Eur Biophys J 21, 129–136 (1992)

European Biophysics Journal

Contrast variation studies of clathrin coated vesicles by small-angle neutron scattering

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Received January 28, 1992/Accepted in revised form March 3, 1992

Abstract. Structural information on clathrin coated vesicles has been obtained by small angle neutron scattering using contrast variation. A characteristic peak in the neutron scattering profile, which is apparent in 75% D_2O, as well as in H_2O, disappears when contrast matching the protein component of the coated vesicles in 42% D_2O. Neutron, as well as dynamic, light scattering give a coated vesicle size of about 900 Å in H_2O and D_2O, but for neutron scattering the diameter decreases when matching out the protein coat of the clathrin coated vesicles. From the match point for the clathrin coated vesicles it is demonstrated that the clathrin cages do contain internal membrane. The mass of 34 MD and composition of 75% protein and 25% lipid found from the analysis of the small-angle scattering data are both in good agreement with the values reported in the literature. Electron microscopy gives an average outer diameter of 880 Å for the coated vesicles and an average diameter of 460 Å for the vesicle itself.

Key words: Clathrin – Vesicles – Neutron scattering and light scattering

Introduction

In a living cell, the protein clathrin (Pearse 1975) is polymerised and has a spherical or barrel shaped polygonic structure, which encloses a bilayer membrane vesicle. The clathrin network is made up of 12 pentagons and a number of hexagons (Crowther et al. 1976).

Attached to the clathrin molecules are 'clathrin accessory polypeptides', usually termed CAPs (see e.g. Pearse and Crowther 1987). This suggests a model with a multi-shell structure of clathrin/CAPs/vesicle for the clathrin coated vesicle. The existence of this multi-shell structure has been supported by electron microscopy, which has provided the main part of the structural information about the clathrin coated vesicles in previous papers (see e.g. Vigers et al. 1986 and Pearse and Crowther 1987).

The measurements described in this paper are an extension of earlier studies of the structure and stability of clathrin coated vesicles using small angle X-ray and neutron scattering, as well as dynamic light scattering (Bauer et al. 1991). As previous studies of clathrin coated vesicles were done using techniques requiring the measurements to be performed in a less than physiological environment we thought that small angle scattering studies might supplement these studies by allowing more physiological conditions for the measurement. By undertaking contrast measurements we have been able to extract the contributions to the scattering from the membrane and protein part of the intact coated vesicles separately and this gives additional information on the structure of the coated vesicles. The ability to extract these different contributions is specific to neutron scattering, owing to the distinct difference in scattering densities between the protein coat and the internal membrane vesicle.

Materials and methods

The biochemical preparations and the cryo-electron microscopy were done in the Biological Support Laboratory at Daresbury Laboratory. The dynamic light scattering experiments were performed at the Royal Veterinary and Agricultural University in Denmark, and the neutron experiments were carried out at Risø National Laboratory in Denmark.

Sample preparation

All chemicals used were of analytical grade. D_2O was from SIGMA. The buffer used throughout, except for a change in D_2O percentage, was 0.1 M MES, 0.2 mM magnesium chloride, 0.02% sodium azide and 1 mM EGTA, hereafter denoted buffer A. The preparation of the coated vesicles was done as described in Bauer et al. (1991). The

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only difference in the procedure was that the final purification on a S-1000 loaded column was performed with the column equilibrated in buffer A with a D_2O percentage of 100. This was done because of the stabilizing effect of D_2O on the coated vesicles when high concentration is necessary (about 5 mg/ml). The changes to 0%, 42% and 75% D_2O were done by dialysing the coated vesicles in buffer A with the appropriate D_2O percentage and with a 100-fold buffer to sample volume.

The concentrations of the samples were measured by UV absorption at 280 nm using an absorption value of 1.5 ml · mg^{-1} · cm^{-1} in a cuvette with a 1 cm light path. This gives the weight concentration, including the lipid part set to 25% of the total mass (Pearse 1975). Using an absorption coefficient of 1.1 ml · mg^{-1} · cm^{-1} at 280 nm for clathrin (Winkler and Stanley 1983), subtracting a background proportional to λ^{-4} from light scattering (derived by extrapolating the λ^{-4} part of the absorption spectrum between 350 and 400 nm to 280 nm) and multiplying the result by 1.3333 to include the lipid mass gave the same weight concentration as the direct use of the value of 1.5 ml · mg^{-1} · cm^{-1}. The concentration of the coated vesicles used for neutron scattering were determined after the neutron scattering experiments.

