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Aggregation behaviour of a phospholipid based on \( \alpha \)-(−)-threitol

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Abstract. The aggregation behaviour of a synthetic \( \mathrm{C}_4 \) phospholipid, containing two phosphate groups and two stearoyl groups, has been studied. The surfactant formed different types of aggregates in water depending on the concentration, the temperature and the treatment of the samples. Tilted bilayers were found at high surfactant concentrations, whereas at low surfactant concentrations intercalated bilayers could be identified. The intercalated structures could be converted into very small vesicles upon sonication. Tube-like aggregates were formed after storage at low temperature, which coagulated into thread-like superstructures.

Introduction

The properties and structure of biomembranes have attracted interest from chemists and biologists for many years. The structural complexity of these membranes requires a systematic approach to understand their function. Therefore, researchers have isolated membrane components and studied their properties. Since phospholipids are the main constituents of cell membranes, much attention has been focussed on models which describe the aggregation behaviour of these molecules. In more recent years, synthetic amphiphilic molecules with self-organising properties have been described. It has been shown that the aggregates formed by these amphiphiles can be used as models for some biomembranes.

Surfactant systems able to form chiral aggregates have been described in the literature. With the aim of obtaining aggregates with a high degree of dissymmetry, which can be applied as chiral matrices for catalytic systems, we designed a phospholipid, 1, containing two adjacent chiral centres. Although many synthetic phospholipid derivatives and other artificial amphiphilic phosphates are known, only a few of them contain more than one stereogenic centre. The (2S,3S) stereoisomer of 1, derived from \( \alpha \)-(−)-threitol, was synthesised starting from \( \alpha \)-(−)-tartaric acid. A study of its properties and aggregation behaviour in water by different techniques including electron microscopy, polarisation microscopy, differential-scanning calorimetry, \( ^{31} \text{P} \) nuclear magnetic resonance, X-ray diffraction, and Brewster angle microscopy on monolayers is presented here.

Determination of \( pK_a \) values

The \( pK_a \) values of 1 were determined, in order to select a buffer system in which the charge of the molecules during aggregation experiments could be controlled. The values for \( pK_{a1} \) and \( pK_{a2} \) in water were determined by titrating a sonicated dispersion of fully protonated 1 with an aqueous sodium hydroxide solution. The \( pK_{a1} \) and \( pK_{a2} \) values were found to be 1.8 and 10.2, respectively. The fact that the compound showed only two \( pK_a \) values for four acidic protons, implies that both phosphate head groups are deprotonated simultaneously. A similar effect has been observed in deprotonation studies of \( \alpha \)-myo-inositol hexaphosphate, where only two \( pK_a \) values are observed for the abstraction of the second protons of six phosphate groups. The \( pK_a \) values of phosphatidic acids have been determined by titration (\( pK_{a1} = 3.9 \pm 0.1 \); \( pK_{a2} = 8.6 \pm 0.3 \)) and \( ^{31} \text{P} \)-NMR experiments (\( pK_{a1} = 3.5 \); \( pK_{a2} = 9 \)). The fact that the \( pK_a \) values for 1 differ from those determined for phosphatidic acids may be caused by a difference in the aggregation behaviour, which has been found to influence these values.

In view of the above findings, further experiments were carried out at pH = 7 where each phosphate group is in its monoprotonated state.

Electron microscopy

The aggregation behaviour of compound 1 was studied in 2mM PIPES (1,4-piperazinediethanesulfonic acid) buffer (pH 7) using freeze-fracture electron microscopy. Samples were prepared by vortexing the suspension of 1 in a buffer solution at 70°C. The results of this study are presented in Figure 1. Stacked bilayer structures were observed at high amphiphile concentrations (20–80% w/w), whereas at low concentrations (0.1–1.0% w/w) small platelets were visible.
**Polarisation microscopy**

A sample containing very small amounts of water (< 5%) was examined with crossed polarisers, in the temperature range 30–80°C. Under these conditions one distinct texture was observed. The surfactant as such did not show any birefringence. After addition of water a second texture type appeared at the water–surfactant interface in the form of oily streaks [18], suggesting a second type of aggregation in the more hydrated region (Figure 2).

