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Insights into the self-reproduction of oleate vesicles

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Abstract

In view of the importance of vesicles as models for early cells, several groups have started work looking for conditions under which vesicles can undergo growth and division. Evidence for growth and division has been obtained with the help of ferritin-labelled vesicles; furthermore, it has been shown that in such processes the vesicle size distribution is largely conserved. In both cases, the data suggest that the process under study is mainly characterized by vesicle growth and eventually division into daughter vesicles. However, direct evidence for vesicle division has not been obtained. In this paper, mostly based on freeze-fracture electron microscopy, we describe conditions under which for the first time division intermediates can be trapped in the form of twin vesicles. This finding, together with supporting dynamic light scattering and fluorescence investigations, permits us to establish some additional points in the mechanism of vesicle self-reproduction.

1. Introduction

The formation of compartments in the early prebiotic times represents a key step in the origin of cellular structures [1–5]. Following the original reports of Deamer [6] and Cistola [7, 8], several authors investigated fatty acid vesicles as protocell models. This interest was born out of the observation that fatty acids have been found in meteorites and they are therefore to be considered prebiotic [1, 2]. In the last few years, several aspects of these systems have been studied and reported in the literature, like their self-assembly [9, 10], physico-chemical properties [11–13], interaction with lipid vesicles [9, 14–17], self-reproduction [18–20] and competition processes [21, 22].

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The external addition of a fresh surfactant precursor to a vesicle population can simulate a prebiotic scenario in which a membranogenic compound, synthesized *in situ*, binds to pre-existing parent vesicles, thus leading to their growth and eventually division into daughter compartments.

Recently we investigated this process by feeding oleic acid/oleate vesicles (for short, 'oleate vesicles') with oleate micelles, in order to study whether and under which conditions vesicle division can occur in the absence of finely tuned biochemical cellular control [9, 23].

In this paper we report on the electron microscopy observation of a particular vesicle structure, trapped during the transitory phase of oleate vesicle formation. The existence of such structure strongly supports the vesicle division model.

2. Materials and methods

2.1. Materials

The sodium oleate (>99%), bicine (>99.5%), calcein (>99%) and sodium cholate (>99%) were from Fluka (Buchs, Switzerland).

2.2. Preparation of the pre-formed vesicles

Extruded oleate vesicles were prepared as described elsewhere [9]. Calcein-containing vesicles were prepared similarly using calcein-containing buffer; free calcein was removed by gel filtration spin column chromatography, using Sepharose 4B (Pharmacia) as the stationary phase.

2.3. Vesiculation of oleate micelles

Oleate micelles were prepared by dissolving sodium oleate in ultrapure water (18 MΩ Milli-Q type), and then injected into bicine buffer (0.22 M, pH 8.5). In a typical matrix effect experiment, oleate micelles are added to a solution of pre-formed extruded vesicles in bicine buffer; the volume injected is generally $\leqslant$10% of the total volume. The experiments were carried out directly in the measurement cell, generally kept at 25.0 °C.

2.4. Dynamic light scattering (DLS) and freeze-fracture electron microscopy (ffEM) analysis

DLS measurements were carried out with an ALV home-assembled light scattering spectrometer made of a 25 mW He–Ne laser (Model 127, Spectra-Physics Lasers, Mountain View, Canada), an ALV DLS/SLS-5000 Compact Goniometer System (ALV, Langen, Germany), two SPCM-AQR avalanche photodiodes (Perkin-Elmer Optoelectronics, Vaudreuil, Canada) and an ALV-5000 Multiple-tau Digital Correlator (ALV, Langen, Germany). All the experiments were performed at the scattering angles 60, 90 and 120°; other settings were: solvent viscosity 0.899 mPa s, solvent refractive index 1.33. Great care was taken to avoid the presence of dust in every step of the vesicle preparations, which were analysed—unless specified otherwise—without any pre-treatment. Intensity weighted size distributions were calculated by the CONTIN algorithm, using programs provided by the manufacturer.