Electron microscopy

The structure of the coated vesicles was examined by cryo-electron microscopy (Dubochet et al. 1982) by withdrawing 10 µl of a solution containing the coated vesicles, placing it on a "holey" carbon grid and immediately transferring it to liquid ethane. The cryotransfer system (Gatan model 626, Pleasanton, C.A., USA) was used to transfer the specimen to a Philips EM 400 fitted with a minimum-dose kit. Images taken at magnification of 43 200 were recorded on film. The coated vesicles were also examined by staining with uranyl acetate (1% solution) after placing 10 µl on a carbon coated grid (air dried for two minutes). The electron microscopy was performed with a Phillips EM 300 microscope operated at 60 kV. The electron microscopes were calibrated with a standard grid from Agar Scientific with 2160 lines per mm.

Dynamic light scattering

The dynamic light scattering instrument consists of a 35 mW He–Ne laser (Spectra Physics), a Langley-Ford autocorrelator and a single-photon counting photomultiplier (Hamamatsu, red sensitive). The hydrodynamic diameters were calculated as

$$D_h = \frac{kT}{3\pi\eta D} \tag{1}$$

where D is the diffusion coefficient and D_h the hydrodynamic diameter. The samples used for light scattering were diluted by a factor of 100 in buffer A (filtered through a 0.2 µm filter) relative to the samples used for small angle scattering. The molar weight and hydrodynamic diameter were derived from the spectra as described in Bauer et al. (1991).

Small angle neutron scattering

The small angle neutron scattering instrument was used in the q-range of 0.003–0.15 $Å^{-1}$ (q is the length of the scattering vector and is given by: $q = 4\pi \sin(\theta)/\lambda$, where θ is half the scattering angle and λ the wavelength of the neutrons). The data presented were obtained with a sample-to-detector distance equal to 3 meter and a wavelength of 6 Å, and a sample-to-detector distance equal to 6 meter and a wavelength of 14 Å. The monochromator system is a mechanical velocity selector, which in the setting used has a wavelength spread $\Delta\lambda/\lambda \approx 18\%$. The samples were contained in quartz cuvettes (Hellma) of 2 mm light path for 75% D_2O and 1 mm light path for 42% D_2O as well as 0% D_2O. The raw scattering data were corrected for background from buffer, cuvettes and zero-flux background according to conventional procedures, and the spectra were normalized and corrected for possible variations of detector efficiency by dividing with data obtained from a H_2O sample.

SANS data analysis

The scattering $I(q)$ from a dilute collection of particles as a function of the scattering vector q is (see e.g. Glatter 1982):

$$I(q) = N \left| \int \Delta\varrho(\bar{r}) \exp(i\,\bar{q} \cdot \bar{r})\, \mathrm{d}\bar{r} \right|^2 \tag{2}$$

where N is the number density of the scattering particles and $\Delta\varrho(\bar{r})$ is the excess scattering length density. For randomly oriented particles (2) can be written as:

$$I(q) = N \cdot 4\pi \int p(r) \frac{\sin q r}{q r}\, \mathrm{d}r \tag{3}$$

where $p(r)$ is the distance distribution function, which is related to the excess scattering length density $\Delta\varrho(\bar{r})$ by

$$p(r) = \left\{ \int_V \Delta\varrho(\bar{r}+\bar{r}')\, \Delta\varrho(\bar{r}')\, \mathrm{d}\bar{r}' \right\} r^2 \tag{4}$$

where V is the volume of the scatterer, $\mathrm{d}\bar{r}'$ is a volume element and $\{.\}$ means averaging over all orientations of the particle. The distance distribution functions were obtained using the indirect Fourier transformation method described by Glatter (1977). In this method $p(r)$ is defined on the interval $[0; D_{max}]$ where D_{max} is the maximum distance within the particle. The distribution function $p(r)$, as well as D_{max}, is obtained by a constrained least-squares method. The resolution function of the neutron spectrometer was included in these calculations as described in Skov Pedersen et al. (1990) and Hansen and Skov Pedersen (1991).

The radius of gyration R_g was calculated using the distance distribution function and

$$R_g^2 = \int p(r) r^2\, \mathrm{d}r / (2 \int p(r)\, \mathrm{d}r) \tag{5}$$

The values for the scattering intensity at zero angle $I(0)$ were calculated from the distance distribution functions according to

$$I(0) = \int p(r)\,dr \qquad (6)$$

The error estimates on $p(r)$ were derived as described by Glatter (1977), including covariances (Moore 1980). However, when calculating the errors on the derived parameters, R_g and $I(0)$, the contribution to the error from the uncertainty in the estimate of D_{max} is not included in the calculation when following Glatter and Moore. The error estimates therefore tend to be too small. To get more realistic error estimates we have made a least-squares fit assuming $I(q) \approx I(0)\exp(-R_g^2 q^2/3)$ for $q < 0.006\,\text{Å}^{-1}$ and used conventional error analysis (Bevington 1969). For 0 and 75% D_2O the errors determined in this way agree with those from $p(r)$ whereas for 42% D_2O they are a factor of 3–4 larger.

