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Encapsulation of compartmentalized cytoplasm mimic within lipid membrane by microfluidics†

Marta Sobrinos-Sanguino,a‡ Silvia Zorrilla,a‡ Christine D. Keating,b Begoña Monterroso,a‡ and Germán Rivas*a

There is a growing interest in analyzing the effect of microenvironments, that may be mimicked through liquid-liquid phase separation (LLPS), on the reactivity of biological macromolecules. We report the encapsulation by microfluidics of the division protein FtsZ and a LLPS system inside microdroplets and their conversion into permeable vesicles (allowing ligand uptake), with higher yield, homogeneity and biomolecular compatibility than those previously described.

All living cells contain microenvironments and/or compartments where certain molecules specifically localize to optimize their functions. The most obvious compartments are organelles separated from the cytosol by lipid membranes, but both eukaryotic and prokaryotic cells contain membraneless organelles that act as defined functional entities. These microenvironments can be mimicked using polymers at concentrations high enough for phase separation to occur. Studies of the reactivity and localization of proteins and nucleic acids in these synthetic systems evidenced the deep influence that microenvironments can have on biological processes. Some of these studies report procedures to better mimic the cellular compartimentation by encapsulation of LLPS systems inside lipid containers as giant unilamellar vesicles (GUVs) or microdroplets.

One of the most successful strategies for the encapsulation of LLPS systems involves passive encapsulation of the two composing polymers during vesicle formation by gentle hydration or electroformation under conditions in which they do not phase separate and subsequent induction of their segregation by shifting experimental conditions. This procedure leads to a distribution of different polymer concentrations and hence different compositions and relative volumes of phases within a population, generating substantial vesicle-to-vesicle variability in protein localization. Another strategy is the production of water in oil emulsion droplets stabilized by surfactant or by lipid monolayers, which presents advantages as high yield of droplets containing the LLPS system with no need to alter the conditions after encapsulation to induce phase separation. However, the lipid monolayer-coated water/oil interface fails to capture key structural and functional features of bilayer membranes. Also, from a practical standpoint, all reactants must be included upon encapsulation, in contrast to GUVs, which can allow introduction of small ligands through for example pore-forming molecules or packing defects, as those induced by the lipid DMPC, in the bilayer. Despite these advances, the controlled encapsulation of LLPS systems within model membranes remains challenging and there is a need for new procedures to improve the yield and uniformity of the containers in terms of size and composition under mild conditions compatible with biomolecules.

Microfluidic methods have been recently used for the generation of compartmentalized structures as two-droplet multisomes, nested liposomes and for surfactant stabilized single-phase droplets where separation was subsequently induced. Here we report a microfluidics based method for the direct encapsulation of two aqueous phases together with a protein and a physiological ligand inside droplets stabilized by a lipid monolayer. In addition, we have produced GUVs from these droplets containing the two-phase system and the protein by a modified droplet transfer method. Finally we show the incorporation of small molecules into the permeable GUVs. To optimize the procedures we have selected a LLPS system widely used in previous studies, PEG 8/dextran 500, and the central bacterial division protein FtsZ, which behaviour we have recently studied in bulk LLPS systems. In the presence of GTP, FtsZ forms filaments sufficiently large under crowding conditions to be visualized by confocal fluorescence microscopy. We have triggered FtsZ polymerization with GTP either immediately before droplet formation or by external addition of the nucleotide to the

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*a Centro de Investigaciones Biológicas, Consejo Superior de Investigaciones Científicas (CSIC), 28040, Madrid, Spain. E-mail: silvia@icb.csic.es, monterroso@cib.csic.es, grivas@cib.csic.es

*b Department of Chemistry, Pennsylvania State University, University Park, Pennsylvania 16802, USA

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permeable vesicles. Our method could aid in the generation of artificial cells more faithfully resembling functionalities, as it enables the inclusion of some of the features vital for living systems\(^\text{21}\). The semipermeable boundary allows the exchange of molecules, and can be used as the basis for developments in tunable permeability promoting adaptivity. Membraneless phase separated systems modulate macromolecules dynamic localization. The tight control of the concentrations of all reagents (and hence in the generation of synthetic compartments) is of most importance for the control of reaction networks.