Microscopy analysis: 100 μl of 111 mM sodium oleate micelles in water were injected, by means of a Hamilton microsyringe, into: (i) the control experiment: 1 ml of bicine buffer (0.22 mM, pH 8.5); (ii) the matrix effect experiment: 1 ml of 11 mM pre-formed oleate vesicles (extruded to a final size of 100 nm) in bicine buffer (0.22 M, pH 8.5). Samples were taken after different time intervals from the injection and immediately frozen in a propane jet freezer [24].

The specimens were fractured at −150 °C and this was followed by immediate platinum/carbon shadowing in a Balzers BAF 300 Freeze-Etcher.

2.5. Fluorescence determination of calcein release

In a typical experiment, 10 μl of 20 mM oleate micelles were added to 200 μl of calcein-containing '100 nm' oleate vesicles (2 mM). Complete release was obtained by destroying calcein-containing vesicles by addition of sodium cholate (cholate/oleate 40/1 mol/mol). The oleate micelle-to-vesicle transition was followed using a Perkin-Elmer LS50B spectrofluorimeter, exciting the sample at 490 nm and collecting the emitted radiation at: (i) 490 nm to monitor the scattered light, as a probe for the vesicle formation; (ii) 514 nm to monitor the concentration-dependent calcein fluorescence. The measurements were performed at 25.0 °C using a fluorescence microcell (Hellma); other settings were: excitation slit: 5 nm; emission slit: 3 nm.

3. Results and discussion

When oleate micelles (pH ∼11) are added to a buffer solution having pH in the range 8–9, there is a spontaneous micelle-to-vesicle transition leading to a morphologically heterogeneous sample with a broad particle size distribution, ranging from 50 nm to about 5 μm in size. In contrast, when the same experiment is performed in the presence of pre-formed oleate vesicles of a certain homogeneous size, scattering measurements indicate that oleate micelles interact with pre-formed vesicles. Although these data have been partly published, we show a typical example in figure 1(A), mostly with the aim of permitting a comparison with the corresponding electron microscopy data taken under the same conditions.

The higher vesiculation rate (faster than in the control experiment; see figure 1(B)) and the analysis of the particle size distribution (biased to the distribution of the pre-formed vesicles; see figure 1(A)) indicate that, in the presence of pre-formed vesicles, the process of autonomous (or *de novo*) vesiculation is strongly reduced, and the new vesicles resemble the pre-added ones. The phenomenon, called the 'matrix effect', is a unique example of structural information transfer among large supramolecular structures [9, 14, 19, 20].

As already mentioned, recent mechanistic studies on these processes have been based on dynamic light scattering (DLS). Earlier studies based on cryo-transmission electron microscopy should also be mentioned [11, 20]. In that case, POPC (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidylcholine) vesicles were labelled with internalized ferritin, an iron-rich protein. After the addition of oleate micelles, the analysis of the distribution of ferritin-containing vesicles suggests the division of some of the parent vesicles [20], because the vesicles that contained ferritin molecules were smaller than the 'parent' vesicles. No evidence of intermediate states could be obtained.

In order to shed more light on these mechanisms, and in particular with the aim of trapping some intermediates, we carried out a freeze-fracture electron microscopy (ffEM) analysis of the sample in the initial, transitory and final states of the micelle-to-vesicle transformation.

Oleate micelles were injected into bicine buffer (pH 8.5) to induce the spontaneous vesiculation in the absence of pre-formed oleate vesicles (control experiment) and in a suspension of pre-formed 100 nm extruded vesicles. For the electron microscopy analysis, samples were taken before and after different time intervals from the injection and immediately frozen in a propane jet freezer [24].

Figure 2(A) is a micrograph of oleate vesicles obtained by spontaneous vesiculation in the buffer. Large and heterogeneous particles are present. Vice versa, when oleate micelles

