2C. Consistent with the elution profile shown in Fig. 1B a multimodal, non-Gaussian size distribution was obtained. The weight average radius derived from this bar histogram is in good agreement with the mean Stokes' radius derived from gel filtration (Table I).

The same membrane dispersions which were subjected to size analysis by gel filtration and electron microscopy were also analyzed by quasi-elastic light scattering (for experimental details,

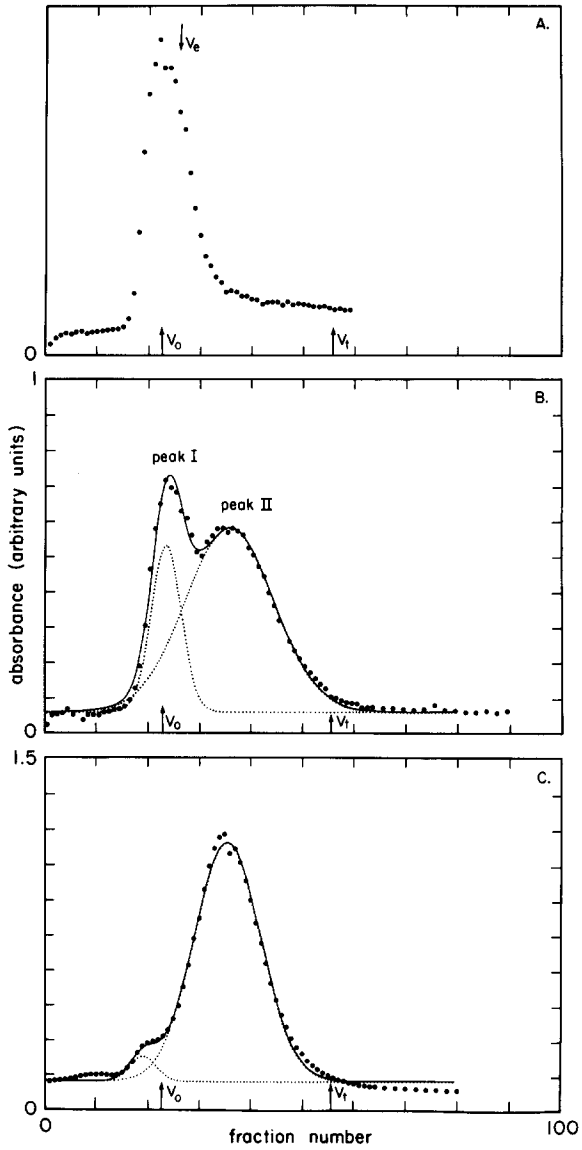


Fig. 1. Elution profiles from Sephacryl S-1000 of brush-border membrane vesicles from rabbit small intestines (A) and chromatophores prepared from a carotenoid-free mutant of *Rhodospirillum rubrum* G9 (B). The column (37×1.1 cm) was equilibrated with 0.15 M NaCl, 10 mM Hepes (pH 7.5), 0.02% NaN_3; 0.6–1 ml of brush-border membrane vesicles (approx. 10 mg protein/ml) or chromatophores (approx. 50 mg protein/ml) were applied to the column and eluted with the same buffer (flow rate 3–9 ml/h); 0.59 ml fractions were collected and analysed for protein (●). The column was calibrated as described previously [3]. The void volume $V_0 = 13.5$ ml and the total volume $V_t = 33$ ml are indicated by arrows. Fractions 36–39 of (B) were pooled, concentrated and rechromatographed (C). The chromatograms in (B) and (C) were fitted by the sum (solid line) of two Gaussian distributions (dotted line) using a non-linear least-squares program.

