The Inverse Contrast Variation in Small-Angle Neutron Scattering: A Sensitive Technique for the Evaluation of Lipid Phase Diagrams

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Abstract
Small-angle neutron scattering experiments have been performed with aqueous dispersions of binary lipid mixtures. One component was protonated, the other was partly deuterated. By varying the mole fraction of the deuterated species the mean scattering-length density of the lipid lamellas and hence the contrast between liposomes and the solvent was changed. It is shown that this inverse contrast variation has the advantage of (i) a simpler data analysis, (ii) an increased resolution of homogeneous and heterogenous lipid distributions and (iii) a considerably increased sensitivity for the evaluation of phase diagrams in segregated lipid mixtures. Phase boundaries can now be determined to an accuracy of better than 1 mol%.

1. Introduction
Many studies in small-angle neutron scattering (SANS) of biopolymers in aqueous solvents employ the well-established contrast variation technique (Stuhrmann, 1973; Jacrot, 1976): By performing scattering experiments in buffers of different H2O/D2O ratios the contrast between the solute and the solvent can be varied over a wide range. Besides information about the size and the shape of the dissolved particles the different scattering curves in particular at low contrasts give also valuable insight into the internal structure of the molecules (Kneale, Baldwin & Bradbury, 1977; Stuhrmann & Miller, 1978).

In recent years, we have shown that this contrast variation technique can also be applied successfully to the investigation of the thermotropic polymorphism of aqueous lipid dispersions (Knoll, Haas, Stuhrmann, Füldner, Vogel & Sackmann, 1981; Knoll, Ibel & Sackmann, 1981). By the analysis of the scattering curves in terms of the Kratky–Porod model for lamellar systems (Kratky & Porod, 1948; Porod, 1948), the theoretical concepts developed for three-dimensional particles are applicable also to the study of the quasi-two-dimensional membrane lamellas. For example, the zero-angle intensity, $I_{00}$, and its dependence on the D2O volume fraction of the buffer yields information about the temperature-dependent mean scattering-length density of a lipid molecule, from which molecular volumes of the lipids in different physical states may be obtained (Knoll, Haas, Stuhrmann, Füldner, Vogel & Sackmann, 1981).

By deuteration of one component in a binary lipid mixture very high contrasts between segregated phases can be achieved. It is thus possible to distinguish clearly between homogeneous mixtures and systems that undergo a lateral phase separation. This is the basis for the evaluation of composition–temperature phase diagrams of binary lipid alloys (Knoll, Ibel & Sackmann, 1981). The method allows the determination of phase boundaries and excess volumes but is equally well suited to study dynamical heterogeneities in fluid membranes like critical concentration fluctuations (Knoll, Schmidt, Sackmann & Ibel, 1983).

This paper reports on the inverse mode of operation: At a constant H2O/D2O ratio in the solvents the amount of deuterated lipid in the membrane lamellas is changed, thereby varying the contrast between the solute and the solvent. We will show that this method has several advantages over the conventional contrast variation technique, among which are: (i) an easier and more reliable correction of the raw scattering data, (ii) together with a mass densitometric measurement (Knoll, 1981) of the lipid dispersion a considerably more accurate determination of molecular volume and degree of deuteration, (iii) and a by far more sensitive evaluation of phase boundaries in segregated lipid mixtures.

In passing we note that the proposed method has some common features with techniques based on the specific deuteration of some of the subunits in complex biopolymers (Koch & Stuhrmann, 1979). In our case, however, a continuous variation of the mean scattering-length density of a liposome composed of...
some $10^7$ lipid molecules can be achieved. Hence the term inverse contrast variation.

2. Experimental

(a) Materials and methods

1,2-dimyristoyl-sn-glycero-3-phosphorylcholine (DMPC) was obtained from Fluka (Buchs, Switzerland), 1,2-dimyristoyl-d$_{27}$-sn-glycero-3-phosphorylcholine (DMPC-$d_{24}$) was from Avanti (Birmingham, Alabama) and cholesterol was from Sigma (Heidelberg, Germany). All lipids gave a single spot on a thin-layer chromatography plate and were used without further purification. A more sensitive quality test for the two phospholipids was the temperature width of their main phase transition (Schmidt, 1984). This was measured with a densitometer (Paar Heraeus, model 602 HP/DMA 60). DMPC only showed the instrumental resolution limit ($\Delta T \approx 0.3$ K). DMPC-$d_{24}$, however, was slightly asymmetrically broadened ($\Delta T \approx 0.5$ K).

