An Autocatalytic Reaction Leading to Spontaneously Assembled Phosphatidyl Nucleoside Giant Vesicles

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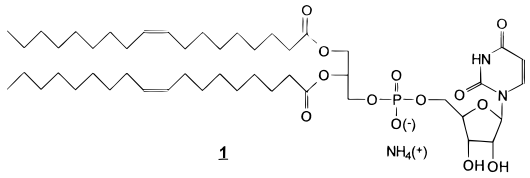
During the last years we described the self-reproducing properties of fatty acid micelles[1] and vesicles.[2] Vesicles prepared from oleic acid, for example, can catalyze the hydrolysis of the surfactant precursor oleic anhydride. In particular, when a droplet of the water insoluble oleic anhydride is layered on a basic buffer solution, anhydride hydrolysis is at the beginning very slow, but as soon as the first oleic acid/oleate vesicles are formed, it sharply accelerates and a dramatic increase of the vesicle number is observed. This is mainly due to the fact that the first vesicles can solubilize the water insoluble precursor, which is then efficiently hydrolyzed by the vesicles themselves, a process which brings about the formation of more vesicles, in a typical autocatalytic fashion.

This kind of work has been until now carried out only with fatty acids via a hydrolysis reaction.[1,2] The aim of the present investigation is to extend the self-replication scheme to different chemical reactions and to more complex systems, in particular to phosphatidyl nucleoside vesicular systems.

Phosphatidyl nucleosides are a class of synthetic surfactants in which a hydrophobic diacylglycerol is linked to a hydrophilic nucleotide. When dispersed in aqueous medium, phosphatidyl nucleosides self-assemble to form liposomes or other supramolecular structures.[3]

We now report conditions under which a water-insoluble lipid, compound **2**, can be converted into phosphatidyl nucleoside **1**, which spontaneously self-assembles to form vesicles.



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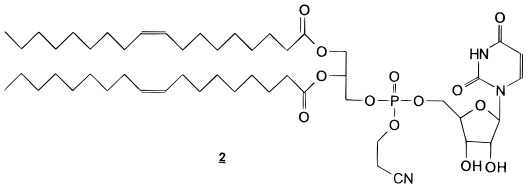

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We show that this reaction is autocatalytic, i.e., the surfactant which is generated gives rise to structures which catalyze its own production. This process, leading to a rapid increase of the vesicle number, represents the first step toward the design of compartimented self-replicating systems, carrying the potential for molecular recognition and information chemistry.

Compound **2** was prepared in two steps. Reaction of 1,2-dioleoyl-*sn*-glycero-3-phosphocoline with uridine in the presence of phospholipases D from *Streptomyces* sp. AA 586 as a catalyst,[4] afforded **1**, which was than coupled to 2-hydroxyproprionitrile using 1-(2-mesitylenesulfonyl)-3-nitro-1,2,4-triazole (MSNT) as a coupling reagent[5] to obtain **2**.[6,7] The 2-cyanoethyl group is a base labile protecting group that can be easily cleaved off via β-elimination in mildly basic conditions to release the related phosphate diester in quantitative yield.[8] The idea behind this work is to perform the cleavage reaction in a mildly basic ammonia solution to generate the ammonium salt of surfactant **1** in situ under the conditions required to promote its spontaneous self-organization into vesicles.

The reaction was started by preparing a two-phase reaction system obtained by layering compound **2** as a lipid film on the glass walls of a reaction vessel and then exposing this film to a mildly basic ammonium buffer solution.[9] Under these conditions, the film gradually disappeared, and in the meantime, the liquid phase became opalescent due to vesicle formation. Isolation of the product, followed by TLC and NMR analysis, confirmed that the reaction had taken place as expected, leading to surfactant formation.

The development of the reaction with time was monitored by measuring the optical density at 500 nm, which reflects the turbidity change of the system caused by vesicle formation, and measuring simultaneously the amount of surfactant **1** and of precursor **2** released from the film, by means of TLC densitometry measurements.[10]

Typical reaction profiles are shown in Figure 1. No significant optical change is noticed for about 2 h, a time interval in which no trace of **1** or of **2** can be detected, in analogy to what already observed for carboxylates vesicles[2] or micelles.[1]

After this time, however, the optical density of the reaction mixture increases dramatically (Figure 1a), and in the meantime, a strong increase of **1** (Figure 1c) and **2** (Figure 1b) concentrations is observed in the liquid phase. The curve related to the change in turbidity then levels off, and no more changes in optical density can be observed.