TABLE I

SIZE ANALYSIS OF BRUSH-BORDER MEMBRANE AND CHROMATOPHORE VESICLES CARRIED OUT BY THREE DIFFERENT METHODS

	Mean radius r (nm)	
	brush-border membrane	chromatophores
Gel filtration	97 [b]	42 ± 4
Electron microscopy [a]		
Number average	74	38
Weight average	82	41
z-average	81	43
Quasi-elastic light scattering		
Unfractionated	$125 \pm 4\begin{pmatrix} 68 \pm 3 \\ 190 \pm 20 \end{pmatrix}$ [c]	$46 \pm 2\begin{pmatrix} 38 \pm 4 \\ 106 \pm 20 \end{pmatrix}$ [c]
Membrane dispersion		
Peak I	–	116 ± 4
Peak II	–	$44 \pm 1\begin{pmatrix} 36 \pm 1 \\ 75 \pm 10 \end{pmatrix}$ [c]

[a] The number average radius is defined as $r_n = \Sigma N_i r_i / \Sigma N_i$, the weight average radius $r_w = \Sigma N_i M_i r_i / \Sigma N_i M_i$ and the z-average radius $r_z = \Sigma N_i M_i^2 P_i / \Sigma N_i M_i^2 P_i r_i^{-1}$ where N_i, M_i, P_i are the number, the molecular weight and the form factor, respectively, of membrane vesicles of radius r_i. M_i and P_i were derived as described in Ref. 3.

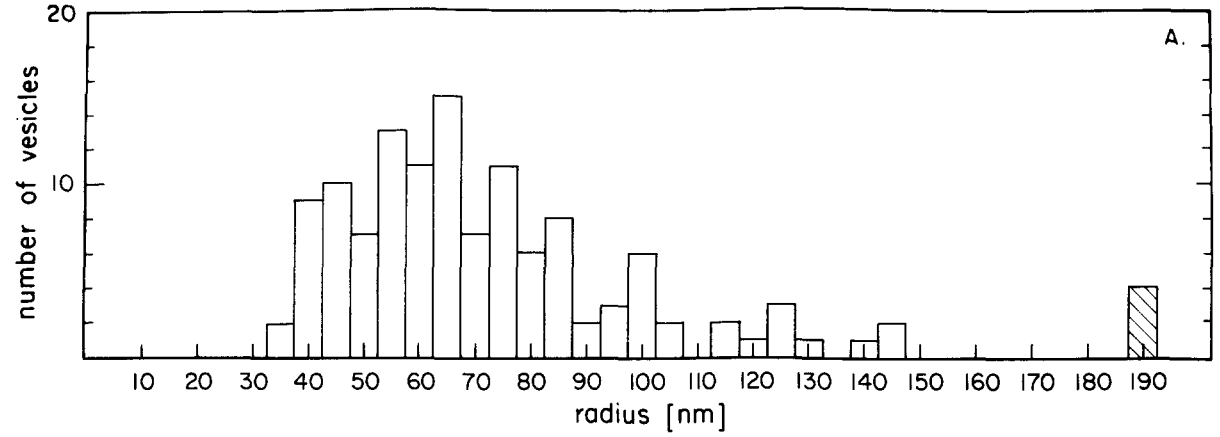
[b] V_e was so close to V_0 that V_0 was practically within the error margin of the V_e measurement. Hence the error of the determination of the Stokes' radius was very large indeed spreading from about 75 nm to 125 nm, the exclusion limit of the column.