Results

The results of the small angle neutron scattering experiments on the clathrin coated vesicles 8 °C are shown in Fig. 1 for the different contrasts used. Dynamic laser light scattering experiments performed on the samples used for neutron scattering gave identical molar weights and hydrodynamic diameters before and after the neutron experiments.

The scattering profiles obtained in 75 and 0% D_2O both a show a peak at $q = 0.025\,\text{Å}^{-1}$ in accordance with earlier measurements (Bauer et al. 1991). However the 42% D_2O measurement shows no such peak. As the protein contribution to the scattering profile vanishes at this contrast, and as the major component of the coated vesicle is protein (Crowther et al. 1976), the sample scatters only weakly.

Figure 1 shows fits to the measurements calculated from an indirect Fourier transformation as described above, giving the distance distribution functions as shown in Fig. 2. In all cases $D_{max} < 2\pi/q_{min}$ for the scatterer.

Table 1. SANS contrast variation

% D_2O	D_{max} (Å)	R_g (Å)	$I(0)$ (cm^{-1})	c (mg/ml)
75	1000	336±6	91.1±2.2	3.6
42	800	352±60	3.7±1.0	4.1
0	900	299±10	50.9±2.0	3.9

The results of the measurements are summarized in Table 1. The light scattering data were also for determining the hydrodynamic diameter D_h and the molecular mass M_w of the coated vesicles. The values obtained were $D_h = 900 \pm 25$ Å and 24±4 MD respectively. There is

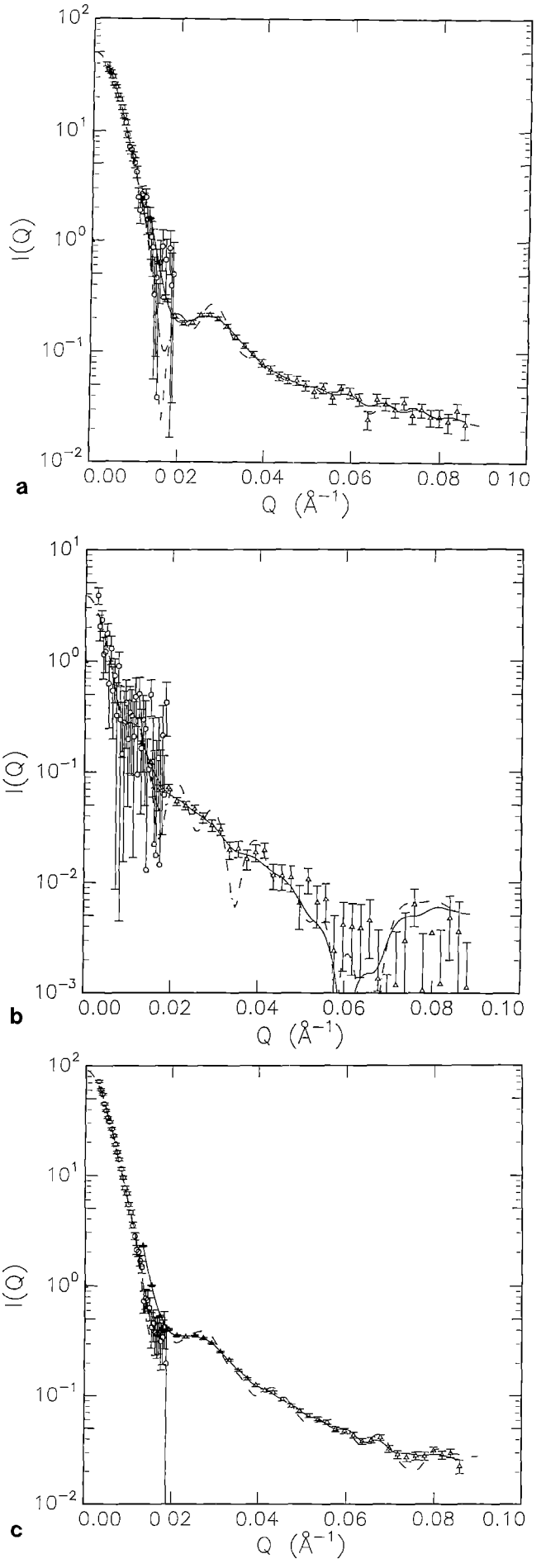

Fig. 1 a–c. SANS data for clathrin coated vesicles in 0, 42 and 75% D_2O respectively. The full line is a fit of the data (shown as errorbars) and the *dashed-dotted* line shows the corresponding deconvoluted scattering curve