The inset of Figure 1 shows that complete conversion of **2** into surfactant **1** requires several days. The inset shows also that the increase in rate reaches a plateau, which corresponds to the

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(6) The reaction conditions were adjusted so that no protection of other functional groups present on the molecule was necessary. A 10:1 pyridine/2-hydroxyproprionitrile mixture, in the presence of an excess of MSNT (4 equiv), proved to be particularly effective to promote the coupling.

(7) The new compound was purified by chromatography. Purity and chemical structure were assessed by ^{1}H NMR, ^{31}P NMR, MS (FAB), and TLC.

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(9) In a typical reaction, the film was prepared dissolving 0.9 mg of compound **2** in 1.5 mL of $CHCl_3$ in a cuvette, gently evaporating the solvent under reduced pressure, and then leaving the film under high vacuum overnight. The film-containing vessel was placed in the thermostated cell holder of a UV spectrophotometer and thermostated at 25 °C; 3 mL of NH_4Cl 0.1338 M were then added, and the mixture was stirred gently, so that the two phases remained separated during the reaction.

(10) Samples (10 μL) were taken from the mixture during the reaction progress, and layered on a silica TLC plate. The TLC plate was eluted, and the UV-active spots relative to compounds **1** and **2** were analyzed with a densitomer.

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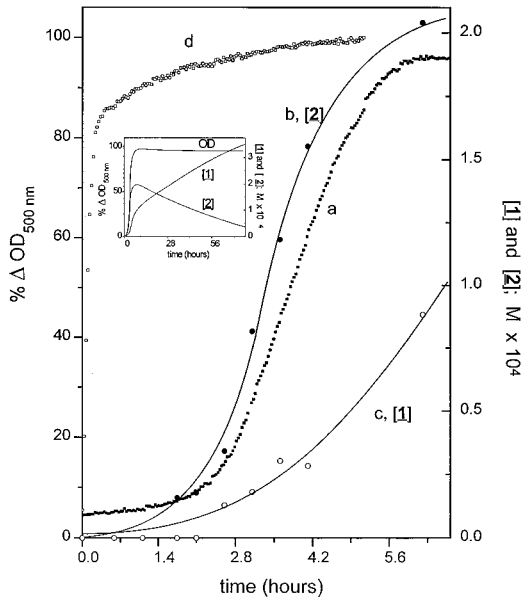

Figure 1. (a) Relative change in optical density measured at 500 nm as a function of time (■), left axis. (b,c) Concentration change of compounds **2** (●) and **1** (○), right axis, observed in a system consisting initially of a lipid film of compound **2**, exposed to a pH = 9, 0.134 M NH_4Cl aqueous solution, at 25 °C. The concentration of the two compounds was determined as a function of time from samples taken from the aqueous mixture during the reaction progress. The inset reports the tendency lines referred to the change in optical density and, in **1** and **2**, concentrations observed monitoring the reaction progress for a longer time scale. (d) The time-dependent change in optical density, observed when starting from a film mixture of **2** and **1** (□), left axis, is also reported.

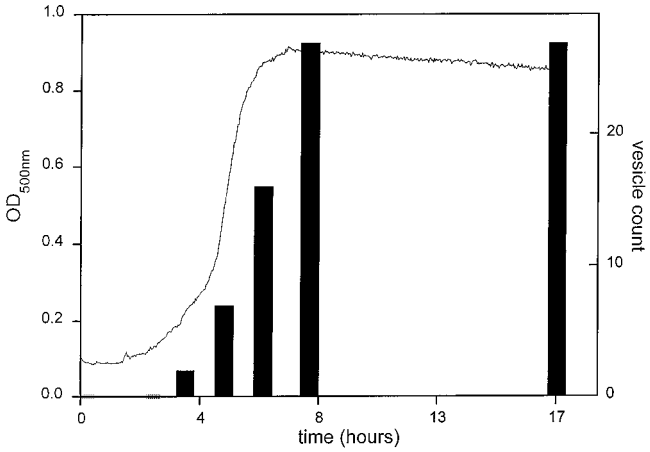

Figure 2. Microscopic analysis of samples taken from the reaction mixture. Change in optical density (continuous line, left axis) and change in vesicle number (column bars, right axis) observed in the course the reaction. The vesicle number was determined by light microscopy analysis of samples taken from the mixture during the reaction progress. The mean number of vesicles per micrograph is reported. Five micrographs were analyzed for each sample. For vesicles containing another vesicle in the interior, only the outer vesicle was counted.