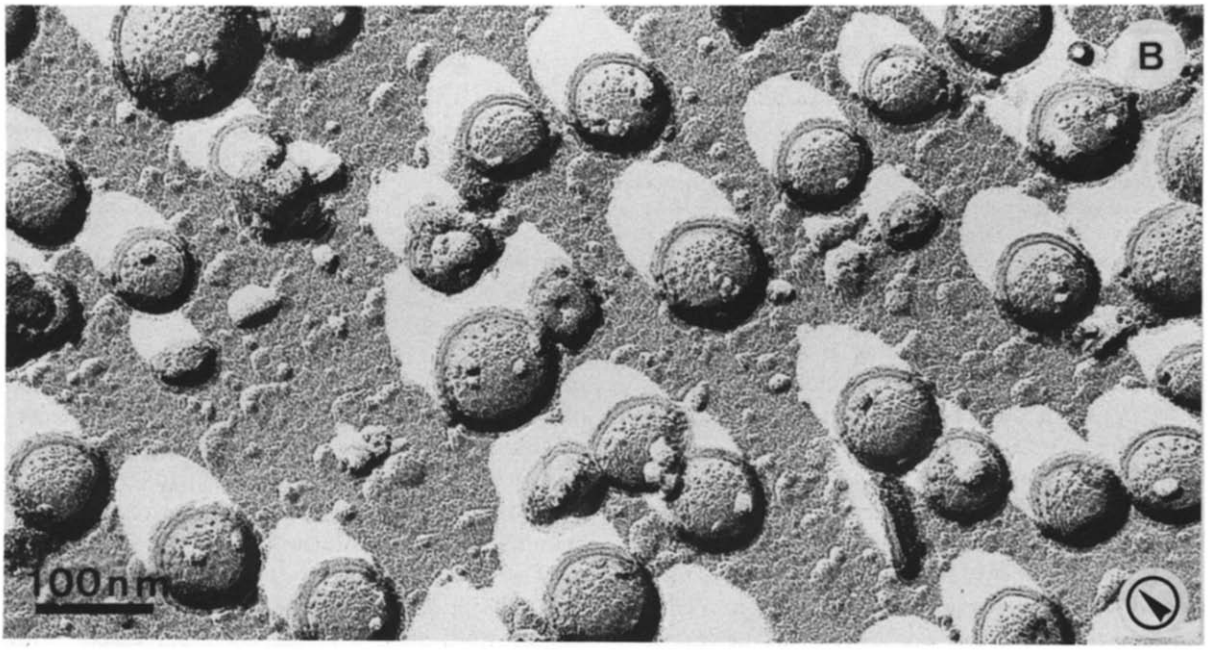
[c] The two values were derived from an analysis of the normalized intensity autocorrelation function in terms of two discrete particle sizes [3].

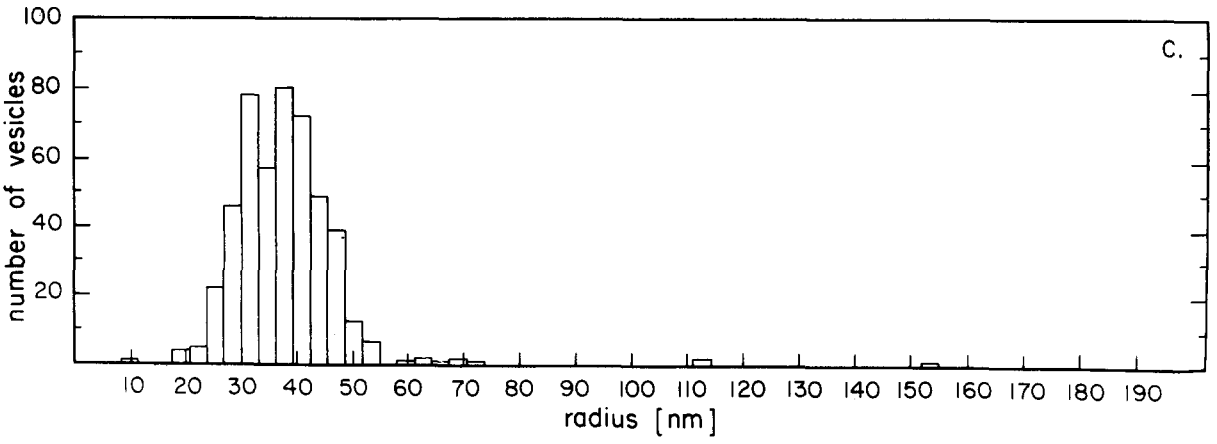
instrumentation used and data evaluation see Refs. 3, 9–11). The results are summarized in Table I. The mean hydrodynamic radius of brush-border membrane vesicles derived from a cumulant analysis is in reasonably good agreement with the mean Stokes' radius considering the large error of the gel filtration experiment. It is, however, significantly larger than the z-average radius derived from electron microscopy. This discrepancy may be due to the greater polydispersity of these vesicles compared to chromatophores (see discussion below) for the mean hydrodynamic radius determined by light scattering is known to be highly sensitive to even trace amounts of large particles. It may, however, also reflect some bias of the electron microscopist towards small vesicles. Based on the multimodal distribution found by gel filtration and electron microscopy (Figs. 1A, 2A) the light scattering data were analyzed in terms of two discrete particle sizes [3]. The mean hydrodynamic radius of the small-vesicle population $r = 68$ nm (Table I) is close to the radius of the peak fraction of the bar histogram ($r = 65$ nm, Fig. 2A). It is interesting to note that the mean hydrodynamic radius of the large-vesicle population $r = 190$ nm (Table I) is identical with that of the bar outside the main peak of the bar histogram (Fig. 2A). This population of large membrane vesicles is probably a contamination of the brush-border membrane.