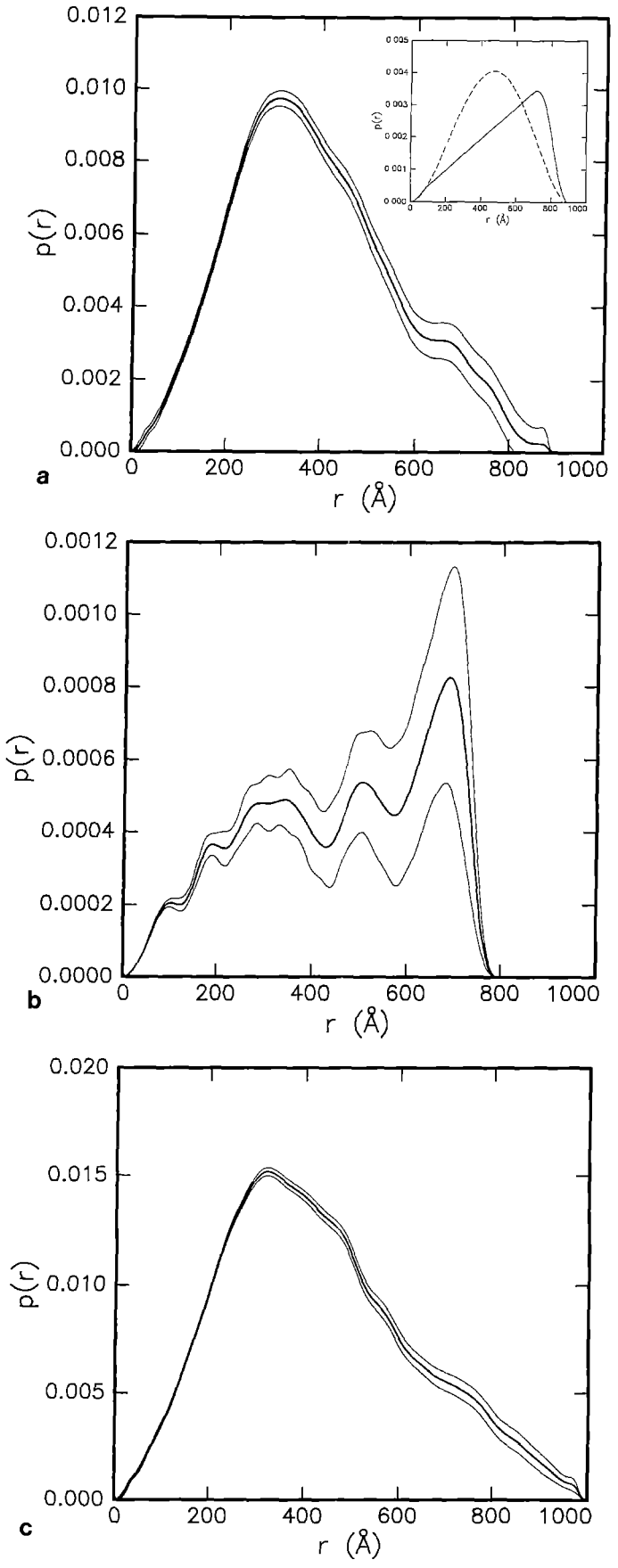

Fig. 2 a–c. Distance distribution functions for 0, 42 and 75% D_2O respectively. The upper and lower curves represent the error limit. Insert in Fig. 2a shows the distance distributions for sphere and shell as described in text

agreement between the maximum diameter D_{max} determined by SANS and D_h. Also shown in the table are the concentrations of the samples used for SANS. The errors on these values are about 5%.

Figure 3a shows an EM picture of the coated vesicles used for neutron scattering obtained as described under materials and methods and Fig. 3b shows cryo-EM pictures of clathrin coated vesicles. From these pictures average diameters were measured for the clathrin coated vesicle as well as for the vesicle component inside the coat. This gave 880 Å for the clathrin coat and 460 Å for the vesicle. The estimated error for these measurements is about 10%.

In the forward direction ($q = 0$) the phase factor in (1) is equal to unity and the dependence of $\Delta\varrho(\bar{r})$ on $\bar{r}$ disappears. The equation can be rewritten in terms of $\Delta\varrho_{av}$, the average excess scattering length per unit mass (D). Taken into account the linear dependence of $\Delta\varrho_{av}(x)$ on the D_2O concentration x one has:

$$\Delta\varrho_{av}(x) = \varrho_{av}(x_{0,av} - x), \tag{7}$$

where $\varrho_{av} \cdot x_{0,av}$ is the excess scattering length per unit mass in H_2O and $x_{0,av}$ is the match point for the particles. Using this and $N = N_A \cdot c/M$, where N_A is Avogadros number, c is the (mass) concentration of scattering material and M is the mass of the particles, (1) can be written as:

$$\frac{I(0)}{c} = N_A \cdot M \cdot [\varrho_{av}(x_{0,av} - x)]^2 \tag{8}$$

Thus plotting $\sqrt{I(0)/c}$ versus x gives a straight line and the match point $x_{0,av}$ can be determined. This is done in Fig. 4 for the present data and the match point is determined to be $x_{0,av} = 0.32 \pm 0.02$.

