# Vesicle Formation by Enzymatic Processes

J. Chopineau,*,§ S. Lesieur,‡ and M. Ollivon‡

*Laboratoire de Technologie Enzymatique*
*CNRS URA 1442, 60206 Compiègne, France*
*Equipe Physicochimie des Systèmes Polyphasés*
*CNRS URA 1218, 92296 Châtenay-Malabry, France*

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Because of their size and molecular organization, liposomes are elementary volume units for transport of water-soluble compounds in their internal compartments while lipophilic molecules are conveyed in the bilayer.[1] They are used for research on membrane protein reconstitution[2] and for commercial applications from topical preparations to drug delivery.[3] Nonionic surfactant vesicles (NSV) composed of a monoalkyl amphiphile and cholesterol are similar in many respects to liposomes.[4]

Liposomes can be prepared by mechanical and/or chemical procedures.[5] Processes based on elimination of surface-active agents or surfactants from lipids containing mixed micelles are commonly used for substance encapsulation when mild conditions are required. Surfactant removal is generally performed by dialysis, dilution, and gel filtration chromatography.[5]

Recently, a self-evolving system has been developed for studying the dynamic interactions of enzymes and colloidal aggregates.[6] Enzyme-induced self-replicating systems, such as micelles, reversed micelles, and liposomes have been investigated and related to autopoiesis.[7] Specific enzyme-induced decapsulation of vesicles has been reported for chemotherapeutic purposes.[8] Two distinct extemporaneous enzymatic fabrications of liposomes from micelles composed of classical amphiphilic compounds are described in this paper. Enzymatic reaction removes the water-interacting head group of the surfactant, which leads to micelle-vesicle transition.

System I: Dipalmitoylphosphatidylcholine (DPPC) vesicles are formed from dodecyl β-D-maltoside (DM)-DPPC mixed micelles. An enzymatic solution composed of β-D-glucosidase and amyloglucosidase[9] hydrolyzes DM according to the following successive reactions:

$$\text{DM} + \text{amyloglucosidase} \rightarrow \text{dodecyl-}\beta\text{-D-glucoside} + \text{glucose} \quad (1)$$

$$\text{dodecyl-}\beta\text{-D-glucoside} + \beta\text{-D-glucosidase} \rightarrow \text{dodecanol} + \text{glucose} \quad (2)$$

In a typical experiment, 20 μL of an enzymatic solution containing β-D-glucosidase ($4 \times 10^{-7}$ M) and amyloglucosidase ($1.5 \times 10^{-6}$ M) is added to 2.0 mL of DM-DPPC (0.75-0.5 mM) mixed micelles in 145 mM NaCl and 10 mM Na-HEPES pH 7.4 buffer. This solution is placed in a spectrophotometer (Perkin-Elmer lambda 2) cuvette, and the optical density (OD) is monitored at 400 nm according to a procedure used in a study of vesicle reconstitution.[10] After 50 min, the initial low turbidity of the solution (OD = 0.02), characteristic of mixed micelles,[10] increases drastically because of the formation of large aggregates (Figure 1). Then, the turbidity stabilizes at an OD value of about 0.8. Quasielastic light scattering (Coulter electronics nanosizer) measurements indicate the presence of particles with apparent mean diameters of about 600 nm.

To demonstrate that vesicles are formed, the enzymatic reaction was performed in the presence of 1 mM calcein, a water-soluble fluorescent probe. After reaction, the aggregates are chromatographed on a gel exclusion column (290 mm length, 16 mm internal diameter, Sephacryl S1000) coupled with online fluorescence detection (excitation at 489 nm; emission at 509 nm) using a Spex spectrofluorimeter (F1 T11 I apparatus). Elution profiles plotted in Figure 2 consist of two distinct peaks at respective elution volumes of 12.2 and 28.4 mL, which correspond to entrapped and free calcein.[11]

Together these results demonstrate that large aggregates are formed by DM hydrolysis by enzymes (eqs 1 and 2). These aggregates are probably vesicles of dodecanol-containing DPPC bilayers. Indeed, Lee[12] found that DPPC bilayer can accommodate up to 66% (mol/mol) dodecanol. Glucose should be completely solubilized in water.