**Differential-scanning calorimetry (DSC)**

Thermograms were recorded using suspensions of 1 in buffered solutions of varying surfactant concentrations in the range from 1 to 99% (w/w) (Figure 3). Highly concentrated samples showed an endothermic peak at $T_m$, 45°C ($\Delta H = 30 \pm 1$ J/g), indicating a phase transition from a gel to a liquid–crystalline state [19]. Lowering the concentration gave rise to a second phase transition at 38°C. This became the main transition when the amount of amphiphile was approximately 50%. Further dilution of the sample caused the endotherms to shift to lower temperatures, which is common for phospholipid samples [20]. Finally, a 1% dispersion of 1 again showed a single phase transition. This appeared at 20°C ($\Delta H = 88 \pm 3$ J/g). These results are in good agreement with the data from the electron-microscopy experiments (see above), where two distinct morphologies were observed depending on the concentration of the surfactant molecules used.

The transition entropies calculated from the data obtained from these experiments can be compared with that calculated for distearoylphosphatidic acid (DSPA) [21] (Table I).

From these values, one may conclude that the packing of the molecules of 1 at low water content is less efficient than that of DSPA molecules, whereas at high water content the reverse is true.

**31P-NMR spectroscopy**

31P-NMR studies were performed on aqueous dispersions containing various concentrations of surfactant in the temperature range from 10 to 70°C (Figure 4). At 70°C...
the sample containing 80% (w/w) surfactant showed an anisotropic signal, similar to signals obtained previously \(^{18,22}\) for phosphatidic acid, with a maximum at \(-6\) ppm. This chemical shift anisotropy is due to restricted motion of the phosphate head groups and indicates the presence of bilayer structures \(^{22,23}\). When the temperature of the samples was lowered, a second signal appeared at \(-1.9\) ppm, which indicates the formation of a different type of aggregate \(^{24}\). On further decreasing the temperature and passing the phase transition this isotropic signal remained, superimposed on a faint, very broad peak.

The effect of varying the surfactant concentration was studied at different temperatures. Lowering the concentration from 80 to 1% (w/w) at 70°C led to a downfield shift of the signal originally present at \(-6\) ppm, and to a decrease in the chemical shift anisotropy. At all the concentrations used, the same apparent sharpening of signal with decreasing temperature was observed, as described for the sample containing 80% (w/w) of the surfactant.

The \(^{31}\)P-NMR results support the conclusions from the DSC measurements, polarisation-microscopy and electron-microscopy experiments, that the amphiphile concentration determines the type of aggregate formed. Low water contents seem to favour the formation of large bilayer structures, whereas at higher water concentrations the aggregates are smaller and therefore less restricted in the averaging of chemical-shift anisotropy of the NMR signal.

The distribution of the molecules over both types of aggregates also depends on the temperature of the samples. Lowering of the temperature favours the formation of the smaller aggregates exhibiting relatively low chemical-shift anisotropy. Raising the temperature will increase the number of gauche conformations in the hydrocarbon chains, and therefore increase the volume of the hydrophobic parts of the molecules. This change of temperature may cause the molecules to adopt a different form of aggregation, which is a well-known phenomenon for various classes of lyotropic phospholipids \(^{13,25}\). The transition between the two types of aggregates appears to occur gradually, and, therefore, could not be detected in DSC experiments.

**Table I** Data derived from calorimetric experiments.

<table>
<thead>
<tr>
<th>Sample</th>
<th>(T_m) (°C)</th>
<th>(\Delta H) (J/g)</th>
<th>(\Delta S) [J/(g·K)]</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (99%)</td>
<td>45.1</td>
<td>30</td>
<td>0.09</td>
</tr>
<tr>
<td>1 (1%)</td>
<td>19.7</td>
<td>88</td>
<td>0.30</td>
</tr>
<tr>
<td>DSPA(^{22})</td>
<td>72.5</td>
<td>59</td>
<td>0.17</td>
</tr>
</tbody>
</table>

**X-ray diffraction**

Powder diffraction patterns were recorded on samples of compound 1 having different levels of hydration. Reflections between 3 and 60° were analysed and interpreted in terms of high-order reflections of stacked bilayer structures. The diffraction pattern of a sample containing 98% (w/w) surfactant showed six higher order reflections \((n = 3, 4, 5, 6, 8, 11)\) corresponding with a bilayer periodicity of \(d = 57.5 ± 0.3\) Å. The lipid bilayer thickness was calculated to be of the same magnitude within these experimental errors (Eqn. 1, see below).

