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Reinforced vesicles withstand rigors of microfluidic electroporation

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Abstract

Living cells synthesize and utilize femtomole and picoliter amounts of material, and an important goal of analytical chemistry is to develop artificial interfaces to efficiently study substances on this scale. This could be achieved with a picoliter container that could be controllably loaded, transported, and unloaded, most desirably in a microfluidic environment. Phospholipid vesicles – surfactant multilayers that can form 10 μm spheres – have been studied for this purpose, but they suffer from fragility and high deformability, which have made them difficult to handle and have limited their application. We present an approach in which a gel is formed in vesicles shortly after they are created. Microfluidic mechanical testing of these vesicles shows that, in the absence of gel, vesicles are difficult to maintain in a trapped state, but the reinforced vesicles exhibit a wide window of pressures under which they can be trapped and manipulated. This improvement is likely to be an essential feature of practical applications of vesicles as microfluidic cargo containers.

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1. Introduction

Most methods for establishing a chemical interface to living cells involve holding the cell with a macroscopic instrument such as a pipet and perforating the cell to cause exchange between it and an external environment. The concentration of material leaving the cell easily becomes much lower and less uniform, a problem that is exacerbated when attempts are made to transport the material to other locations [1,2]. In a much more desirable arrangement, substances extracted or exuded from a cell would be rapidly collected into a container of similar volume for transport and subsequent analysis.

The technology for making and handling containers in this volume range is still in its early development. Perhaps the most impressive approaches involve uniformly sized water droplets surrounded by a thick shell of an oil phase, stabilized by surfactants, formed using concentric microfluidic channels or other means [3–5]. These may prove useful in the transport of preconcentrated analytes, but it may prove challenging to load and unload these in close proximity to cells. Further, analytes with hydrophobic character may partition into or be denatured by the oil phase. Block copolymer vesicles have many useful and tunable properties, but face similar challenges [6].

Another approach is the use of phospholipid vesicles. Natural phospholipids such as soy lecithin, and their synthetic analogs, readily form sheets of molecular bilayers or multilayers, and these sheets can form 10-μm-scale spheres in an aqueous environment [7–11]. While water can diffuse across the lipid layers, larger or charged molecules essentially cannot, so they can be used as closed containers [12–15]. To open a typical 10 μm diameter container in suspension, it is exposed to an electric field of about 100 mV/μm [16–18]. Under such conditions, pores form in the lipid membrane, allowing relatively large molecules to diffuse across it [19]. The same phenomenon can cause adjacent vesicles to fuse and combine their contents [20–22]. Such manipulations have been much more widely demonstrated for phospholipid vesicles than for the other methods described above.

Several disadvantages of phospholipid vesicles have limited their use, including challenges with efficient preparation of vesicles of uniform diameter and composition; property changes or instability as a function of salt concentration, temperature, pH, and other parameters; mechanical fragility and deforma-

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bility, making them difficult to handle [23–25]. The use of polymer-based or polymer-grafted membranes widens the range of solution environments that can be used, and the inclusion of charged polymers in the interior can improve mechanical properties by modulating the internal osmotic pressure and membrane tension [6,26]. In a microfluidic environment, design and cost constraints result in channels with sharp or rectangular edges, on which vesicles cannot be trapped as easily as they can with glass micropipets. Thus, even with recent improvements in vesicle formulations, microfluidic handling of vesicles has proven challenging. In a previous report, we were able to trap vesicles, but only with low yields, short durations, and careful handling, requiring hours of work for each experiment [27].

Even though vesicles are reasonably good approximations of cell membranes, living cells are much easier to handle using either micropipets or their microfluidic equivalents, being much more resistant to deformation and failure than vesicles [28–30]. This is because the interior of a cell is much more rigid due to the presence of structural proteins that form a cytoskeleton, as well as other cell contents—a fact suggesting that further improvements to vesicle mechanical properties could be obtained by adjusting the interior volume of the vesicle rather than just the membrane, in effect creating an artificial cytoskeleton.

A simple instance of this would be an aqueous polymer gel. Bulk gels can be made that resist shear despite consisting of more than 99% water and that can be easily loaded with small molecules as well as biological macromolecules [31]. A gel-filled vesicle would be much less likely to deform into the narrow channel of a microfluidic device or pipet than a vesicle containing higher concentrations of small-molecule or small-polymer additives. Several approaches to this have already been presented, including injection of gel precursors into a preformed vesicle, and release of gel precursors and lipids from electrodes. These formulations were not studied with respect to mechanical properties [32–34].