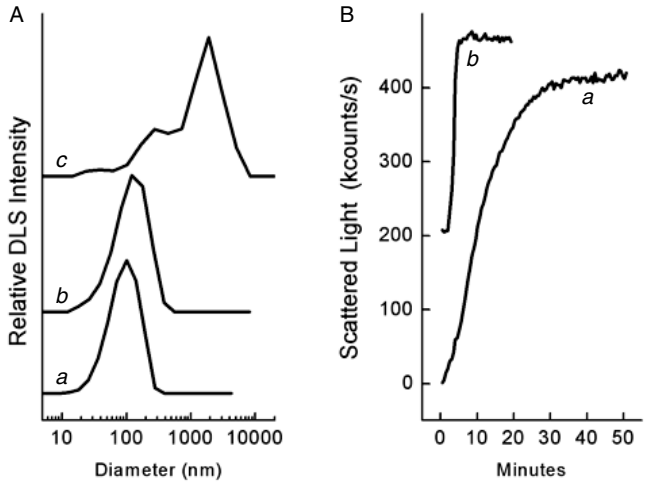

Figure 1. (A) DLS CONTIN analysis (90°) of oleate vesicles. Pre-formed oleate vesicles were produced by the extrusion technique, providing a rather homogeneous population of '100 nm' oleate vesicles (curve *a*); then micelles were injected into the suspension, at micelle-to-vesicle ratio 1:1 (curve *b*). When micelles were injected into the buffer, i.e., without the presence of pre-formed vesicles, a broad size distribution was obtained (control, curve *c*). (B) Scattered light profiles for the vesiculation of oleate micelles in bicine buffer (curve *a*), and in the presence of an equimolar amount of pre-formed extruded vesicles (curve *b*). Notice the dramatic rate enhancement produced by pre-formed vesicles (curve *b*): the final state was reached after about 30 min in the control experiment, whereas in the case of the matrix effect it was in less than 3 min.

are injected into a suspension of pre-formed and homogeneous extruded oleate vesicles (figure 2(B)), the final size distribution is closely related to the initial one (figure 2(C)). These results are in good agreement with DLS analysis (figure 1(A)).

By quick sampling and freezing of the reactive mixture during the relatively short transitory phase (<3 min), we were able to trap an intermediate vesicular structures of the process under study. The micrographs, shown as figure 3 (and enlarged as figure 4), clearly indicate the presence of characteristic structures, 'twin vesicles', that are present during the process. Notice that these particular structures are relatively abundant: approximately 20% of the objects depicted in the electron micrographs are twin vesicles. This is a quite high value, suggesting that such particles represent a common intermediate in the pathway of the observed process.

As regards the interpretation of the results, first of all we rule out the possibility that such structures are due to vesicle aggregation: in fact, if vesicle aggregation was an important phenomenon under our conditions, one would have observed also higher oligomers, i.e. aggregates with three, four or more units. Furthermore, aggregates are not present at the end of the process. Therefore, these twin vesicles most probably represent vesicles in the act of division. This confirms that the 'new' vesicles derive from the parent vesicles, so a process of vesicle self-reproduction takes place.

Discussing now the mechanism of vesicle self-reproduction, our general assumption was based on a preliminary growth of the vesicles (at the expense of oleate micelles), followed by a division of the larger, unstable vesicle structure. No evidence of intermediate larger structure has been obtained by ffEM. However, it is quite possible that these larger intermediates, if they exist, have a very short lifetime, and therefore they are not detectable with this experimental set-up. Now, the enlargement of the original parent vesicles, instead of being a homogeneous