Multilamellar liposomes were prepared as described previously (Knoll, Ibel & Sackmann, 1981) by rinsing solvents containing H$_2$O (mfillipore quality) and D$_2$O (Merck, 99-67% isotopic purity as checked by densitometry) in the desired ratio across thin layers of the lipid mixtures, which were deposited onto glass walls by solvent evaporation. The D$_2$O content of the water mixtures was controlled by a density determination with a mass densitometric balance (Paar Heraeus, model 602 HP/DMA 60). DMPC and DMPC-$d_{24}$ differ by less than 1% within the temperature interval of interest so that the difference can be neglected. One only has to modify (3) if one performs experiments between the two phase-transition temperatures, which differ by 6 K, where the volumes change by about 4% (Knoll, 1981). (ii) The degree of deuteration has to be known accurately. This can be obtained by combining a scattering-length density determination with a mass densitometric measurement (Knoll, 1981).

The SANS experiments were performed with the D11 camera (Ibel, 1976) at the Institut Laue–Langevin (Grenoble, France) at a sample–detector distance of 10 m and a neutron wavelength of $\lambda = 10$ Å. The range of momentum transfer was $0.23 \times 10^{-2} < k < 2.2 \times 10^{-2}$ Å$^{-1}$. Scattering data were collected within 20 min for the samples with a low contrast. Temperature control of the thermostatted sample holder was better than 0.3 K.

(b) Data analysis

Analysis of the scattering curves was performed in a similar way to that discussed elsewhere (Knoll, Ibel & Sackmann, 1981). Since the samples of an inverse contrast variation (ICV) series all contain the identical buffer, the transmission correction of the coherent scattering contribution and the subtraction of the empty cell (which is also transmission dependent) and of the incoherent background intensity are the same for all samples. This reduces considerably the error of the coherent scattering intensity $I(k)$ especially for low contrast and hence low-intensity samples. Therefore, also the zero-angle intensities, $I_{d0}$, which are obtained from the Kratky–Porod plots according to

$$I(k)k^2 = I_{d0}\exp\left\{-R_g^2k^2\right\},$$  \hspace{1cm} (1)

are more accurate and allow for a more reliable determination of the mean scattering-length density of the lipids, $\bar{\rho}_p$, according to

$$I_{d0} \propto (\bar{\rho}_p - \rho_s)^2,$$  \hspace{1cm} (2)

where $\rho_s$ = scattering-length density of the solvent. $R_g$ is the thickness of gyration. In a contrast variation series, $\rho_s$ is varied until for a homogeneous lipid dispersion $I_{d0} = 0$, whence $\bar{\rho}_p = \rho_s$ (Knoll, Ibel & Sackmann, 1981). The ICV experiment is characterized by a constant $\rho_s$ while $\bar{\rho}_p$ is varied by replacing protonated lipid by deuterated (or vice versa). In this case, $\bar{\rho}_p$ depends linearly on the content, x, of deuterated lipid according to

$$\bar{\rho}_p(x) = \frac{1}{V} \left\{(1-x)b_p + xb_d\right\},$$  \hspace{1cm} (3)

where $V$ is the volume per lipid molecule, $b_p$ stands for the sum over all atoms (each having a coherent scattering length $b_i$) comprising a lipid molecule in its protonated form, and $b_d$ is the corresponding quantity of a (partially) deuterated lipid. Two aspects are noteworthy: (i) For a contrast variation experiment it was necessary to ensure that the molecular volume did not depend on the D$_2$O content of the buffer (Knoll, 1981). The analysis of the ICV is simplified if the replacement of a protonated lipid molecule by a deuterated one does not change the mean molecular volume, i.e. $V = \text{constant}$ in (3). It has been shown previously (Knoll, 1981) that the molecular volumes of DMPC and DMPC-$d_{24}$ differ by less than 1% within the temperature interval of interest so that the difference can be neglected. One only has to modify (3) if one performs experiments between the two phase-transition temperatures, which differ by 6 K, where the volumes change by about 4% (Knoll, 1981). (ii) The degree of deuteration has to be known accurately. This can be obtained by combining a scattering-length density determination with a mass densitometric measurement (Knoll, 1981).