We present a convenient formulation in which vesicles form spontaneously from a surface (which could potentially be a microfluidic chamber) in the presence of aqueous buffer, with components that spontaneously crosslink to form a gel. Mechanical testing in a microfluidic trap shows that these vesicles are a dramatic improvement over those in which gelation is suppressed. This result shows that vesicles are much closer to being practical tools in the manipulation of substances on the picoliter scale than previously thought.

2. Preparation of vesicles

Our procedure builds upon the approach of Yamashita et al., in which vesicles form from a dry film of polymer-grafted phospholipids [26]. In our work, we modify not only the lipid membrane but also the vesicle interior in order to improve vesicle properties, and we handle materials in less hazardous solvents. Lipids were purchased from Avanti Polar Lipids: soy-derived L-α-phosphatidylcholine (lecithin) and 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[methoxy(polyethylene glycol)-2000], ammonium salt (PEGDOPE). An approximately 100 mM lipid stock solution was made by adding 30 mg PEGDOPE and 155 mg lecithin (5 mol% PEGDOPE) to 2 mL ethanol. In another stock solution, a sub-milligram amount of fluorescein DHPE (Invitrogen) was added and dispersed with sonication. A stock solution of 0.4 M poly(allylamine) in ethanol was prepared by rotary evaporation of 285 μL 20% aqueous poly(allylamine) (M_w 17 kDa, Aldrich) at 50 °C followed by sonication of the film into 2.5 mL ethanol. Also prepared were stock solutions of 1 M acetic acid in ethanol and 0.6 M sodium acetate in water. To gain an understanding of pH effects, 10 mL solutions of 25 mM poly(allylamine) in deionized water or 0.6 M sodium acetate were titrated by adding 5 μL drops of 4 M hydrochloric acid or acetic acid, respectively, and monitoring pH with an ISFET meter (IQ Scientific Instruments).

To a cylindrical vial with an inside diameter of about 12 mm was added 20 μL lipid stock solution, 60 μL poly(allylamine) stock solution, and 14–20 μL acetic acid stock solution, amounting to 0.6–0.8 equivalents of acetic acid per amine. The solution was mixed, and then the ethanol evaporated by rotating the vial under a gentle nitrogen stream to form a smooth film, followed by exposure to vacuum (less than 10 Torr) for 5 min.

To release vesicles from the film, it is first hydrated by placing a 2-μL water drop on the side of the vial and then heating the capped vial for 10 min at 37 °C. At the same time, a buffer vial composed of 1 mL 0.6 M sodium acetate, 15 μL poly (ethylene glycol) diglycidyl ether (M_n 526, Aldrich) or poly (ethylene glycol) monomethyl ether (M_w 550, Fluka) and 10 μL ethanol was warmed to 37 °C [35]. The buffer was added to the lipid film, which was agitated gently, forming vesicles in the 10–40 μm diameter range. These were kept at 37 °C overnight, during which time the crosslinking reaction occurs between the poly(allylamine) and poly(ethylene glycol) diglycidyl ether, as illustrated in Fig. 1.

Bulk gelation reactions were performed by preparing solutions consisting of poly(allylamine) as 1 M amine, varying amounts of acetic acid near 0.8 equiv., 0.6 M sodium acetate, and varying amounts of poly (ethylene glycol) diglycidyl ether below 1 vol%, and heating at 37 °C overnight.

2.1. Pressure and electrical measurement

The microfluidic test platform, outlined in Fig. 2, is built around a silicon chip (0.5 mm thick) with a 1 μm silicon nitride film, which is plasma etched to produce a single 6 μm diameter orifice. The silicon underlying the orifice is wet etched to expose a 1 mm × 1 mm membrane area [36]. The chip is clamped in a manifold that is open above the membrane and provides a fluidic connection to the closed chamber below the membrane. One platinum electrode and one Ag/AgCl electrode (In Vivo Metric, Healdsburg, CA) are in contact with liquid on each side of the membrane. Tubing connects the bottom chamber to a pressure transducer (Storm Series, Senstronic USA, San Francisco, CA) and a hand-controlled syringe pump, which is used to control pressure. The electrical signals are processed by op amps and connected to a National Instruments PCI-MIO-16E-4 card for computer control and measurement, as previously described [27]. Another previously described chip was used for the imaging in Fig. 3(d and e) [37].