Water in oil droplets containing the LLPS system stabilized by the \textit{E. coli} lipid mixture (EcL) were produced in flow focusing microfluidic chips\(^\text{22}\) (Fig. 1 and ESI†). Two streams of dispersed aqueous phases were mixed in an approximately 1:1 ratio prior to the droplet formation junction, one stream containing dextran 500 and the other one PEG 8, prepared as described elsewhere\(^\text{10}\) and in ESI†. Encapsulation of both aqueous phases was verified by fluorescence microscopy including Alexa-647 labelled PEG (ESI†). FtsZ, added to the two aqueous phases, was monitored by including a tracer amount labelled with Alexa 488 (ESI†). No difference was observed when adding the protein in either phase. The third stream delivered the EcL mixture in mineral oil. The typical flow rates were 120 μL/h for the continuous phase and 5 and 7 μL/h for the dextran and PEG aqueous phases, respectively, controlled by automated syringe pumps (ESI†) rendering uniform droplets (∼2 pL). Production of droplets was more laborious than when a single phase is encapsulated (i.e. when both aqueous solutions have the same composition), likely due to the different viscosity of the two crowding solutions.

To analyse the resulting droplets, we imaged them directly in the chip (ESI†). Despite some polydispersity in droplets size due to the poor surfactant capacity of EcL\(^\text{22}\), the formation of a large number of droplets narrowly distributed in size, virtually all of them containing in similar proportion the two aqueous phases, was observed (Fig. 1). The distribution of diameters for a representative population of over 100 droplets from 3 independent experiments is shown in Fig. 1C, with an average diameter for the main population of ∼16 μm. Slight variations in the size were found depending on the flows tested. The overlap between the intensity profiles in the red and green channels for different microdroplets, reflecting the distribution of PEG-Alexa 647 and of FtsZ-Alexa 488, evidenced the uniform distribution in the encapsulation of the two phases and in the protein partition among the droplet population (Fig. 1D). FtsZ was found preferentially in the dextran phase, in good agreement with previous observations\(^\text{10}\) (See ESI†).

In order to test the performance of the method for the coencapsulation of a protein and one of its physiological ligands, we encapsulated the LLPS system with FtsZ and GTP, which triggered the polymerization of the protein just before droplet formation. To this aim we included FtsZ with FtsZ-Alexa 488 in one aqueous solution and GTP in the other, with similar results irrespective of their individual addition to either the PEG or dextran solutions. Imaging of the chip revealed the formation of droplets of similar sizes, containing comparable amounts of phases and FtsZ polymers. The distribution of FtsZ
polymers, preferentially located in the dextran phase and at the interface, was compatible with prior work. The microfluidics based method we report allows the generation of a high number of virtually equal microdroplets containing LLPS together with a protein, with or without physiological ligands, enabling to perform studies of protein reactivity and distribution.

Once we achieved the microfluidics encapsulation of the LLPS system with the protein of interest inside the microdroplets, we optimized a method to convert them into GUVs, as the bilayer boundary provides both a better cell-like system and the possibility of external addition of small ligands. The procedure was based on the droplet transfer method in which the droplets acquire the bilayer upon transition from an oil phase to an aqueous solution when the interface between them is coated with oriented lipids. The first step was the collection of the droplets by introducing the outlet tubing from the microfluidic chip into oil phase lying on top of the outer solution (Fig. 2A and ESI†). Different outer solutions were assayed and the composition was chosen to be the same as that of the inner buffer with a sucrose concentration rendering an osmolarity slightly above the highest osmolarity of the encapsulated solutions (ESI†), which we found enhanced vesicle integrity. It should be noted that, to avoid any possible influence on FtsZ polymerization, we removed the sucrose usually contained in the encapsulated solution in the original method. Different droplet collection times were tried and 30 min was chosen to improve the yield of the procedure (ESI†).