Assuming that the clathrin coats are composed only of protein and lipids, $\Delta\varrho_{av}(x)$ can be written as:

$$\begin{aligned}\Delta\varrho_{av}(x) &= \varrho_{av}(x_{0,av} - x) \\ &= \varrho_P(x_{0,P} - x) f + \varrho_L(x_{0,L} - x)(1 - f),\end{aligned} \tag{9}$$

where ϱ_P/ϱ_L are the excess scattering length per unit mass of protein/lipid in H_2O, $x_{0,P}/x_{0,L}$ are the respective match points and f is the mass fraction of the proteins (mass of protein to total mass). By inserting the value for the match point $x_{0,av}$ in the equation and using known values for ϱ_P, ϱ_L, $x_{0,P}$ and $x_{0,L}$ the mass fraction f can be determined. The following values of scattering length densities of protein and lipid were calculated from Cusack et al. (1985): $\varrho_P = 7.14 \cdot 10^{-14}$ cm/D, $x_{0,P} = 0.42$, $\varrho_L = 10.9 \cdot 10^{-14}$ cm/D and $x_{0,L} = 0.12$. The protein mass fraction f was determined to be $75 \pm 5\%$. This protein mass consists of the clathrin coats (including CAP's), the protein in the membrane of the vesicle as well as the protein bound to the receptors inside the vesicle (owing to the r^2-term in the expression for R_g the latter two contribute little to R_g).

The total mass (protein + lipid) was determined using (8) and the mass fraction determined. Using the 0% and 75% D_2O data the value $M = 34 \pm 5$ MD was obtained. The main contributions to the uncertainty are the uncertainty in the absolute scale and the uncertainty in the concentrations c.