System II: The surface-active agent was chosen to be transformed into cholesterol, a naturally occurring membrane component. Diglycerol hexadecyl ether ($C_{16}G_2$) and its homologs are known to form bilayers and vesicles in association with cholesterol, but they are self-associated and form interdigitated lamellar structures in the absence of cholesterol.[4] Two separate buffered solutions containing $C_{16}G_2$-dicetyl phosphate (DCP) and poly(oxyethylene) cholesteryl sebacate diester[13] (Chol-POE) are mixed at 50 °C to give 5 mM total lipid concentration (1/0.07/1 mol/mol/mol). An isotropic clear solution is obtained (the OD value is about 0.2 at 400 nm) at this temperature. Chol-POE analogs are known to solubilize $C_{16}G_2$ and form an open lamellar phase or large disk-shaped structures.[14] A 50 μL aliquot of esterase solution[15] is added to 1.15 mL of the surfactant solution. The Chol-POE is converted into water-soluble poly(oxyethylene) (POE) and sebacic acid and lipid-soluble cholesterol, which forms unilamellar vesicles when associated with $C_{16}G_2$.[16] The progress of the reaction was monitored as in system I, and a similar turbidity evolution was observed. Encapsulation of calcein was successfully performed. Figure 3 shows a negative staining electron micrograph of the vesicle products.

In conclusion, elimination of surface-active agent from mixed micelles or similar "open" structures by enzymatic reactions

§ Laboratoire de Technologie Enzymatique.
‡ Equipe Physicochimie des Systèmes Polyphasés.

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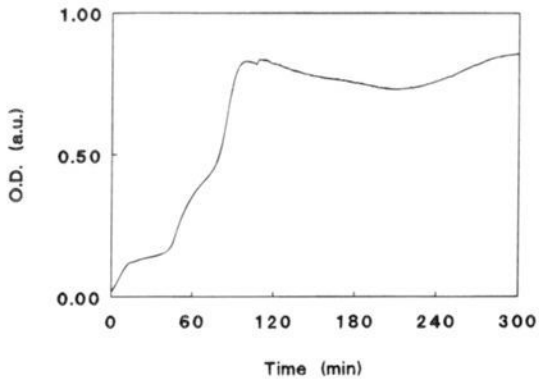


**Figure 1.** Turbidity monitored at 400 nm after rapid addition of enzymes to DM−DPPC (0.75−0.5 mM) mixed micelles at 37 °C. Enzymatic hydrolysis of DM leads to larger aggregates (OD increases from 0.02 to 0.8), probably vesicles composed of DPPC bilayers and dodecanol.

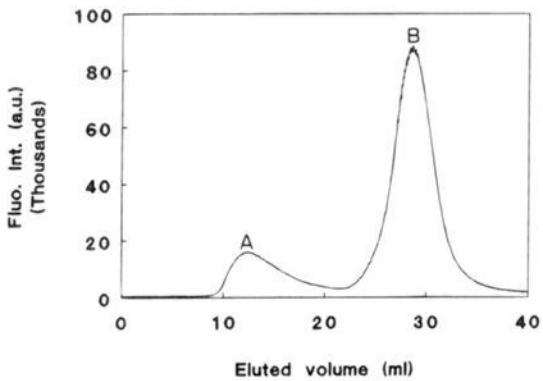


**Figure 2.** Gel exclusion chromatography profile of aggregates enzymatically formed in the presence of initial concentrations of 0.5 mM lipid and 1 mM calcein (0.33 mL elution rate, sample load 0.5 mL). Peaks A and B correspond to calcein-containing vesicles and unentrapped calcein, respectively.

produces liposomes in aqueous media under mild conditions. The process described here is simple and reliable and is based upon well-established analytical techniques. Two completely different systems have been investigated. One leads to the formation of phospholipidic vesicles while the second yields single-chain nonionic vesicles. The modulation of the rate of vesicle formation and its influence on particle size are presently under study in our laboratories.

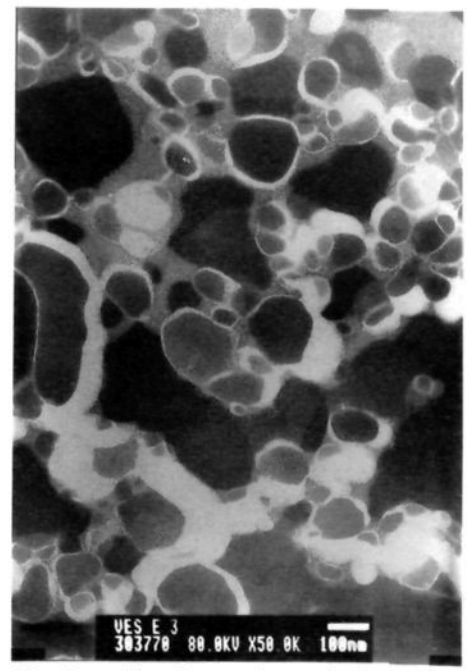


**Figure 3.** Negative staining electron micrograph of vesicles made from system II. (One drop of aggregate suspension was deposited on a carbon grid. After drying with optical paper, one drop of 1% uranyl acetate was used for staining). Stained samples were observed in a Jeol 1200× microscope at 80 kV. Closed vesicles, mean size about 120 nm, are clearly visible and similar to reference vesicles made by ultrasonication.[17]

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