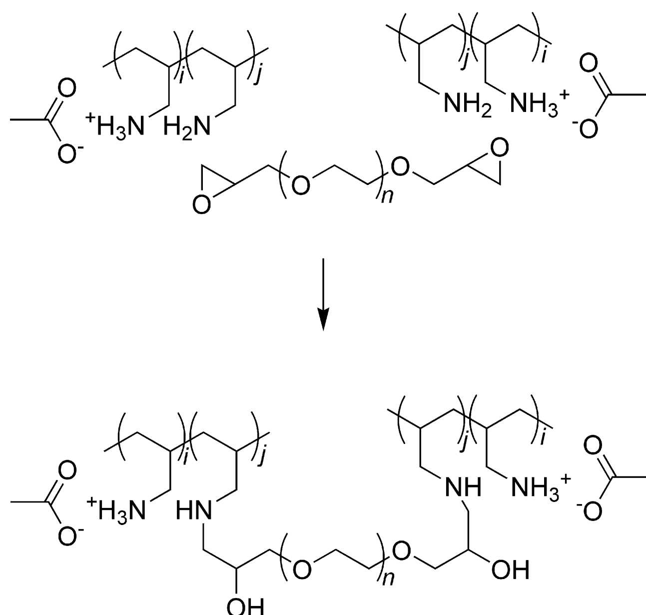

Fig. 1. Crosslinking reaction. Poly(allylamine) chains, partially titrated with acetic acid, react with a poly(ethylene glycol) digycidyl ether crosslinker in water to form a gel. The number of ethylene glycol monomers *n* per crosslinker is about 10, the number of amines per backbone molecule is about 300, and the ratio of *i* to *j* is 0.6:0.4 or 0.8:0.2.

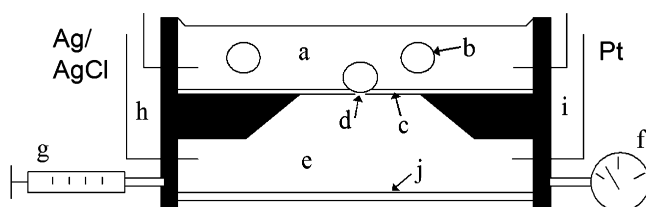

Fig. 2. Microfluidic device consisting of open top chamber (a) containing vesicles (b), silicon-supported silicon nitride membrane (c) with 6 μm orifice (d), closed bottom chamber (e) with pressure measurement (f) and control (g), four electrodes for measurement (h) and control (i) of current and voltage, and a bottom window (j) allowing microscope observation.

In a typical experiment, the device is loaded with buffer solution, including 10 mM sodium chloride to stabilize the electrodes. A drop of the vesicle solution is added to the open top chamber of the device, and the pressure of the bottom chamber is slowly decreased until a vesicle traps on the orifice, at which point the pressure is recorded (the trapping pressure). Then, the pressure is further decreased slowly until the vesicle falls through the orifice (the pull-through pressure). Alternatively, a voltage staircase is applied and the current monitored. The transparent bottom of the manifold allows the vesicle be observed and recorded on an Olympus IMT-2 inverted microscope.

3. Results and discussion

Fig. 1 illustrates the expected crosslinking reaction within the vesicles. One reactant is included in the lipid film, so we expect that, inside at least some of the vesicles, it will be present in significantly higher concentrations than that of the bulk solution. A minimum concentration of reactants is required to achieve the degree of crosslinking necessary for gelation: in bulk experiments, titration of free amine groups above 0.8 equiv. with acetic acid prevented gelation. At 0.8 equiv., reducing crosslinker concentration from 0.8 to 0.4% prevented gelation. The bulk concentration of reactants is far below that necessary for gelation, but we will present evidence that the reaction does occur within the vesicles.

Other vesicle properties are expected to be affected by changes in formulation parameters. According to our titration data in buffer, the pH of a poly(allylamine) solution with about 0.7 equiv. acetic acid is about 9.3, although the pH in a vesicle formed during the vapor hydration step could be as low as 7.2, according to our titration in the absence of other salt. We thus expect some variation in properties depending on how early a vesicle forms a sealed volume. Increasing the amount of acetic acid has several effects in addition to reduction of the pH. For vesicles formed with deionized water, it increases the ionic strength (and thus the osmotic pressure, making the vesicle more rigid) and also decreases the adhesive properties of the polymer, which are counteracted by the small amount of ethanol added to the buffer. Concentrations of either poly(allylamine) or poly(ethylene glycol) much higher than those specified result in poorer yields, sizes, and size distributions of vesicles. The procedure documented here represents the results of attempts to optimize these parameters.