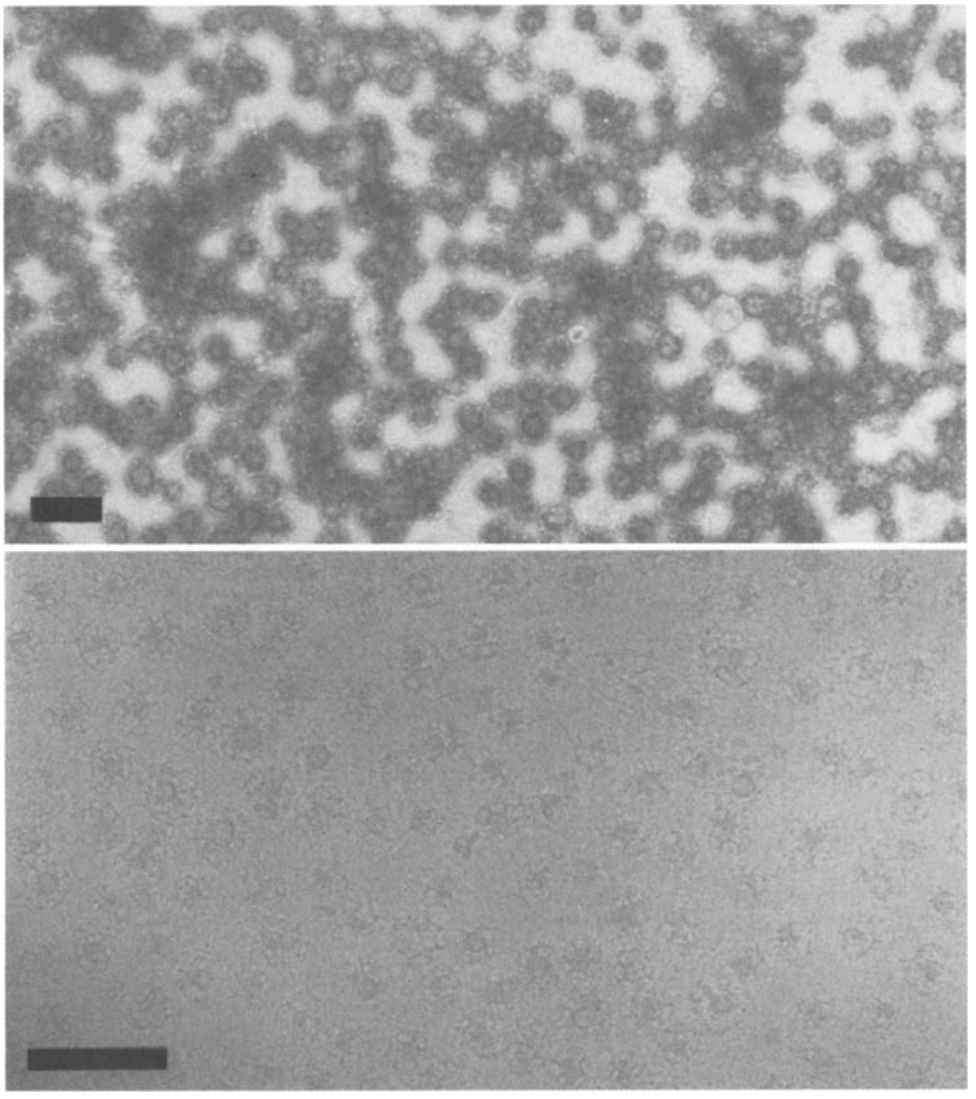

Fig. 3. a Micrographs (TEM) of clathrin coated vesicles. **b** Micrographs (Cryo-EM) of clathrin coated vesicles. (The bars in a) and b) are 2000 Å)

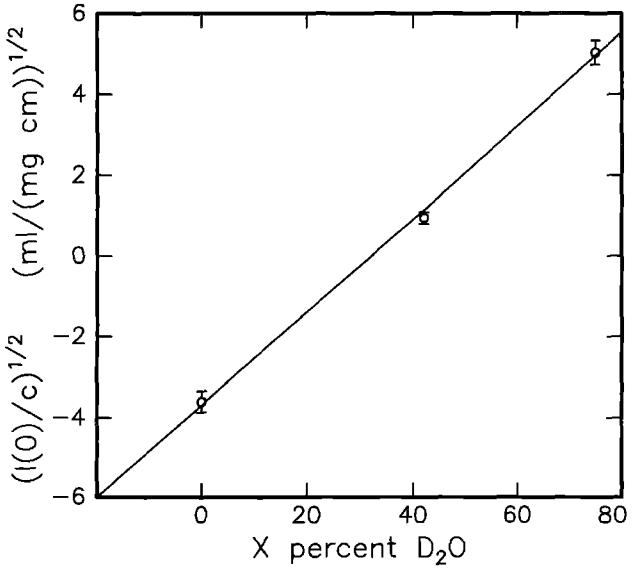

Fig. 4. Plot of $\sqrt{I(0)/c}$ versus the D_2O concentration

The mass distribution of 75% protein and 25% lipid allows the contribution to the scattering of the two components at the different contrasts to be determined. We consider the integrated scattering length $F_P(x)$ and $F_L(x)$ for protein and membrane respectively, and define arbitrarily the scattering of protein in H_2O as $F_P(0)=1.0$. Then $F_L(0)=0.14$, $F_P(0.42)=0$, $F_L(0.42)=0.36$, $F_P(0.75)=0.78$ and $F_L(0.75)=0.76$. Thus the membrane contributes only 14% of the scattering length in H_2O whereas it gives rise to about half the scattering length in 75% D_2O.

The scattering from the particles can be written as

$$I(q,x)=N\{|\int[\varrho_P(\bar{r},x)+\varrho_L(\bar{r},x)]\,e^{i\bar{q}\,\bar{r}}\,d\bar{r}|^2\}$$
$$=I_P(q,x)+I_L(q,x)\pm 2\sqrt{I_P(q,x)}\sqrt{I_L(q,x)}, \qquad (10)$$

where $\{.\}$ means averaging over all orientations and spherical symmetry has been assumed. $I_P(q,x)$ and $I_L(q,x)$ respectively, are the scattering from the protein and lipid when the other component is absent. The last term is an interference term.

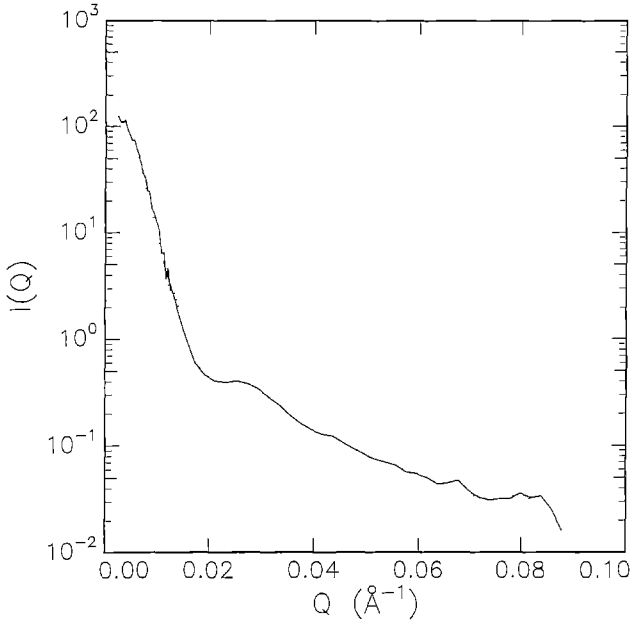

Fig. 5. *Dashed line:* SANS data at 75% D_2O. *Dotted line:* spectrum constructed as described in text