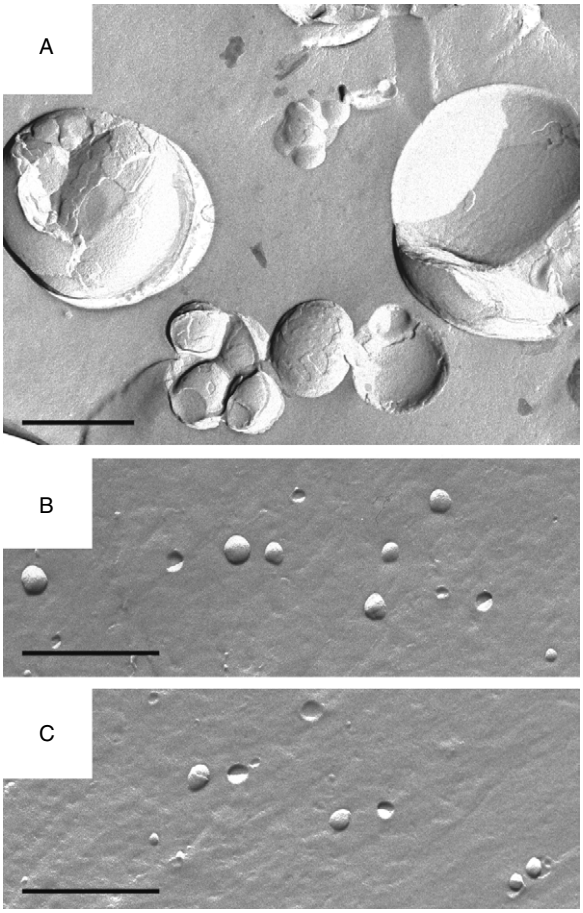

Figure 2. Freeze-fracture electron micrographs of oleate vesicle samples. (A) Structures formed after the vesiculation in the buffer (control experiment) of oleate micelles. (B) Pre-formed '100 nm' oleate vesicles (prepared by extrusion) before the addition of oleate micelles. (C) Sample (B) after the addition of an equimolar amount of oleate micelles. The overall oleate concentration is 10 mM in (A) and (B); and 20 mM in (C). In all cases a sodium bicine buffer (0.22 M, pH 8.5) was used. Bars represent 500 nm.

process, may start and proceed from a single surface region of the parent vesicle. This may lead to a budding mechanism, from which namely the new vesicle may originate. In fact, the twin structures of figures 3 and 4 appear to testimony of a budding process. Again, one cannot yet exclude the possibility that these structures derive from a homogeneously enlarged vesicle intermediate, namely that there is a preliminary growth of the structure. Evidence for the parallel existence of a growth process comes from the work of Berclaz *et al*, based on ferritin as label for the water pool of vesicles [11, 20]. On the other hand, the work by Berclaz *et al* was based on mixed vesicles, oleate being added to POPC vesicles.

One can exclude however, on the basis of this EM evidence, alternative mechanisms. For example, in some growth processes documented with giant vesicles [25], two concentric giant vesicles are formed, and than the internal one flips out, giving rise to a kind of duplication. As mentioned, this kind of concentric vesicle mechanism can be excluded on the basis of the twin structure of figure 3.

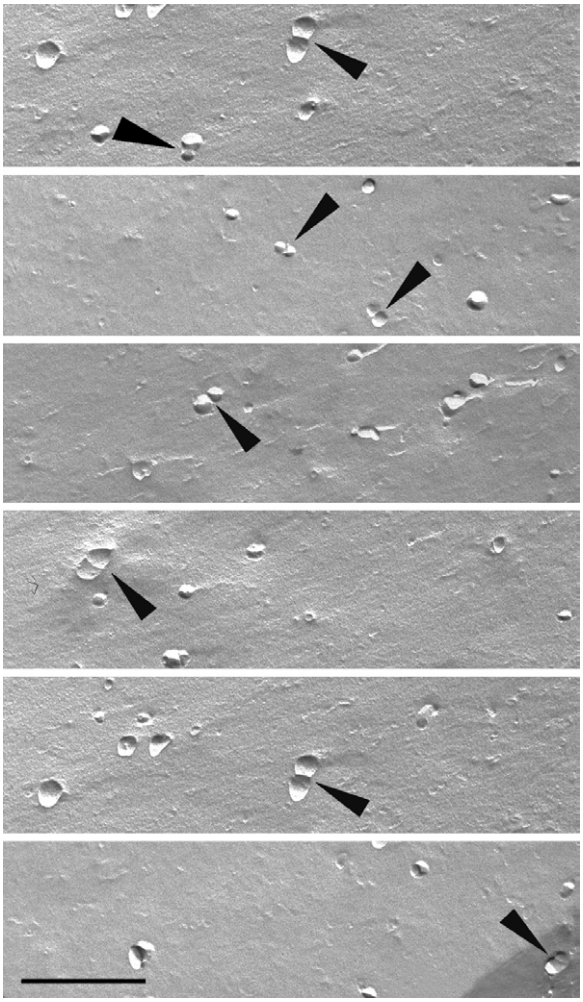

Figure 3. Freeze-fracture electron micrographs of intermediate structures formed during the vesiculation of oleate micelles in the presence of pre-formed extruded oleate vesicles. In particular, 0.1 ml of a solution of 110 mM sodium oleate micelles in water were injected into 1 ml of 11 mM oleic acid–sodium oleate vesicles in 0.22 M bicine buffer, pH 8.5. Samples were taken 40 s after the injection of oleate micelles. The bar represents 500 nm.