In the case of a contrast variation study with phase-separated binary lipid mixtures the zero-angle intensity can be described by the sum of the contributions from the two phases (Knoll, Ibel & Sackmann, 1981)

$$I_{d0} \propto \frac{x_2-x_1}{x_2-x_1} (\bar{\rho}_1 - \rho_s)^2 + \frac{x_1-x_2}{x_2-x_1} (\bar{\rho}_2 - \rho_s)^2,$$  \hspace{1cm} (4)

where $x_i$ is the initial mole fraction of the mixture, $x_1$ and $x_2$ denote the mole fraction of the two separated phases and $\bar{\rho}_1$ and $\bar{\rho}_2$ are their respective scattering-length densities. The same expression holds for the ICV, the only difference being that $\rho_s$ = constant and both $\bar{\rho}_1$ and $\bar{\rho}_2$, which are composed of component A and component B, respectively, contain the former partly in protonated and partly in deuterated form.
The dependence of $I_{d0}$ on $x$ is therefore a more complicated expression:

$$I_{d0} \propto \frac{x_2 - x_1}{x_2 - x_1} (\rho_p^2 - \rho_s + \Delta \rho_1 x)^2 + \frac{x_2 - x_1}{x_2 - x_1} (\rho_p^2 - \rho_s + \Delta \rho_2 x)^2$$

with

$$\rho_p^2 = \frac{x_i b_p^2 + (1 - x_i) b_A^2}{V_i}$$

and

$$\Delta \rho_i = \frac{(1 - x_i)(b_A^2 - b_p^2)}{V_i}.$$ 

It depends on the scattering lengths of the protonated component $B$, $\rho_B^p$, and of the protonated and deuterated forms of component $A$, $\rho_A^p$ and $\rho_A^d$, $V_1$ and $V_2$ are the molecular volumes of phase 1 and phase 2, respectively. Both quantities are known from mass densitometric measurements. As we will show in the following, the dependence of $I_{d0}$ on the mole fraction, $x$, of deuterated component $A$ can then be fitted very sensitively by the proper choice of $x_1$ and $x_2$, the desired phase boundaries. As in the case of the usual contrast variation two series are necessary to determine both phase boundaries independently.

3. Results and discussion

(a) Homogeneous lipid distribution

Earlier experiments with lipid dispersions have shown that protonated and deuterated DMPC molecules mix ideally (Knoll, Haas, Stuhrmann, Füldner, Vogel & Sackmann, 1981). The simplest ICV experiment is therefore the replacement of some of the deuterated DMPC-d$_{54}$ molecules in the liposomes by protonated DMPC. The result is depicted in Fig. 1.

The sample temperature was 308.2 K, where the lipid is in its fluid state. The D$_2$O content of the solvent was 72.8%, which corresponds to a scattering-length density $\rho_s = 447 \mu m^{-2}$. $x$ denotes the mole fraction of DMPC-d$_{54}$. Shown are raw scattering data, which are only corrected for the empty cell contribution, but still show the incoherent buffer background $I_{inc}$. For dilute samples in identical aqueous solvents this background should be constant for all spectra. This can be seen in Fig. 1 only for the three samples with the lowest contrast (labeled $x = 0.88$, 0.90 and 0.92) because only for these samples is the coherent intensity contribution sufficiently low in the presented $x$ range. For better clarity, the different curves are shifted relative to each other by the constant $I_{inc}$ value and by $\kappa = 0.003 A^{-1}$.

As expected for the chosen D$_2$O content, the coherent scattering intensity decreases with increasing DMPC content until at $x = 0.9$ virtually no intensity in addition to the incoherent solvent signal can be detected. Upon further decrease of $x$ the coherent intensity reappears again.