Assuming the fatty-acid chains to have an all-trans conformation, the thickness of the bilayer was estimated to be 60 Å from CPK models of two molecules oriented parallel to the bilayer normal. The measured value of 57.5 Å suggests, therefore, a tilted orientation of the molecules with respect to the bilayer normal.

In the diffraction pattern a broadened reflection representing a periodicity of 4.11 Å and a faint reflection representing a periodicity of \(4.11/(\sqrt{3})\) Å were observed. These reflections are typical for the hexagonal packing pattern of the hydrocarbon chains in lipid bilayers. The broadening of the reflections indicates a distorted packing commonly found in tilted bilayers \(^{26,28}\).

The occupied area per surfactant molecule and the area per hydrocarbon chain were calculated to be \(S = 47.5\) Å\(^2\) and \(\Sigma = 19.7\) Å\(^2\), respectively \(^{29}\). The tilt angle of the bilayer can be determined from \(\cos\theta = 2S / \Sigma\), which results in \(\theta = 34.0\)°. The \(S/\Sigma\) ratio of 2.4 shows that the occupied area per molecule is large enough to accommodate 2.4 hydrocarbon chains. This indicates that under the conditions used, this area is determined by the head groups of the molecule rather than by its hydrophobic part. This additionally suggests a loosely packed hydrocarbon region, which is consistent with the thermodynamic parameters derived from DSC experiments (Table I).

The wide-angle diffraction pattern derived from a 1% (w/w) suspension of the surfactant did not show any characteristic peaks or bands \(^{30}\). This suspension was subsequently dried below its phase transition temperature (preventing the reorganisation of the aggregates) until the water content was approximately 1.5%. This dried sample displayed many high-order reflections from which two periodicities could be derived, as well as a weak, but sharp reflection representing \(d = 4.12\) Å. One d value (19 reflections) was calculated to be \(40.2 ± 0.3\) Å, and the other (20 reflections) was \(d = 38.7 ± 0.3\) Å. These values may be rationalised as the thickness of the hydrated and the dehydrated forms of the aggregate, respectively. From these values, using the Luzzati formalism \(^{26}\) (Eqn. 1), the lipid bilayer thickness can be calculated. Assuming a local water content of 2% (w/w) and using the d value of 40.2 Å, Eqn. 1 resulted in a bilayer thickness of \(d = 38.6\) Å, which is in agreement with the assumption that the d value of 38.7 ± 0.3 Å represents the thickness of a dehydrated bilayer. This extraordinarily small value implies that the bilayer has a fully intercalated structure \(^{31}\), indicated schematically in Figure 5.

\[
\begin{align*}
\frac{d_1}{d_1 + (\nu_2/\nu_1) \cdot (1 - C)/C} &= 1 + (\nu_2/\nu_1) \cdot (1 - C)/C \\
\end{align*}
\]

\(d_1\): the thickness of the bilayer
\(d\): the bilayer periodicity
\(\nu_2\): the partial specific volume of water
\(\nu_1\): the partial specific volume of the surfactant
\(C\): the surfactant concentration

Calculation of the area occupied per surfactant molecule and the area per hydrocarbon chain from these data,
resulted in $S = 70.7 \text{ Å}^2$ and $\Sigma = 19.6 \text{ Å}^2$, respectively. The $S/\Sigma$ ratio was calculated to be 3.6, which implies that the molecular area in these aggregates is enough to be occupied by 3.6 alkyl chains. Additional small reflections were also detected in the sample, representing $d$ values of 8.8 Å, 1/2 (8.8 Å), 1/3 (8.8 Å), and 1/4 (8.8 Å), together with three sharp reflections representing periodicities of 2.81 Å, 1.62 Å, and 1.61 Å. No lattice dimensions could, however, be assigned to those numbers.