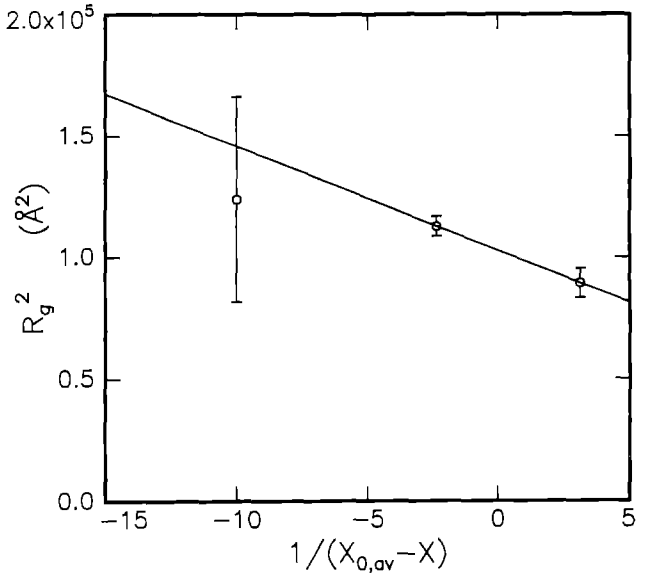

Fig. 6. Plot of $R_g^2(x)$ versus the contrast

As the contribution to the scattering from the lipids at 0% D_2O is small, $I_P(q, x)$ can be estimated from the measured scattering data at this D_2O concentration and $I_L(q, x)$ is given by the scattering curve in 42% D_2O. Using this we can construct and estimate of the scattering at 75% D_2O and compare it to the measured spectrum, assuming a positive sign for the interference term in (10). This is valid at small q-values because the protein and lipid scattering lengths have the same sign. The comparison of the curve constructed in this manner to the data at 75% D_2O is shown in Fig. 5. The curves deviate for larger scattering vectors ($q > 0.015$ Å^{-1}) indicating different radial positions for the membrane and protein respectively.

The radii of gyration given in Table 1 can be used for determining the radius of gyration $R_{g,\infty}$ at infinite contrast, as described by Ibel and Stuhrmann (1975). The radius $R_{g,\infty}$ is the radius of gyration of the particle with the same shape but with uniform scattering density. Assuming spherical symmetry of the particles and neglecting inhomogeneous D_2O exchange one has:

$$R_g(x)^2 = R_{g,\infty}^2 + \alpha/[(x_{0,av} - x)\,\varrho_{av}] \tag{11}$$

where α is calculated from the fluctuation $\varrho_F(r) = \Delta\tilde{\varrho}(r) - \varrho_{av}$ in excess scattering length per unit mass ($\Delta\tilde{\varrho}(r)$ is the excess scattering length per unit mass):

$$\alpha = \frac{1}{V} \int \varrho_F(r) \cdot r^2 \cdot \mathrm{d}^3 r \tag{12}$$

A plot R_g^2 versus $1/(x_{0,av} - x)$ (Fig. 6) gives the value $R_{g,\infty} = 320 \pm 6$ Å. Assuming the coated vesicle to be a compact sphere the radius would give $R = 413 \pm 10$ Å.

Discussion

From the extensive work from EM studies on coated vesicles and cages it is known that clathrin exists in the form of triskelions which unite to a polygonic "sphere" (Pearse and Crowther 1987; Crowther et al. 1976). In the study of Crowther et al. (1976) on coated vesicles from pig brains two main classes of polygonic coats of clathrin were observed. One with 108 clathrin molecules and another with 84. By performing ultracentrifugation on the coated vesicles a weight average molar mass of 28 MD was determined. Assuming a protein to lipid mass ratio of 3 (Crowther et al. 1976) this gives an average of 100 clathrin molecules in the polygonic sheet. However, these studies were made with coated vesicles from pig brains with average sizes smaller than those observed in the present studies. In Vigers et al. (1986) the polygonic sheet was determined to be close to 200 Å in thickness. For a coated vesicle with a diameter of 900 Å, as observed in the present study, this would give a surface area of about $2.6 \cdot 10^6$ Å^2 or about 130 clathrin molecules, equivalent to a molar mass of clathrin equal to 24 MD, calculated by using a length of 150 Å in the hexagonal structure of the coat. Both the mass from light scattering as well as neutron scattering agrees with this number of clathrin molecules. Because of the uncertainty in the determination of the molar mass in both techniques we can only say that the presence of CAP proteins could account for up to about 30% of the protein mass. Note that the molar mass from the light scattering is dominated by the contribution from the protein part of the coated vesicle (the refractive index increment used is taken for proteins which contributes about four times as much as a polyunsaturated lipid chain).