If $I_{inc}$ is subtracted from each spectrum, the analysis of the scattering curves according to the Kratky-Porod model allows the determination of the zero-angle intensity, $I_{d0}$ (data not shown; see, however, the example given in Fig. 5). According to (2) and (3) a plot of $I_{d0}^{1/2}$ versus $x$ should give a straight line for a homogeneous lipid distribution. Within the experimental error this is fulfilled for the data presented in Fig. 1 and is shown in Fig. 2 (full circles). The dashed line is a least-squares fit and cuts the abscissa at $x = 0.897$. At this DMPC-d$_{54}$ mole fraction the mean scattering-length density of the mixed membrane is

![Fig. 1. Raw scattering data of multilamellar liposomes in aqueous buffers each containing 72.8% (vol/vol) D$_2$O. The samples were composed of protonated DMPC and different mole fractions, $x$, of deuterated DMPC-d$_{54}$. The spectra are shifted relative to each other for better clarity, Temperature was 308.2 K.](image)

![Fig. 2. Plot of $I_{d0}^{1/2}$, obtained from Kratky-Porod analysis of the scattering curves in Fig. 1. versus the mole fraction, $x$, of DMPC-d$_{54}$. Shown are data points for two different temperatures: - $\bullet$, $T = 308.2$ K, - $\circ$, $T = 280.2$ K.](image)
masked by the mixed solvent, which contains 72.8% D_2O. For higher x values positive contrasts, (\rho_p - \rho_s), are prepared; for lower x values (\rho_p - \rho_s) is negative. Therefore, it is reasonable to present the data with positive slope contrary to the usual contrast variation plots. Judged from the statistical error of the raw scattering data (see Fig. 1) a lower limit for the detection of any coherent scattering intensity in addition to the incoherent buffer contribution can be estimated. This is indicated in Fig. 2 by the shaded area.

Next, the samples are measured at T = 280.2 K (where the lipid is in the condensed state). If one again analyses the obtained scattering curves according to the Kratky–Porod model, one finds different zero-angle intensities, I_{00}. Their square roots are also plotted in Fig. 2 (open circles). The full line (again a least-squares fit) is parallel to the broken line through the data at T = 308.2 K and cuts the abscissa at x = 0.849.

As discussed earlier (Knoll, 1981), one needs in addition to a contrast variation experiment also a mass densitometric measurement in order to be able to obtain the molecular volume and the degree of deuteration, \eta, of a DMPC-d_{54} molecule independently. The same considerations hold for the results found by an ICV series. As one is here concerned with a mixture of DMPC and DMPC-d_{54} one needs a mass densitometric characterization of both molecules (Schmidt, 1984). We obtained \eta = 0.907 for T = 308.2 K and \eta = 0.897 for T = 280.2 K. The latter value was calculated taking into account the temperature-dependent scattering-length density of the solvent. As an average we take \eta = 0.902 and calculate from that \delta = 53.83 \times 10^{-12} \text{ cm} for DMPC-d_{54}. This value will be used in the analysis of the experiments with heterogeneous lipid distributions, which are presented in the next section. For the molecular volume of DMPC-d_{54} we obtain V = 1025 Å^3 at T = 280.2 K and V = 1093 Å^3 at T = 308.2 K.

With these values mean scattering-length densities, \bar{\delta}, of the different samples can be calculated as a function of the mole fraction, x, of the deuterated lipid. One obtains for x = 0.7: \bar{\delta} = 376 \mu\text{m}^{-2}; and for x = 1.0: \bar{\delta} = 525 \mu\text{m}^{-2}. This means that the two samples could be matched by 62% D_2O and 84% D_2O, respectively. The presented range of the ICV corresponds, therefore, to a variation of the D_2O content in a classical contrast variation by 22%.

(b) Heterogeneous lipid distribution

In order to demonstrate the advantages of the ICV over the conventional contrast variation for the evaluation of lipid phase diagrams we have performed experiments by both methods with a mixture of DMPC/DMPC-d_{54} and cholesterol. This system is partly immiscible and undergoes a phase separation. The full composition–temperature phase diagram will be described elsewhere (Knoll, Schmidt, Sackmann & Ibel, 1984). Here the results obtained with a lipid mixture at T = 280.2 K containing 10% cholesterol are presented.