The appearance of the vesicles does not vary as a function of the variables described in our procedure. Fig. 3(a–c) shows vesicles of three different compositions (with and without crosslinker, and with varying degrees of titration), showing no more variation between compositions than within them, and

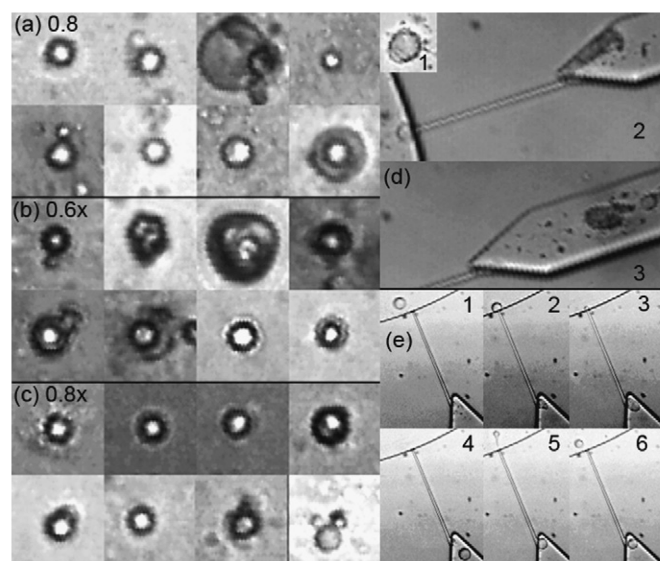

Fig. 3. (a–c) Optical micrographs of vesicles trapped on 6 μm orifice, prepared with: (a) 0.8 equiv. acetic acid and no crosslinking groups, (b) crosslinking groups and 0.6 equiv. acetic acid, and (c) crosslinking groups and 0.8 equiv. acetic acid. (d and e) Optical micrographs of vesicles being pulled through a 4 μm poly(dimethylsiloxane) orifice. (d) Gel-filled vesicle before (1), during (2) and after (3) being pulled through, showing its disintegration. (e) Vesicle without crosslinker being pulled through, remaining intact on the first pass and breaking in two on the second.

this is also observed within larger populations. Specifically, no significant change is seen as the amount of acetic acid is varied or in the presence or absence of epoxide crosslinking groups. The vesicles are multilamellar, which is easy to see from the presence of several that consist of multiple chambers. Through inclusion of fluorescein-containing lipids, we were able to confirm the presence of the lipid membrane after reaction by observing its fluorescence. Inclusion of 0.9 M sodium iodide would be expected to quench the fluorescence significantly if the vesicles are unilamellar [38,39], but no change is observed, confirming that even single-chambered vesicles are multilamellar. This fact is not a direct concern for practical purposes, as long as transport into or out of a single-chambered vesicle is achievable, by electroporation or other means. Another notable result of this experiment is that these vesicles are very tolerant of high salt concentrations and large changes in salt concentration, as can be expected from previous results [26,27].

Despite the similarity in appearance of each type of vesicle, their mechanical properties are drastically different. This can first be noticed in Fig. 3(d and e), in which vesicles are trapped on an orifice parallel to the plane of the device. In Fig. 3(d), a vesicle containing crosslinker fragments upon failure, whereas, in Fig. 3(e), a vesicle without crosslinker easily deforms through the narrow channel, breaking into smaller vesicles only on a second pass through it.

This difference is quantified in Fig. 4, which plots the trapping and pull-through pressures of three types of vesicles. For vesicles without crosslinker, there is only a very narrow window between the two pressures. In fact, for most, the pressures were the same, because a vesicle would only temporarily remain trapped at a certain pressure before falling through the orifice. As a result, the data points for these vesicles are all clustered in the lower left corner of the graph, and near the stability threshold. In contrast, the difference between the pressures for gel-filled vesicles can be as high as 60 kPa, or about 2 μN acting on the orifice, and the majority of vesicles measured are stable. However, in some cases the difference is still low, suggesting that there is variation in the composition of the interior of vesicles, and that some do not contain gel. When using internally dyed vesicles, we have observed similar variations in fluorescence intensity [27]. The likely cause of this variation is the time at which the vesicle forms, as noted above; in a vesicle that does not seal shut prior to addition of a large volume of buffer, it would be expected that dye or gel reactants would leak out, and buffer would leak in. Because the gelation reaction is sensitive to concentration, it is clear that this would result in a variation in mechanical properties. Further tuning of the duration and temperature of the hydration step may vary the fraction of vesicles that seal early, allowing this effect to be confirmed and the fraction to be maximized.

