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Controlled Microfluidic Encapsulation of Cells, Proteins, and Microbeads in Lipid Vesicles

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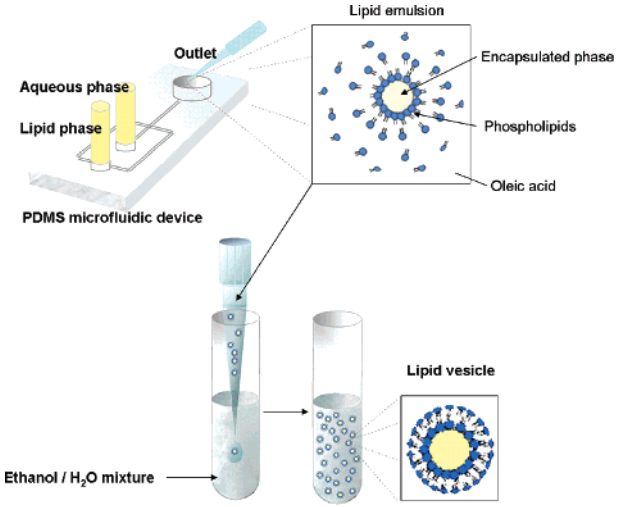
Phospholipids are amphiphilic molecules that self-assemble into vesicles when dispersed into aqueous environments. In nature, large lipid vesicles function as membranes in biological cells to protect the intracellular components from the extracellular environment, and small lipid vesicles (nanometers in size) function to transport biomolecules intracellularly. The compartmentalizations by lipid membranes ultimately give rise to the complexity and functionality of biological systems. The artificial synthesis of nanosized lipid vesicles can be tailored with different surface properties for various applications (e.g., targeted drug delivery).[1] Upon encapsulating reactive agents, these vesicles can be modified into biosensors to respond to a variety of environmental stimuli.[2] Encapsulation of biomolecules into cell sized vesicles have been used as bioreactors to mimic molecular responses.[3] The development of even larger vesicles to retain cells inside vesicles is a promising approach in creating cellular bioreactors that mimic cellular and tissue responses. Nevertheless, due to the fragile nature and the size of cells (ranging from a few microns to greater than 50 μm), encapsulation inside lipid vesicles has not been reported.

While a vast number of techniques have been developed for lipid vesicle synthesis, there still exist many drawbacks. The preparation procedure generally involves dissolving lipids in a transfer medium followed by the removal of the medium in an environment that favors self-assembly of vesicles.[1c,4] In the coacervation and ethanol injection techniques, the encapsulation efficiency is low (25−27%)[5a] and the choice of encapsulated species is limited by the small aqueous core of the created vesicle that formed spontaneously when a lipid ethanol mixture is injected into an aqueous buffer;[5a,5b] however a recent microfluidic alcohol injection technique has shown the ability to control vesicle sizes.[5c] Alternatively, a reversed phase evaporation method provides higher encapsulation efficiency (65%) through first emulsifying the aqueous core phase in a lipid-rich organic phase followed by the evaporation of organic solvents, and the subsequent addition of aqueous buffers yields vesicles by phase inversion.[6] In this latter method the lipid solvents used are generally toxic and not suitable for pharmaceutical use. Furthermore, the formations of these vesicles require many steps that are difficult to streamline and automate.

Recently, droplet based microfluidic systems have demonstrated versatility not only in processing and sampling reagents[7] but also in the encapsulation of biological substances in droplets.[8] Here we present a novel lipid vesicle formation method that controls the vesicle encapsulation through a shear-focusing based droplet generation system,[7b] and we have used it to encapsulate large biological substances ranging from cancer cells and yeast cells to micron sized beads and nanosized proteins. The vesicles are generated within minutes of formulation without the use of toxic solvents, and the encapsulated species are protected inside the aqueous phase during the encapsulation process. The vesicles prepared have a long stability of >26 days with controlled encapsulation efficiency.

