Encapsulation

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Sequestration of Proteins by Fatty Acid Coacervates for Their Encapsulation within Vesicles

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Abstract: *Encapsulating biological materials in lipid vesicles is of interest for mimicking cells; however, except in some particular cases, such processes do not occur spontaneously. Herein, we developed a simple and robust method for encapsulating proteins in fatty acid vesicles in high yields. Fatty acid based, membrane-free coacervates spontaneously sequester proteins and can reversibly form membranous vesicles upon varying the pH value, the precrowding feature in coacervates allowing for protein encapsulation within vesicles. We then produced enzyme-enriched vesicles and show that enzymatic reactions can occur in these micrometric capsules. This work could be of interest in the field of synthetic biology for building microreactors.*

Encapsulating biological materials in vesicles constitutes a step towards the production of minimal and synthetic cells and is therefore of significant interest in the field of synthetic biology and for our understanding of the origin of life.[1,2] Spontaneous overcrowding of biochemicals can occur in some cases when producing vesicles but it remains rare.[3,4] With classical procedures, upon hydration of lipids, a small amount of biological materials can be spontaneously encapsulated within vesicles when they are produced.[5] An encapsulation method known as the "droplet transfer method" has been developed and further successfully been used for producing artificial cells.[6–8]

Coacervates, which can form from polyelectrolytes and/or countercharged or neutral polymers dispersed in water, for example,[1,9,10] spontaneously sequester biomolecules. A hybrid model based on polyelectrolyte-based coacervates covered by a fatty acid bilayer has recently been developed and combines the advantages of both coacervates and vesicles.[11] However, such coacervates form only at high polymer concentrations ($\geqslant 1$ wt %) and/or require expensive polyelectrolytes (e.g., ATP, polylysine).

Surfactant and lipid systems can form coacervate-like droplets under particular experimental conditions (clouding phenomenon).[12] Surfactant coacervation has mainly been studied in the field of colloid science,[10] but to the best of our knowledge, there are no known examples of utilizing such systems as bio-compartments. We have recently developed a system based on fatty acids that forms coacervates[13] and have also independently shown that these fatty acids can form stable vesicles.[14] Herein, we first show that reversible transitions from coacervates into vesicles can be achieved by varying the pH. Next, we demonstrate that proteins are spontaneously sequestered into the membrane-free coacervates. This precrowding feature allows for the encapsulation of proteins within vesicles in high yields using the coacervate→vesicle transition. Finally, vesicles with encapsulated enzymes were produced to conduct reactions within the vesicles.

According to the literature in this field,[15–17] vesicles can be readily obtained from coacervates (10 mg mL^{-1} myristic acid (My), My/NaOH/guanidine hydrochloride molar ratio: 1:1:2, pH 9.5; see the Supporting Information, Section S1) by mild changes in the conditions, by either decreasing the pH value (to pH 8) or by adding an alkanol to the coacervate dispersion (Section S1 and Figure S2). The transition between coacervates and vesicles was visualized by microscopy as a change of size occurs, coacervates exhibiting a diameter of about 5–50 μm whereas that of vesicles does not exceed 5 μm (Figure 1). The variation of the pH value allows for reversible transitions between coacervates and vesicles and the realization of up to ten transitions (not shown). The formation of vesicles starting from coacervates was confirmed by solid-state NMR spectroscopy (Figure 1F); a broad resonance was observed, which is characteristic of lipids embedded in bilayer assemblies.[14,18] As both coacervates and vesicles have previously been separately characterized by various techniques,[13,14] we can suggest in the present study that coacervates formed at pH 9.5 and vesicles at pH 8.

The use of alkanol did not allow for reversible transitions as the alkanol was inserted within the fatty acid bilayers of the vesicles (Figures 1 and S2). However, it was still of interest because the presence of alkanol is well known to increase the range of pH values in which fatty acid vesicles form.[14,16] The present system then 1) combines advantages of both coacervates and vesicles in a unique system and 2) enables transitions between both these structures.