**Monolayer experiments**

Pressure–area isotherms of 1 were recorded under various conditions. Simultaneously, the compressed monolayers were observed with a Brewster Angle Microscope (BAM)\textsuperscript{32}. The $\Pi$–$A$ diagrams, recorded at pH 7 and at temperatures of 10, 20 and 30°C, in all cases showed a phase transition from a liquid-expanded (LE) to a liquid-condensed (LC) state\textsuperscript{33} (Figure 6). Brewster angle micrographs of the different phases at 20°C clearly showed the increasing degree of hydrocarbon-chain packing which takes place when the system proceeds from the LE phase, through the LE–LC coexistence phase, to the LC phase. This phase transition is pseudo-first order, as assessed from the close-to-zero slope in the transition area\textsuperscript{34,35}. At 30°C the phase transition occurs at relatively high area per molecule, reflecting the expansion of the hydrophobic region, due to an increased number of gauche conformations. The compressibility of the monolayer at this temperature is low, as is evident from the fact that both the LC–LE transition and the collapse occur at lower pressure.

The isotherms recorded at 10 and 20°C are very similar, except for the phase-transition pressure, which is lower at the latter temperature. This suggests that, at these temperatures, unlike at 30°C, conformational motions in the hydrocarbon chains play a minor role in determining the area per molecule. The molecular area is dependent on the size of the head group, as follows from experiments at different pH values of the subphase (Figure 7).

At pH 1, where both phosphate groups of 1 are fully protonated, the molecular area is relatively small as a result of the reduction of the electrostatic repulsion. At pH 12, where the molecules bear a fourfold charge, the area per molecule in the liquid-expanded phase is larger. At this pH no transition to the liquid-condensed state was observed. This can be explained by the assumption that, in its fully deprotonated state, the compound dissolves in the subphase at pressures over 36 mN/m. The isotherms in Figure 7 were compared with those reported in the literature for distearoylphosphatidic acid (DSPA)\textsuperscript{31}. No significant differences were observed for surface pressures below $\Pi$ 30 mN/m. No data at higher pressures have been reported. This prevents the comparison of the phase transitions which, in all the cases examined here, occurred at pressures between 30 and 40 mN/m. The area per molecule, as deduced from the isotherm at pH 7 and $T$ 20°C by extrapolating the slope of the isotherm in the LE phase to zero pressure, amounts to 70 Å\(^2\)/molecule. This value may be regarded as the molecular area of a completely hydrated surfactant molecule and can therefore be compared to the value of $S = 70.7 \text{ Å}^2$/molecule, derived from X-ray experiments. Extrapolation of the slope of the isotherm in the LC phase, where the head groups are believed to be closely packed, leads to a molecular area of 45 Å\(^2\)/molecule, which is in agreement with the value of 47.5 Å\(^2\)/molecule derived from the diffraction data.

**Vesicle formation**

Freeze–fracture electron micrographs showed that 1 forms vesicles when its dilute dispersions were sonicated for 1 h at 70°C (Figure 8). The dimensions of these vesicles (300–500 Å) suggest a highly curved surface. The critical-packing radius ($R_c$) for vesicles can be estimated using the relationship\textsuperscript{3,36} in Eqn. 2. An $R_c$ value of 204 Å was calculated when an $a_0$ value of 70.7 Å\(^2\) was used for the molecular area at low surfactant concentration. This is in good agreement with a radius of 150–250 Å obtained from electron microscopy observations.

$$R_c = \frac{l_c}{\frac{1}{V_0}a_0 \cdot l_c}$$

$l_c$: the maximum length of the molecule
$V_0$: the volume of the hydrophobic part of the molecule
$a_0$: the area of the polar head group

Using the approximation\textsuperscript{3} in Eqn. 3, the number of molecules per vesicle can be estimated. For a bilayer thickness of 40 Å, this number amounts to 20000. The ratio of molecules on the outer and inner side of the
membrane then follows from $R_e^2/(R_c - t)^2$ and amounts to 1.6.

$$N = 4 \cdot \pi \cdot \frac{R_e^2 + (R_c - t)^2}{a_0}$$ \hspace{1cm} (3),

$t$: the bilayer thickness\(^{37}\)

Formation of tubuli

When a 0.1 M ammonium formate buffer of pH 8 was used to prepare a 20% dispersion of 1, instead of PIPES, and kept at −18°C for two months, the formation of thin white threads was observed. Samples of these threads were dried on a microscope grid, shaded with platinum and carbon, and examined with transmission-electron microscopy. The electron micrographs (Figure 9a and b) showed the presence of tubuli\(^{38}\), which probably arise from rolled-up, tape-like aggregates. Scanning-electron microscopy showed that these tubes were assembled in thread-like structures with diameters of approximately 0.5 μm (Figure 10).