Scheme 1. Preparation of Lipid Vesicle Encapsulation



In Scheme 1, the aqueous phase containing the target encapsulated species is emulsified in the liquid lipid phase in the microfluidic channel to produce stable lipid emulsions. The lipid phase consists of dissolved phospholipids in long chain unsaturated lipids, oleic acid, which exists in liquid form at room temperature. Oleic acid is a nontoxic derivative of olive oil and is compatible with many pharmaceutical processes.

The lipid emulsion solution is then injected into an aqueous mixture consisting of ethanol and water, and since oleic acid rapidly dissolves in ethanol, this process removes the excess solvent. Upon reducing the solvent concentration, the phospholipids are disassociated from liquid lipids and are forced to rearrange around the emulsions to assemble into lipid vesicles.

Using this method vesicles have been generated using lipids from ethanol insoluble phospholipids, phosphoethanolamine (DPPE), and ethanol soluble phospholipids, phosphocholine (DOPC, DMPC and DPPC). Shown in Figure 1, GFP (fluorescent proteins) fluorescent beads with a mean diameter of 4.12 μm and fluorescently labeled cells have been encapsulated inside the derived phospholipid vesicles. The GFP, the Hela cell-cervical carcinoma cells (Figure 1b), and the bead encapsulated vesicles have mean diameters of 27.2 μm, 62.4 μm, and 55.9 μm, respectively. The variations of vesicle sizes are ~20% for the GFP and cell encapsulated vesicles and ~10% for the bead encapsulated vesicles.

When GFP is encapsulated inside droplets, the fluorescence is quenched by the acidity of the oleic acid. Although oleic acid is not miscible with water, oleic acid being an amphiphilic molecule exposes the carboxylic end into a water droplet causing change in the overall acidity, thus initially the lipid vesicles containing GFP are nonfluorescent. Upon adjusting the external aqueous phase to pH 10, the GFP vesicles start to fluoresce. This indicates that some ion exchange is allowed between the interior of the vesicle and the

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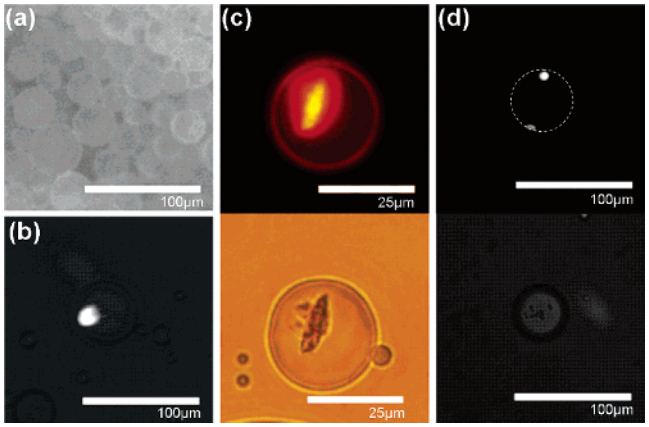

Figure 1. Fluorescent microscopy of (a) GFP fluorescent protein encapsulated in DOPC vesicle and (b) single Hela cell-cervical carcinoma cell (10 μm diameter) encapsulated in DOPC vesicle. (c) Fluorescent microscopy (top) and light microscopy (bottom) of MCF7 breast cancer cell encapsulated in DMPC vesicle and (d) fluorescent beads (4.12 μm diameter) encapsulated in DOPC vesicle. The perimeter of the vesicle is outlined in (d).

external aqueous environment which will be precluded if the inside of the vesicle was separated by an oil layer from the outside. To test the viability of a cell encapsulated inside the lipid vesicle, a live cell staining dye, CMXPoS Red, was used to stain the MCF7 breast cancer cell prior to encapsulation. The CMXPoS dye is fluorescent only after being oxidized in live mitochondria, and the accumulation of cell staining dye is dependent on the membrane potential of the mitochondria. As a result the death of the cell would cause the loss of fluorescence. The fluorescence shown in Figure 1b indicates that the encapsulated cells are viable after encapsulation.