We further questioned whether our fatty acid coacervates could sequester proteins as previously described for poly-

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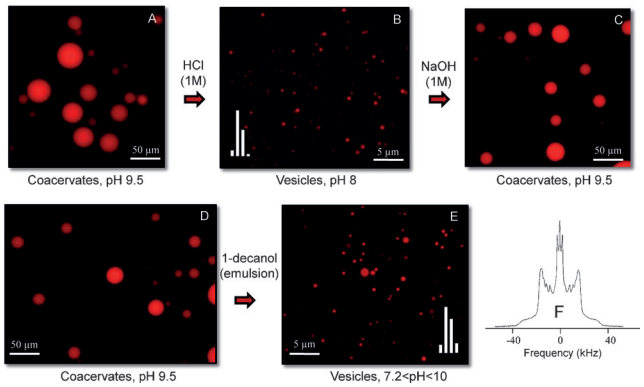

Figure 1. Epifluorescence micrographs of A) coacervates (10 mg mL^{-1} My, My/NaOH/guanidine hydrochloride molar ratio: 1:1:2, pH 9.5; Section S1) labeled with Nile Red. B) Vesicles formed after acidification (pH 8) of the coacervate dispersion. The vertical bars are a histogram of the size distribution, from left to right: <1 μm, 1 μm, 2 μm, >2 μm. C) Coacervates (pH 9.5) re-formed upon addition of NaOH from the previous vesicular dispersion. D) Same dispersion as that in (A). E) Vesicles obtained from the coacervate dispersion upon addition of a 1-decanol emulsion (see Sections S1 and S2). The vertical bars are a histogram of the size distribution (see (B)). F) Solid-state deuterium NMR spectrum of the vesicles in (B).

electrolyte- and/or polymer-based coacervates.[9,10] Coacervates were first produced (see the composition above and Section S1) in the presence of fluorescent proteins, namely self-fluorescent YFP and FITC-labeled ribonuclease A and catalase. Nile Red was also added to label the fatty acid coacervates. Yellow, 5–50 μm large dots, which represent the proteins, were observed by epifluorescence and confocal microscopy (Figure 2A and S3a) and were superimposed with red dots (Figure 2B and S3a), demonstrating that these proteins were indeed crowded into coacervates.

This crowding feature was further quantitatively analyzed for other proteins by making use of the fact that coacervates coalesce with time (1–2 h), further yielding a macroscopic phase separation (Figure 2C).[13] The partitioning of proteins between the upper (fatty acid enriched)[13] and lower phases was determined by UV/Vis absorption spectroscopy (Figure S3b,c). Obviously, whatever the isoelectric point (pI), the proteins were efficiently extracted into the fatty acid enriched phase, showing that they are mainly sequestered ($>70\%$) into the coacervates. This was possible because proteins are zwitterionic biomolecules that contain free, positively

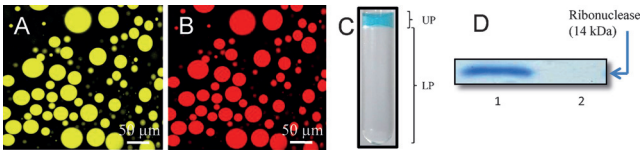

Figure 2. A, B) Confocal micrographs of coacervates containing A) YFP and B) Nile Red. C) Photograph of a sample tube showing macroscopic phase separation, here for the protein markers (blue) used for gel electrophoresis. D) SDS-PAGE gel electrophoresis of coacervates initially produced in the presence of 1) ribonuclease and 2) both ribonuclease and proteinase K. The full gel is shown in Figure S3f.

charged amino acids that are thought to interact with the negatively charged fatty acids within coacervates. This behavior was also observed with zwitterionic organic dyes.[13]

We also tested whether the sequestered enzymes were still functional within the coacervates. Coacervates were produced in the presence of enzymes such as horse radish peroxidase, glucose oxidase (Gox), and alkaline phosphatase (see Section S1). After phase separation, the upper and lower phases were recovered separately, and the enzyme substrates were added to each phase. Enzymatic reactions indeed occurred, mainly in the upper phases (Figures S3d,e). We also prepared a coacervate dispersion to which both ribonuclease and proteinase K (a non-specific protease) were added. Gel electrophoresis showed that the protease fully digested the ribonuclease within coacervates as the band of that protein disappeared (Figure 2D, lane 2). This result confirmed that enzymes are easily sequestered within coacervates and are natively folded and still functional at pH 9.5 in the enriched fatty acid medium.