The cumulant analysis of unfractionated chromatophores and of peak II yielded mean hydrodynamic radii that were in good agreement with values derived from gel filtration and electron microscopy (Table I). For reasons mentioned above the light scattering data were also analyzed in terms of two discrete particle sizes [3]. The hydrodynamic radius of the small-vesicle population $r = 38$ nm is identical with the radius of the peak fraction of the bar histogram (Fig. 2C). The hydrodynamic radius of the large-vesicle population is consistent within experimental error with that of peak I.

In conclusion, gel filtration on Sephacryl S-1000 is an inexpensive, quick and convenient method for the fractionation and routine size analysis of membrane vesicles with a diameter smaller than


A.
number of vesicles
20
10
10 20 30 40 50 60 70 80 90 100 110 120 130 140 150 160 170 180 190
radius [nm]


B
100nm


C.
number of vesicles
100
80
60
40
20
10 20 30 40 50 60 70 80 90 100 110 120 130 140 150 160 170 180 190
radius [nm]

about 250 nm. It also gives a fairly good measure of the size heterogeneity. Tanford and his coworkers [1,2] arrived at similar conclusions working with phospholipid dispersions. The exclusion limit of Sephacryl S-1000 is about 250 nm which is increased by a factor of 3–4 compared to Sepharose 4B, hence Sephacryl S-1000 is a useful extension to existing gel chromatographic media. Calibration of Sephacryl S-1000 is conveniently carried out with polystyrene latex beads of known diameter; Stokes' radii derived for membrane vesicles from such a column are in good agreement with those determined by quasi-elastic light scattering or by electron microscopy. The agreement between the different methods is remarkably good, this is particularly true for a homogeneous vesicle population (see results for peak II, Table I). This is certainly one of the main conclusions of this work. Further, with polydisperse samples meaningful results are obtained from quasi-elastic light scattering provided the measurement is preceded by a fractionation procedure such as gel filtration on Sephacryl S-1000. Our results demonstrate that polydisperse preparations of biological membrane vesicles as usually obtained by standard preparative methods are adequately analyzed in terms of size and size distribution using a combination of gel filtration, electron microscopy and quasi-elastic light scattering. Freeze-drying together with rotary-shadowing gives better results in our hands than the sample preparation by the standard freeze-fracture method. Since freeze-dried samples are not subjected to any fracturing procedure, whole vesicles are seen in electron microscopy. Under these conditions the apparent diameter is very likely to be the true vesicle diameter. The work presented also demonstrates the applicability of electron microscopy to the problem of the routine size analysis of membrane vesicles and substantiates the conclusions from our previous work on aqueous phospholipid dispersions [3].

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Fig. 2. (A) Bar histogram derived from electron micrographs of freeze-fractured samples of the same brush-border membrane vesicle preparation as that chromatographed in Fig. 1A. Freeze-fractured samples were prepared as described previously [12,13]. Vesicle diameters were measured perpendicular to the direction of shadowing. Most vesicles appeared to be fractured equatorially. This is indicated by the observation that approximately half of each fracture face was covered with Pt as would be expected for an angle of shadowing of 45°. The shaded bar outside the main peak probably represents a contamination. About 250 vesicles were measured. (B) Representative electron micrograph of a freeze-dried preparation of chromatophores. The same preparation as that chromatographed in Fig. 1B was used. Freeze-dried samples were prepared as described before [14]. Vesicle diameters were measured perpendicular to the direction of shadowing indicated by the arrow. The bar is 100 nm. (C) Bar histogram derived from electron micrographs of freeze-dried preparations as shown in (B). About 480 vesicles were measured.