The formed GUVs, in which the two aqueous phases are surrounded by a lipid bilayer, were relatively homogeneous in size and composition (Fig. 2B). However, the yield of GUV production among experiments was more variable than that of the microdroplets because it involves many more steps, some of which cannot be as tightly controlled as the production of microdroplets in microfluidics, like the bilayer acquisition step and vesicle washing. The size estimation proved to be harder due to the smaller number of replicates amenable to be measured, attributable to factors occurring upon the centrifugation of the droplets as rupture, and fusion and/or segregation of composing phases into individual droplets rendering a somehow wider size distribution. Also, small differences derived from measurements of non-circular shaped vesicles (see, for instance, bottom images in Fig. 2B) cannot be discarded. The distribution of diameters was determined for 60 vesicles obtained in 2 different experiments (Fig. 2C), with an average diameter for the main population of ~18 μm. Noteworthy, the observed dependence of the average size of droplets with the final flows was maintained in GUVs, i.e. the most abundant population of vesicles within an experiment, representative of the microdroplets produced, is that corresponding to the same average size. As expected, the uniform distribution of the encapsulated phases and partition of the protein, determined by the microdroplet production step, was maintained in the GUVs generated from them, as

![Fig. 2](image-url)

**Fig. 2** Generation of GUVs from microfluidics microdroplets. (A) Schematic illustration of the procedure for collection of droplets and subsequent formation of permeable vesicles. (B) Representative confocal images of the GUVs containing the LLPS system and unpolymerized FtsZ or FtsZ filaments triggered by diffusion of GTP through the DMPC in the *E. coli* lipid bilayer. (C) Size distribution of the generated vesicles (*N* = 60). Average size corresponds to the mean value (± SD) of the most populated sizes, indicated by the horizontal line. (D) Intensity profiles of the red (PEG-Alexa 647, up) and green (FtsZ-Alexa 488, down) channels for several vesicles (disregarding polymerization was triggered or not), obtained across lines drawn as exemplified in the inset.
reflected by the overlapped intensity profiles of PEG-Alexa 647 and FtsZ-Alexa 488 across the GUVs population (Fig. 2D).

With our method the final size of the generated vesicles is significantly reduced, at least 30%, comparing with those fully produced on chip and so far applied for a single aqueous phase[24-26], feature that can be of most importance depending on the process under study, as with cellular systems. Besides, the ability to work with two aqueous streams enables the encapsulation of not only two different phases, but also of several species, as for instance proteins with binding partners or inhibitors, with the obvious applications for the study of biological systems. This is, however, at the expense of reducing the number of comparable GUVs obtained per experiment.

As in many instances the introduction of small ligands after encapsulation of the LLPS systems may improve the experimental design, for example for the analysis of time dependent processes, we tested our procedure for the formation of permeable GUVs. To this end, we produced vesicles containing the two phases and FtsZ, following the above described procedure but including DMPC in the lipid mixture (ESI†). To probe the permeability of the vesicles, FtsZ polymerization was triggered by external addition of GTP (ESI†). Polymers inside vesicles were always observed (Fig. 2B), but the amount of vesicles in which polymerization was triggered somehow varied among experiments and was found to increase with time. In any case, the appearance of FtsZ polymers, again with the expected distribution within phases (Fig. 2), proved the permeability of the bilayer as well as the formation of the bilayer itself and the active state of the protein after encapsulation.

In conclusion, the procedures shown allowed the microfluidics based encapsulation of LLPS systems and active protein into microdroplets stabilized by lipids and the production of permeable vesicles from them, notably increasing the yield when compared with established bulk methods. Moreover, by these methods, the formation of droplets and vesicles stabilized by the E. coli lipid mixture is feasible, and the lack of any osmolyte inside them rules out possible uncontrolled effects. Advantages and drawbacks can be named for both procedures. If all elements participating in the studied process can be simultaneously encapsulated, microdroplets by microfluidics is probably the LLPS encapsulation strategy of choice, as it brings about improvements to the simpler, and faster, encapsulation in bulk emulsion: (1) tight control over the size and content of the droplets, (2) minimal contact of the protein with the oil, and (3) elimination of vortexing prior to protein addition to improve the encapsulation of both phases. GUVs produced from microdroplets, when a lipid bilayer is needed to more closely resemble the physiological conditions and/or it is required a subsequent addition of a metabolite, obviously maintain these features, although a loss of material is unavoidable. This procedure also overcomes the necessity of modifying experimental conditions to induce phase separation after encapsulation, thus avoiding possible damage of susceptible biomolecules.

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Notes and references