We then reasoned that because proteins can be precrowded into coacervates, this could enable encapsulation within vesicles upon the coacervate→vesicle transition (Figure 3A). This was first demonstrated by epifluorescence analysis using fluorescent proteins. As initially described, fluorescent-protein-enriched coacervates (see Section S1) were transformed into vesicles by both decreasing the pH (pH 8) or upon addition of 1-decanol. Epifluorescence images showed red dots with a relative polydispersity centered about 1–2 μm, confirming the presence of vesicles (Figures 3C and S4a). It should be noted that yellow dots representing the protein signal were also observed (Figures 3B and S4a) and were superimposed with the red dots (see Figure 3D). This suggests that these proteins had indeed been encapsulated within vesicles. Vesicles do not have a large enough diameter to be observed by confocal microscopy, which would confirm that proteins have indeed been encapsulated. However, we showed that proteins added once the vesicles had been formed were indeed located in the bulk (i.e., outside the

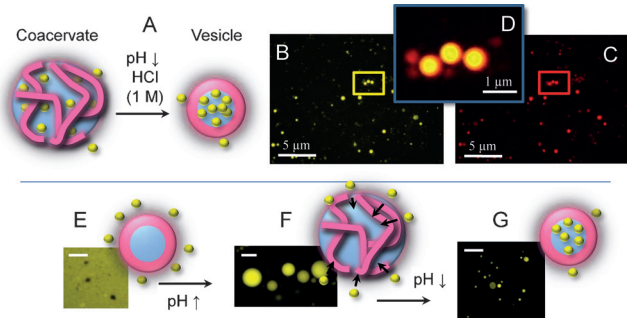

Figure 3. A) Schematic representation of the coacervate→vesicle transition, suggesting that precrowding of proteins enables their encapsulation within vesicles upon the transition. B,C) Epifluorescence images showing vesicles obtained from coacervates enriched in B) FITC-catalase, and C) also labeled with Nile Red. D) An enlarged overlay is shown. E–G) A sequence of epifluorescence micrographs and the corresponding schemes illustrating that YFP added in the bulk can be encapsulated in vesicles by a reversible coacervate→vesicle transition. The scale bars in (E)–(G) correspond to 5, 25, and 5 μm, respectively.

vesicles; Figure S4b) and not adsorbed onto the vesicles nor spontaneously encapsulated within them. This result confirms that the proteins were indeed encapsulated within the vesicles upon the coacervate→vesicle transition.

We then demonstrated that proteins added to the bulk could be further encapsulated in vesicles upon a simple vesicle→coacervate→vesicle transition. Vesicles were formed from a coacervate dispersion (Section S1) upon decreasing the pH value (pH 8); then, the fluorescent protein was added, and located outside the vesicles (Figure 3E). Black dots lacking fluorescence surrounded by the fluorescence of the non-encapsulated proteins were observed (see also Figure S4b). The pH value was then increased to re-form coacervates (pH 9.5), and large yellow dots were observed, confirming that the proteins were now found within coacervates (Figure 3F). The pH value was again decreased (pH 8) to re-form vesicles. Small yellow dots were observed, showing that the proteins were encapsulated inside vesicles (Figure 3G). The fact that the coacervate→vesicle transition is reversible allows for iteratively or sequentially encapsulating proteins inside vesicles.

We then quantified the amount of proteins encapsulated in vesicles. Various protein-enriched vesicles were produced by using the above-mentioned method, and proteinase K was further added to digest non-encapsulated proteins (Figure S4c). Quantitative gel electrophoresis was then performed to determine the amount of non-digested protein. For instance, the initial experiment showed the level of ribonuclease A in the coacervates (Figure 4A, lane 1). After transformation into vesicles and digestion with proteinase K, the band intensity of ribonuclease A decreased. The intensity ratio of both bands (lane 1/lane 2) gives the relative amount of encapsulated protein. About 50% of the protein were not digested and thus had indeed been encapsulated within vesicles (Figure 4A,B). Similarly, the amount of encapsulated catalase was determined to be in the range of 40–60% (Figure 4B). Both methods (pH decrease vs. addition of alkanol) used to form vesicles from a coacervate dispersion yielded the same level of encapsulation (Figure 4A,B).