The tubuli showed a sharp phase transition in DSC at 69°C, proving that they are not merely anhydrous microcrystals, as the pure compound displayed only a very weak transition at 80°C.

Conclusions

The aggregation behaviour in water of a new chiral phosphatidic acid analogue 1 derived from $\alpha$-(-)-threitol has been investigated using a variety of techniques, which provided a set of consistent results. Electron microscopy revealed the presence of different types of aggregates in suspensions of 1, which were dependent on the concentration, the temperature, the buffer and the treatment of the sample. Suspension of 1 at different concentrations led to two different types of structures: stretched bilayers were predominantly formed at higher concentrations, whereas smaller particles were observed at lower concentrations. The latter could rearrange to very small vesicles upon sonication above the phase-transition temperature. Tubuli were formed when a 20% (w/w) suspension of 1 in a 0.1M ammonium formate

buffer was kept at $-18^\circ$C for two months. These coagulated to form large thread-like superstructures.

The two types of aggregates that are initially formed after suspension of the surfactant 1 in a buffered solution, were characterized using various experimental techniques. The aggregates formed upon dispersion of high concentrations of 1 in a buffered solution, were identified as bilayers in which the surfactant molecules had a tilted orientation. From powder-diffraction data a bilayer thickness of 57.5 Å and a tilt angle of 34° were calculated. The area occupied per surfactant molecule, calculated from the same data, was in agreement with the molecular area derived from monolayer experiments, and amounted to 47.5 Å$^2$. This area was large enough to accommodate 2.4 hydrocarbon chains, which suggested a bilayer in which the molecular area is determined by the size of the head group, and in which, as a consequence, the hydrocarbon chains are loosely packed. These suggestions were confirmed by monolayer experiments and DSC measurements, respectively.

The small platelets, present at low surfactant concentrations, were found to be bilayer structures with a thickness of 38.6 Å, which points to an intercalation of the hydrocarbon chains. The molecular area of 70.7 Å$^2$, calculated from X-ray data, was in good agreement with results derived from monolayer experiments. This area would provide room for 3.6 hydrocarbon chains, which is consistent with intercalation of the alkyl chains. The high degree of hydrocarbon-chain packing required for the formation of this type of intercalated bilayer, could also be derived from the thermodynamic data, obtained from DSC experiments.

The structure of both types of bilayers described above, is represented schematically in Figure 5. The ability of the surfactant to adopt different types of aggregation, depending on the water content of the sample, is remarkable. The size of the phosphate head groups of various classes of phospholipids is known to depend on their charge and degree of hydration. The introduction of a second phosphate moiety probably enhances the effect of hydration on the size of the head group. This effect most probably enables 1 to adopt the head-group size required for the formation of the different types of aggregates, e.g. the intercalated and non-intercalated bilayer structures, favoured by the specific experimental conditions.

Both in electron-microscopy and in monolayer experiments we hoped to find proof for the formation of superstructures that can serve as chiral matrices, as mentioned in the Introduction. Although the compound does contain two adjacent chiral centres, it failed to express its chirality on a macromolecular scale because no chiral aggregates are formed, detectable by microscopic techniques.

### Experimental

**Determination of pK_a values**

Typically 10 mg (0.0117 mmole) of compound 1 was suspended in a 50 ml 0.01M H$_2$SO$_4$ solution by sonication for 30 min at 70°C, and subsequently titrated with a 0.1M NaOH solution in the same medium. pH values were measured using a Chemtrix-60A pH meter with a Orion Ross pH electrode 81-02.

**Freeze-fracture electron microscopy**

Freeze-fractured samples were prepared by incubating and vortexing buffered dispersions of the amphiphile above phase transition for 1 h. A drop of the dispersion was brought onto a golden microscope grid (150 mesh), placed between two copper plates and fixed at supercooled liquid nitrogen. Sample holders were placed in a Balzers Freeze Etching System BAF 400 D at 10$^{-7}$ Torr and heated to $-105^\circ$C. After fracturing the samples were etched for 1 min ($\Delta T$ 20°), shaded with Pt (layer thickness 2 nm) and covered with carbon (layer thickness 20 nm). Replicas were allowed to heat up to room temperature and left on a 20% chromic acid for 16 h. After rinsing with water they were allowed to dry and studied under a Philips TEM 201 (60 kV).