First, we discuss the results of a conventional contrast variation experiment. Some of the scattering curves obtained at different H_2O/D_2O ratios in the solvent are shown in Fig. 3 as Kratky–Porod plots. As

$$\sqrt{I_{00}}$$, plotted as a function of the solvent D_2O content (vol%). The shaded area indicates the resolution limit. Experimental conditions as in Fig. 3.

![Fig. 3. Kratky–Porod plots for liposomes of DMPC-d_{54} containing 10% (mol/mol) cholesterol observed at different H_2O/D_2O ratios in the aqueous solvents: △ 12%; ▼ 50%; ○ 70%; □ 80%; ⊿ 100%. The temperature was 280.2 K. I_{00} is obtained by extrapolating the straight lines to \kappa^2 = 0.](image-3)

![Fig. 4. Square root of zero-angle intensity, I_{00}, plotted as a function of the solvent D_2O content (vol%). The shaded area indicates the resolution limit. Experimental conditions as in Fig. 3.](image-4)
for other contrast variation experiments with lipid mixtures the scattering intensities for \( k^2 > 1.5 \times 10^{-4} \text{Å}^{-2} \) can be fitted by straight lines, all with the same slope. Their interceptions with the ordinate yield extrapolated zero-angle intensities, \( I_0 \). Fig. 4 shows \( I_0^{1/2} \) of all scattering curves, plotted as a function of the \( D_2O \) volume fraction of the solvent. Again, the shaded area indicates the resolution limit as it is judged from the scatter of the neutron intensities of the low-contrast sample (80% \( D_2O \), open squares in Fig. 3). All data points can easily be described by a linear dependence of \( I_0^{1/2} \) on the \( D_2O \) content. From this result one would conclude that DMPC-\( d_{54} \) and 10% cholesterol mix homogeneously, \( i.e. \) that the cholesterol molecules are randomly distributed.

The ICV, however, allows for the examination of samples with still lower contrast between solute and solvent owing to the simplified correction of the raw scattering data. This is demonstrated in Fig. 5 for three samples of mixtures of DMPC/DMPC-\( d_{54} \) and 10% cholesterol. The \( D_2O \) volume fraction in the solvent was 73%. Shown are the Kratky–Porod plots to the same relative intensity scale as the scattering curves of Fig. 3. The direct comparison between the curves in the different spectra shows that with the ICV low-contrast samples down to the \( 10^{-5} \) intensity range can be analysed. This increases the resolution of the ICV by a factor of about 2–3 as compared to the conventional contrast variation. Consequently, smaller deviations from a homogeneous scattering-length density distribution of the lipid layers are detectable. This is shown in Fig. 6. Here, \( I_0^{1/2} \), taken from Kratky–Porod plots like those of Fig. 5, is plotted \textit{versus} the mole fraction, \( x \), of DMPC-\( d_{54} \). Quite evidently, the experimental data show that this lipid mixture is not completely homogeneous: \( I_0^{1/2} \) only shows a minimum as it is characteristic for slightly heterogeneous mixtures. Deviations from a true homogeneous and hence maskable mixture (dashed line) are significant only for \( I_0^{1/2} < 0.01 \). This, however, is just below the resolution limit of the conventional contrast variation. The full line through the data is a fit according to (5) with \( x_1 = 0.08 \) and \( x_2 = 0.24 \). Molecular volumes were taken from densitometric measurements: \( V_1 = 975 \text{Å}^3 \), \( V_2 = 925 \text{Å}^3 \). The same phase boundaries were found by fitting ICV series at \( x_1 = 0.14 \) and \( x_1 = 0.21 \) (Knoll, Schmidt, Sackmann & Ibel, 1984). The sensitivity of the method can be demonstrated by calculating \( I_0^{1/2} \) according to (5) for the same initial mole fraction \( x_i \) but with other phase boundaries \( x_i \). The result is plotted in Fig. 6 for \( x_3 = 0.24 \) and \( x_3 = 0.07 \) (dash-dotted curve) and \( x_3 = 0.09 \) (dotted curve), respectively. Given the experimental error of the data points, a sensitivity of better than 1 mol% for the determination of phase boundaries is achieved.

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References


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