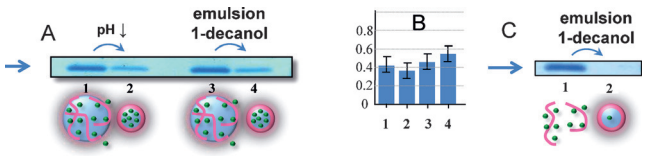

Figure 4. A) SDS-PAGE gel electrophoresis of coacervates prepared in the presence of ribonuclease (lane 1), and of vesicles produced from such coacervates (lane 2; proteinase K was added to digest non-encapsulated ribonuclease). Lanes 3 and 4 correspond to the same processes as above except that the coacervate→vesicle transition was performed by addition of 1-decanol. B) Level of encapsulation within vesicles measured as indicated above, in the case of catalase (1 and 2) and ribonuclease (3 and 4). Vesicles were either produced by decreasing the pH (1 and 3) or by addition of 1-decanol (2 and 4). C) SDS-PAGE gel electrophoresis of 1) a MA dispersion in the presence of ribonuclease and 2) vesicles produced from this dispersion upon addition of 1-decanol (proteinase K was added to digest non-encapsulated ribonuclease). The blue arrow shows the 14 kDa full-length protein. The full gels are shown Figure S4e.

At this point, we wanted to evaluate the efficiency of our encapsulation method in terms of the vesicular volumes. For a dispersion of 5 mL and vesicles with a myristic acid concentration of 10 mg mL^{-1}, the estimated volume of the vesicles is about 10 μL (see Section 4d), which is 500 times lower than the total volume. Therefore, proteins encapsulated at a level of 40–60% are concentrated in the 10 μL internal volume of the vesicles. In other words, the concentration has been increased by a factor of 200–300 (500 × (40–60%)) within the vesicles. Compared to the droplet transfer method, which can achieve protein encapsulation efficiencies of almost 100%,[7] the present system is slightly less efficient but has the strong advantage of producing a large amount of vesicles in a fast, robust, and highly reproducible manner.

To highlight that precrowding proteins into coacervates is crucial for further high-yielding encapsulation into vesicles, vesicles were produced from a fatty acid dispersion (10 mg mL^{-1} My, My/NaOH/guanidine hydrochloride molar ratio: 1:1:1 (without excess guanidine), pH 9.5; freely dispersed worm-like micelles)[19] and not from coacervates. In that case, ribonuclease was not precrowded (Figure 4C, below lane 1). After transformation into vesicles by adding 1-decanol and digestion of non-encapsulated proteins by proteinase K, the band representing the full-length protein completely disappeared owing to the low amount of encapsulated proteins (Figure 4C, lane 2).

We then tested whether enzymes encapsulated within vesicles could catalyze reactions inside the vesicles. Coacervates were produced in the presence of glucose oxidase (Gox), glucose, and non-fluorescent resazurin, which should be reduced into fluorescent resorufin upon glucose oxidation[20] (Figure 5A). We first observed that both glucose and resazurin were dispersed more or less equally within and outside the coacervates (Figure S5a). Consequently, only the enzyme was sequestered within the coacervates. Vesicles were then produced by decreasing the pH value or by addition of 1-decanol. After incubation (see Section S1), epifluorescence images showed yellow dots (Figure 5B) whereas in the blank experiment (Figure 5C, for which vesicles were formed before Gox and substrate addition), black dots lacking fluorescence were observed. This shows that the enzymatic reaction mainly occurred within the vesicles when the enzyme and substrate were initially added in the coacervate dispersion. We also tested a cascade of enzymes using Gox and alkaline phosphatase with glucose-6-phosphate (G6P) as the substrate. In that case, alkaline phosphatase removed the phosphate group of G6P within the vesicles, yielding free glucose, which could further be oxidized by Gox (Figure S5b).