As the distance distribution function is orientally averaged it only contains few features. In the present study counting statistics and polydispersity of the biological sample will smear out the distribution further and apart from confirming that the shape and overall size of the distance distribution function is as expected we only use the radius of gyration calculated from (5) and the value for $I(0)$ determined from (6).

The calculated D_{max} for the 0 and 75% data are in good agreement with the previously found dimensions. But the 42% data is some 200 Å higher than expected from the size of the membrane part of the clathrin coat. However the broad error band for large distances in the distance distribution function for the 42% data indicates that D_{max} is not determined very well owing to the weaker scattering at this contrast. Likewise, the error in R_g is greatest at this contrast (see Table 1).

Some degree of polydispersity is to be expected for the coated vesicles and this evident from the light scattering experiments as well as from the EM pictures (Fig. 3). Both these techniques give a polydispersity of about 30%. Furthermore, it should be noted that the shape of the internal membrane appears to be non-spherical, as can be observed in Fig. 3.

The most significant result of the contrast variation is the reduction of the intensity and the disappearance of the peak at $q = 0.025\,\text{Å}^{-1}$ in 42% D_2O. This was to be expected from the earlier interpretation of the peak as being produced by the interference terms in the clathrin coat internally as well as by interference terms between the clathrin coat (including CAPs) and the membranes. Both these interference terms should vanish in the 42% D_2O measurement.

The molecular mass of 34 MD is in good agreement with the mass estimated by Crowther et al. (1976). From the SANS measurements we also get the same ratio of protein to lipid as that determined by Pearse (1975). A value for the radius of the membrane can be estimated by comparison with the known mass-surface area relation reported in the literature for lipid membranes. From the data of Cusack et al. (1985) on the lipid membrane in influenza virus one finds a value of $18.7\,\text{D}/\text{Å}^2$, which leads to an estimate fo the outer diameter of the membrane of about 400 Å, in agreement with our observation from the electron microscopic picture. The uncertainty in the radius of gyration determined at 42% D_2O makes it meaningless to compare these values. However, the radius determined for the coated vesicles, equal to 413 Å from neutron scattering, agrees well with the diameter derived from dynamic light scattering. It should be noted, however, that a compact sphere of radius 413 Å, corresponding to the size determined from R_g at infinite contrast, would have a molar weight near 200 MD (using a density of $1\,\text{g/cm}^3$ to account for hydration of the protein). We are therefore able to conclude from this that the coated vesicles are far from being compact structures. The measured molecular mass gives a water content in the particles of about 85% compared to compact protein.

Earlier calculations from SANS data gave a molar weight for the clathrin coated vesicles of the order of 100 MD (Bauer et al. 1991), but the present result is in better agreement with the EM work discussed in Pearse and Crowther (1987). However, the previous experiments were performed on coated vesicles of somewhat larger dimensions, which could explain some of the discrepancy.

The distance distribution functions for the different contrasts are shown in Fig. 2. For comparison, the theoretical distance distribution functions for a sphere of radius 450 Å and for a shell of external and internal radii 450 and 350 Å are shown as inserts in Fig. 2a. The calculated distance distribution functions in Fig. 2 for 0 and 75% D_2O are clearly not consistent with assuming a simple sphere or a shell structure for the clathrin coated vesicle.

The interference peak observed at 0 and 75%, but absent at 42% could originate as the secondary maximum for a shell structure or as an interference term between the polygons. However, the negative interference observed in Fig. 5 by the constructed curve lying above the experimental curve for 75% D_2O gives evidence for two shells with different radii equivalent to a clathrin polygonic sheet and a membrane vesicle.

Conclusion and outlook

We have demonstrated that the structural information on coated vesicles can be obtained using neutron scattering and contrast variation. The results obtained by this method are consistent with information from other methods of measurements on clathrin coated vesicles. Contrast variation neutron scattering has demonstrated the presence of membrane vesicles inside the clathrin coat in a non-intrusive manner. Further studies of the conditions under which the vesicles are stable as well as the dynamics of the assembly processes could give important information about the basic mechanisms of the coated vesicles, i.e. the mechanisms governing the creation and breakdown of the clathrin coat. These mechanisms are not known very well, one of the reasons being the instability of the protein coat of the vesicles. The scattering experiments using X-rays, neutrons and light allows the preservation of the coated vesicles under near-physiological conditions and are therefore very well suited for investigation of the fragile protein/membrane-complex. Further studies using these techniques have been initiated with the aim of obtaining additional information about dynamic aspects of the coated vesicles and the basic hormonal regulation of the living cell.

Acknowledgements. The present work has received support from the Danish Natural Science, Technical and Veterinary and Agricultural Research Councils and the Swedish Natural Science Research Councils.

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