One further question is whether this process of self-reproduction via the budding twin structures proceeds with or without leaking of the water pool content. In order to clarify this point, we used calcein-containing vesicles with the aim of following the fate of a water-soluble solute entrapped in the aqueous core of pre-formed vesicles. Oleate micelles were added to a suspension of pre-formed calcein-containing oleate vesicles (micelle/vesicle 1/1), obtained after a spin column chromatographic separation from the free calcein. In the transitory phase, i.e., during the process of vesicle self-replication, only a weak and fast calcein dilution is observed (about 5–25%, depending on the amount of micelles—data not shown), suggesting that the pre-existing vesicles were neither destroyed by the interaction with the added oleate, nor considerably permeabilized. These leakage studies confirm that the integrity of the pre-formed vesicles is largely maintained during the process, which seems to be in keeping with

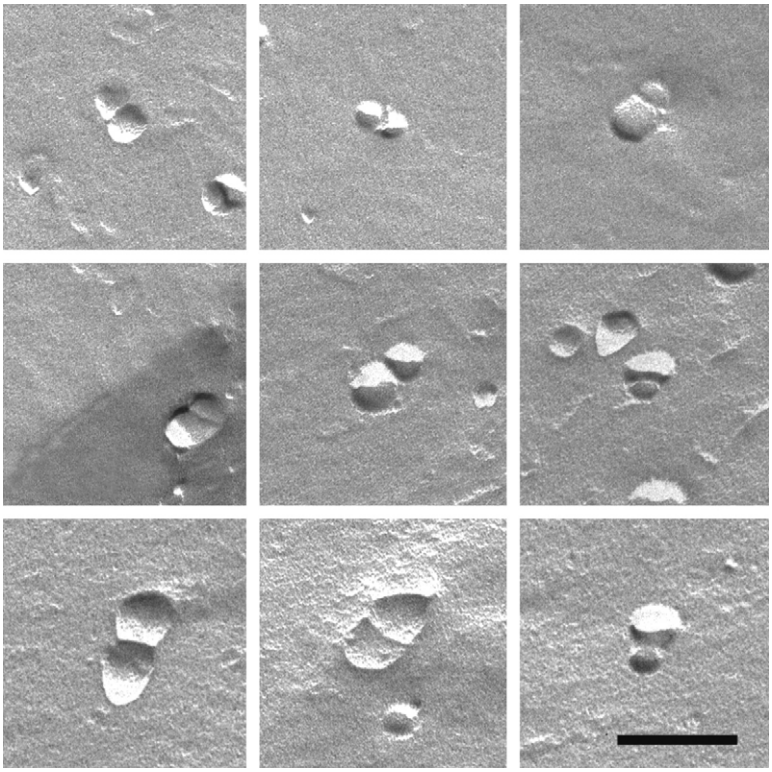

Figure 4. 'Twin vesicles' (an enlargement taken from figure 3). The bar represents 200 nm.

the hypothesis that the new vesicles originate from the parent vesicles without any large rearrangement.

It is worth noting that recent studies by Svetina *et al* [26] provide evidence for vesicle growth and duplication from a theoretical point of view, indicating that a division occurs after a critical state is reached by vesicle growth.

In conclusion, by combining DLS, ffEM, turbidity and fluorescence data, a possible mechanism for the vesicle self-reproduction can be depicted. According to this, freshly added oleate molecules (either as micelles or monomers) are taken up by the pre-formed vesicles, which continue to growth and then divide preferentially via a budding mechanism.

We believe that these findings add considerably to our knowledge about the mechanism of vesicle self-reproduction; however the central question about the mechanism of the matrix effect remains elusive. The main question is why the daughter vesicles should maintain, to a very good approximation, the same dimensions as the parent vesicles. It is as if the twin structure would have a preference for a symmetric growth around a nodal region—and whereas the possible reasons for this are not yet clear, theoretical studies on the matrix effect, now being pursued by other groups (such as those mentioned by Svetina), can help in answering this interesting question.

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