Finally, we made use of the fact that the coacervate→vesicle transition is reversible upon varying the pH to iteratively feed vesicles. Vesicles with encapsulated ribonuclease that were obtained by using the above-mentioned method were fed with a solution of proteinase K, which digested non-encapsulated proteins (see Figure 4A). For comparison with what follows, the electrophoresis gel has been reproduced in Figure 5D (lanes 1 and 2). The pH value was then increased to re-form coacervates (pH 9.5), enabling sequestration of both non-digested ribonuclease and proteinase K within these compartments (Figure 5D). Then, the

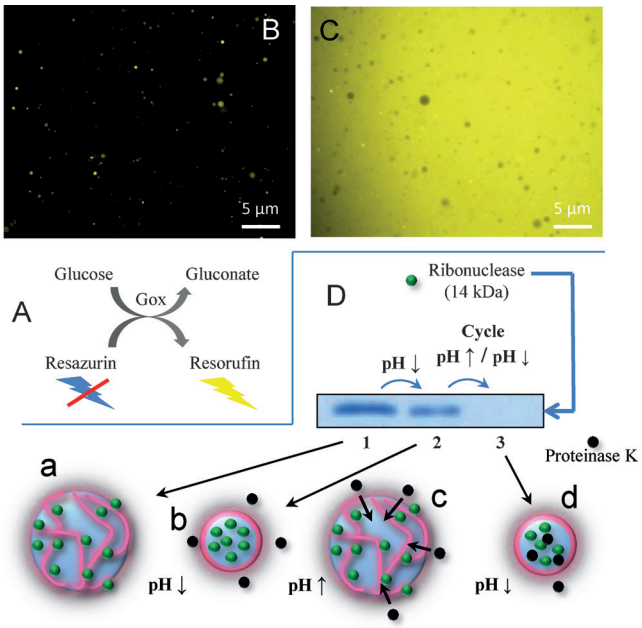

Figure 5. A) Reaction pathway showing the oxidation of glucose into gluconate by glucose oxidase (Gox), accompanied by the reduction of resazurin into resorufin. B) Epifluorescence image of resorufin produced by Gox-enriched vesicles. C) Epifluorescence image of resorufin produced outside vesicles by non-encapsulated Gox. D) SDS-PAGE electrophoresis of ribonuclease in the different states schematically described underneath. From left to right: a) Loading of coacervates with ribonuclease (green dots; lane 1). b) Amount of ribonuclease encapsulated into vesicles (lane 2). c) The pH is increased to re-form coacervates and upload proteinase K. d) The pH is decreased, and both proteinase K and ribonuclease are encapsulated within vesicles, the former being digested (lane 3). The full gel is shown Figure S5c.

pH value was immediately decreased again to re-form vesicles (pH 8). Gel electrophoresis showed that the band representing the full-length ribonuclease completely disappeared, demonstrating that both proteins were encapsulated within vesicles and that proteinase K digested ribonuclease in these compartments. This process leads to vesicles with encapsulated amino acids and peptides resulting from the digestion of the initially encapsulated protein. It is obvious that enzymatic reactions may also occur in the bulk outside vesicles where the solution contains some non-encapsulated enzymes. However, because the enzyme concentration is 200–300 times higher inside the vesicles than in the external environment, these enzyme-enriched vesicles are highly effective microreactors.

Taken together, we have shown that proteins can be encapsulated within fatty acid vesicles in high yields by a simple and robust method. These findings support a new type of microreactor model based on enzyme-enriched vesicles, which could also provide a novel approach for investigating the construction of primitive and artificial cells. Fatty acid vesicles have long been used as models of protocells,[1,2,4,16,17,21,22] the primary cells that first appeared on early Earth. Our fatty acid system could also be used as a protocell model, considering that cycles between coacervates and vesicles induced by pH variations and also fusion between coacervates induced by agitation may have occurred in prebiotic times. The fusion of coacervates would be a plausible explanation for the sharing of ingredients between protocells in the prebiotic period before a mechanism of growing and division of vesicles ensued.[22,23] Whereas our research has focused on fatty acid coacervates and vesicles thus far, catanionic and polymeric systems have also been shown to undergo complex coacervation[24] and are also known to self-assemble into vesicles.[8,25] Therefore, these surfactant and polymeric systems could also be used for precrowding biomolecules within coacervates, to further allow their encapsulation inside vesicles upon a coacervate→vesicle transition.

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