**Polarietation microscopy**

A sample containing 5% of water was placed on a glass plate and studied under a Jeneval microscope, equipped with a THMS 600 hotstage and a Sanyo VHS videocamera, using crossed polarisers. The sample was heated from 27 to 80°C and a drop of water was added.

**Differential-scanning calorimetry (DSC)**

Thermograms were recorded using a Perkin&Elmer DSC7. Samples were prepared in stainless-steel large volume pans (75 µl). After addition of surfactant and buffer solution the pans were sealed and placed in a bath-type sonicator at 70°C. The samples were sonicated for 1 h and left overnight. After incubation at $0^\circ$C for 15 min, heating runs were recorded from 0 to 60°C.

**$^{31}$P-NMR**

Samples were prepared by adding 2.0mm PIPES buffer to a weighed amount of compound 1 in a 10-mm NMR tube. The samples were thermostated at 70°C for 60 min and vortexed frequently. 81-MHz spectra were recorded using a Bruker WM200 NMR spectrometer supplied with an Aspect 2000. Proton-decoupled spectra were obtained from 200–2000 transients, the $\pi/2$ pulse being 22 µs, with an interpulse time of 1 s. Chemical shifts are reported relative to trimethyl phosphate.

**Powder diffraction**

Powder diffractometry was carried out with a Philips PW1710 diffractometer equipped with a Cu LFF X-ray tube at 40 kV and 55 mA. Experiments were carried out between 3 and 60°, using a step width of 0.01°. Dispersions were prepared by sonication for 1 h and left standing for 16 h before use. Samples were prepared on a silicon single crystal and quickly dried at room temperature, under a stream of air.

**Monolayer experiments**

Isotherms were recorded on a thermostatted, home-built trough (140×210 mm). The surface pressure was measured using Wilhelmy plates mounted on a Trans-Tek transducer (Connecticut USA). The surface of compressed monolayers was studied with a Brewster Angle Microscope (NFT BAM-1) equipped with a 10-mW He–Ne laser with a beam diameter of 0.68 mm operating at 632.8 nm. Reflections were detected using a CCD camera and images were recorded on a Panomic super VHS videorecorder. On the subphase (Milli-Q water adjusted to the desired pH) 50–150 µl of a solution of I in chloroform (0.33 mg/ml) was spread and allowed to evaporate for 30 min. The rate of compression was 7.0 cm$^2$/min.

**Scanning-electron microscopy**

A drop of the sample was placed on a glass plate and allowed to dry. The dried sample was covered with layer of gold using a Balzers Gold Sputter Unit and examined at 15°C under a JEOL Scanning Microscope T300 (15 kV).

### Acknowledgements

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References and notes


11. S = 2-d{m, (v/d)/N}-10-24, where m is the molecular weight, d is the partial specific volume of the surfactant, v is the cross-section of the bilayer, and N is Avogadro’s number. From models of the volume I was estimated to be approximately the same as for distearoylphosphatidyl choline: n = 0.958 g/cm² (Ref. 29).

12. S = 2-d{m, (v/d)/N}-10-24, where d is the side of a cell in the hexagonal lattice in which the hydrocarbon chains are packed (Ref. 28). The value of d is generally in the range 4.0-4.5 Å (Ref. 26-30).


17. S = 2-m (v/d)/N}-10-24, where m is the molecular weight, d is the partial specific volume of the surfactant, v is the cross-section of the bilayer, and N is Avogadro’s number. From models of the volume I was estimated to be approximately the same as for distearoylphosphatidyl choline: n = 0.958 g/cm² (Ref. 29).

18. S = 2-d{m, (v/d)/N}-10-24, where d is the side of a cell in the hexagonal lattice in which the hydrocarbon chains are packed (Ref. 28). The value of d is generally in the range 4.0-4.5 Å (Ref. 26-30).


25. v₀ = 27.6 + 26.9 n Å², lₚ = 1.5 + 1.265 n Å, where in this case n = 17. See: C. Tanford, J. Phys. Chem. 76, 3020 (1972).

26. t = 2-tₚ/v₀ (Ref. 3).