The viability of Hela cells was verified through a Trypan blue staining assay. Hela cells were divided into a control and experimental group each with 3 mL of 10^6 cells per millimeter of cell medium. 200 μL of 0.4% Trypan blue were added to both the control and the experimental group. The cells for both groups contained live and dead cells. Dead cells immediately absorb Trypan blue and become distinguishable from live cells. In the experimental group, cells and Trypan blue are encapsulated inside lipid vesicles. The lipid vesicles from the experimental group and cells from the control group were transferred to a hemocytometer and monitored for viability every 4 min for 2 h. Since both Trypan Blue and cells are encapsulated inside the same vesicle, if the cells at anytime become nonviable during the encapsulation process, then the cells would appear blue after encapsulation. Cells that were alive before encapsulation remained free from Trypan blue staining after 2 h as shown in Figure 2, which indicates that cells remained viable throughout the lipid vesicle encapsulation process.

The concentration of ethanol in the aqueous mixture critically affects the vesicle yield. Encapsulations in vesicles occur in concentrations less than 30 wt % ethanol mixture. Dissolving lipid emulsions in an ethanol concentration greater than 50 wt % yields few vesicles and in less than 10 wt % creates many compound drops.

The encapsulation efficiencies of 4.12 μm sized fluorescent beads in emulsions and in the generated vesicles have been quantified. Indicated in Figure 3, lipid emulsions generated from the microfluidic device are monodispersed with sizes controlled by the flow rate of the lipid phase.

Since the lipid emulsions are formed due to the shear viscous interaction on the surface of the liquid thread, the increase of shear stress through increased lipid phase flow rate reduces the droplet sizes. The emulsion generation process showed no dynamic surface

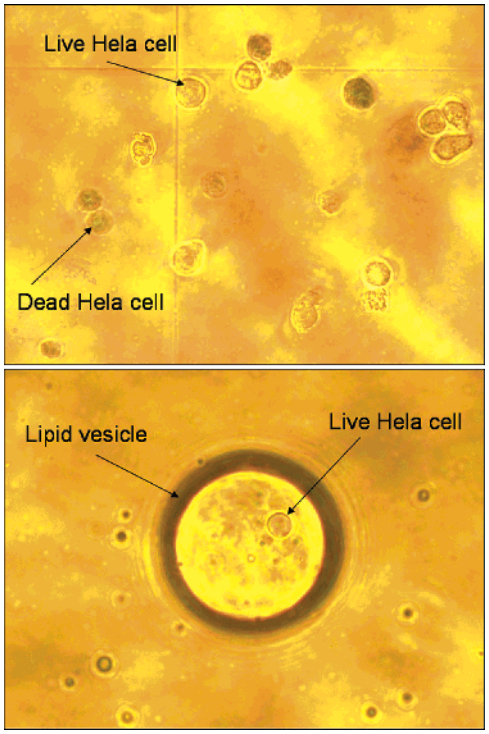

Figure 2. (Top) The dead Hela cell appears blue after Trypan blue staining, and the live cell remains clear. (Bottom) The clarity of the Hela cell inside the lipid vesicle after 2 h indicates that cells are alive after encapsulation.