total conversion of the lipid film into a liposome mixture, and then decreases, probably due to electric repulsive interactions on the bilayer surface.

Freeze-fracture electron microscopy (EM) analysis[11] and light microscopy analysis (Zeiss Axioplan), carried on in the course of the reaction progress, clearly show that the turbidity increase is accompanied by vesicle formation. Surprisingly, aggregate formation is biased toward large multilamellar vesicles, with size from 1−6 μm. Such large vesicular systems, referred in the literature as "giant vesicles" are at the moment subject of intensive investigation.[12]

In fact, very few liposomes are observed by freeze-fracture EM with dimensions below 1 μm. The large size allows one to observe directly under the light microscope the progress of vesicles formation and the increase of their population number during the reaction. A statistical analysis was performed on samples taken from the mixture at different stages of the reaction. The analysis confirms that the turbidity increase of the liquid phase is related to the increase of vesicle number (Figure 2). No vesicles are observed during the first lag-phase. At the end of the lag phase, paralleling the optical density increase, vesicles start to appear, and their number then sharply increases. After the optical density has reached its maximum value, the number and the dimension of the vesicles do not show appreciable variation and remain stable for at least few weeks.

Both the optical density and the concentration versus time plots shown in Figure 1 strongly suggest an autocatalytic mechanism.

The main factor for this is most likely the increase of the active surface: we are dealing in fact with a surface reaction which leads to the formation of more and more active surface. The cleavage yields surfactant **1**, which organizes itself into bilayers, a process attended by the incorporation of precursor **2**. The latter is thus further and further exposed to the basic aqueous solution. This in turn forms more surfactant and consequently a larger bilayer surface: a typical autocatalytic process.

This autocatalytic mechanism is confirmed by the following observation: if the reaction is started from a lipid mixture of **1** and **2** in different ratios, a progressive shortening of the lag-phase is observed by increasing the relative amount of **1**. Also, the higher the **1**/**2** ratio, the greater the acceleration effect (data not shown). When the ratio between **1** and **2** in the film equals 1:5, the curve shape further changes, no lag-phase is observed, and vesicles start to appear in solution immediately after the addition of the buffer (Figure 1d). This is additional evidence that an autocatalytic process is taking place in the system.[13]

In conclusion, we have described a chemical reaction which is capable of yielding a surfactant which spontaneously assembles and undergoes an autocatalytic self-reproduction process. The general autocatalytic mechanism appears to be comparable to that of carboxylic acids,[1,2] in the sense that incorporation of the water-insoluble precursor in the bilayer favors its hydrolysis and that the velocity of hydrolysis increases with the increasing concentration of vesicles. Beyond this general analogy, however, the system described here represents two novels aspects of supramolecular chemistry of surfactant aggregates: complex vesicular systems which possess potentially the information content of nucleic acids can undergo self-reproduction and giant vesicles can be directly formed in this process.

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(13) Note the apparent discrepancy between the notion we are using here ("incorporation of the surfactant monomer into the nascent liposomes") and the previous statement, according to which there is no efficient binding of the surfactant precursor to the liposomes. This is actually an important and interesting point in the physical chemistry of liposomes of this kind: POPC liposomes, for example, once formed do not easily bind cosurfactants such as phosphatidyl serine (PS) or dodecyldimethylammonium bromide (DDAB). However, both PS and DDAB can easily be incorporated into POPC liposomes during the formation of liposomes. Likewise, phosphatidyl nucleoside liposomes do not bind the monomeric surfactant precursor once they have reached their stable energy state, but this compound can easily be taken up by the nascent phosphatidylnucleoside liposomes.