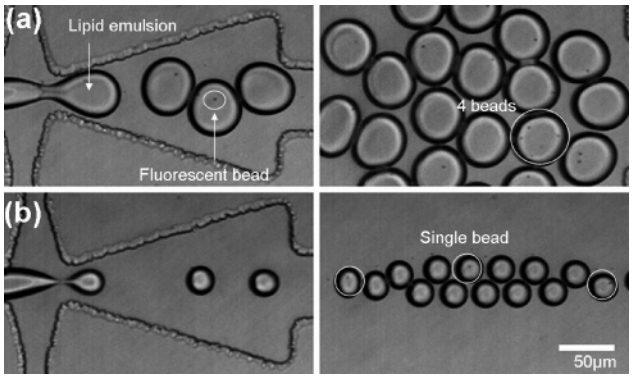

Figure 3. (a) Lipid emulsion generated with 1 μL/min lipid flow rate produced a large emulsion with beads entrapped in ~85% of the droplets but only yielded 5% of beads entrapped in the final vesicles. (b) Smaller emulsion generated with 7−9 μL/min flow rate limits the bead encapsulation efficiency to 15% but yields a bead encapsulation in vesicles from 64% to 91%.

instabilities that would otherwise disrupt the consistency of the droplet generation process. The encapsulated emulsions in Figure 3 contained primarily single beads, but there exist a small number of emulsions that contained two or more beads when a lower flow rate (1 μL/min) is used for emulsification.

Increasing the lipid phase flow rate increases the emulsion production rate, reduces the number of droplets containing beads, and decreases the size of emulsions. Nevertheless, smaller emulsion sizes improve the stability of emulsions during the dissolution process and generate a higher yield of vesicles with encapsulations. For large emulsions (diameter > 77.8 μm) most beads leaked from droplets during the ethanol mixture dissolution process, whereas for smaller emulsions (diameter < 54.8 μm) almost all beads are retained in the final vesicles.

The potential applications for encapsulation cells in lipid vesicles are numerous. Unlike droplets, vesicles are stable for long periods

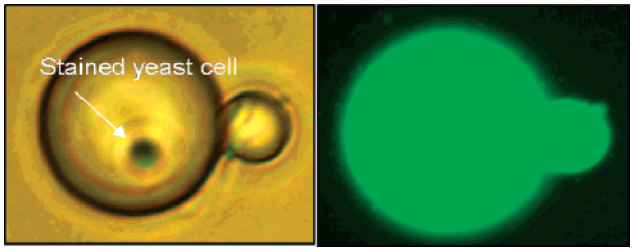

Figure 4. (Left) Microscopy of Trypan blue stained yeast cell encapsulated inside a single lipid vesicle containing GFP fluorescent protein. (Right) The fluorescent image of the same vesicle. The green fluorescence indicates the presence of green fluorescent protein.

of time and can be used to retain cells for long term monitoring of single cell assays. The interior of the vesicle also creates a controlled environment that can be used to study the cellular interaction with biomolecules. As an example we have coencapsulated cell and green fluorescent protein (GFP) simultaneously inside a single lipid vesicle. Shown in Figure 4 the dead yeast cell is stained with Trypan blue and is encapsulated with green fluorescent protein inside a single lipid vesicle. This demonstrates the feasibility to verify GFP and Trypan blue for cell viability experiments.

In conclusion we have developed a novel method for encapsulating biological species ranging from tens of microns sized cancer cells, micron sized fluorescent beads, to nanosized protein molecules in a single-step process using a droplet based microfluidic system to control the encapsulation efficiency. We have determined that the vesicle yield and the encapsulation efficiency depend both on the continuous flow rate applied to the microfluidic system and on the concentration of ethanol contained in the dissolution mixture. The encapsulation of cells, beads, and proteins in lipid vesicles provides a nontoxic, gentle, and efficient means to prepare vesicles for drug delivery, production of biosensors, and engineering of artificial cells. Furthermore, it can be used to design cell based bioreactors, allowing for the study of single cell to multicell interactions inside controlled lipid membranes. By combining the myriad of droplet process technologies (e.g., the fusion and splitting of droplets), the generation of time and spatial concentration gradients can also be incorporated into the lipid vesicle encapsulation system to produce stabilized lipid vesicles with accurate reagent concentrations.

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Supporting Information Available: Supporting Information on the design and fabrication of microfluidic channels, the formulations of lipid vesicles, and the preparation of cells. This material is available free of charge via the Internet at http://pubs.acs.org.

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