Variation in diameter of vesicles is also apparent, but there is no observed correlation between diameter and trapping or pull-through pressure, except that no vesicle much smaller than 10 μm could be trapped long enough to be measured. To correct for the effect of diameter, trapping pressures P can be converted to membrane tensions σ using the formula $\sigma = PR_vR_o/(2R_v - 2R_o)$, where R_v is the vesicle radius and R_o is the orifice radius [40]. Tensions are plotted in Fig. 5. Because the points are scattered about as much as Fig. 4, it is apparent that diameter variations do not account for variations in mechanical properties. Vesicles including crosslinker sustain tensions 10-fold greater than those without, both here and as observed in Ref. [40], and also in polymersomes in Ref. [6]. One would expect variations in lamellarity to result in strength variations of equal magnitude in all types of vesicles, so this is an unlikely explanation. Clearly, the increase in strength of the vesicles is due to something other than the membrane—that is, the vesicle interior, and variation in that strength is due to variations in the properties of the interior. A weak correlation is present between trapping pressure and pull-through pressure, suggesting that the pressure differences are due to their rigidity: a more rigid vesicle would require greater pressure to conform to the shape of the orifice as well as to be pulled through it.

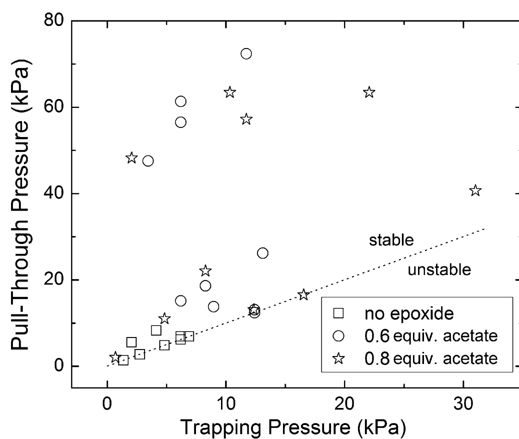

Fig. 4. Maximum pressure across the silicon nitride membrane that a trapped vesicle can sustain before being pulled through the orifice, plotted vs. the minimum pressure required to keep it trapped, for vesicles formed without crosslinking groups and 0.8 equiv. of acetate, and with crosslinking and either 0.6 or 0.8 equiv. of acetate. Vesicles along the stability line remained trapped for only a short duration on the order of 1 min.

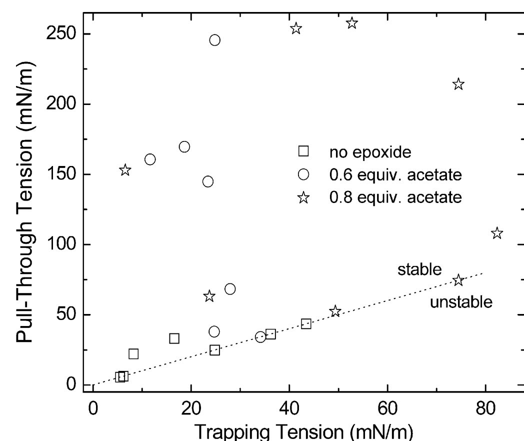

Fig. 5. Maximum tension across the silicon nitride membrane that a trapped vesicle can sustain before being pulled through the orifice, plotted vs. the minimum tension required to keep it trapped, for vesicles in Fig. 4 (all of these also appear in Fig. 3).

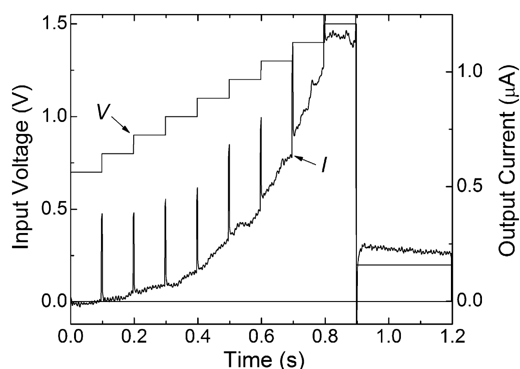

Fig. 6. Current (I) resulting from a voltage (V) staircase applied across a trapped gel-filled vesicle made with 0.6 equiv. acetic acid. Current from a constant 410 kΩ leakage resistance was subtracted out. Abrupt increases in current between voltage steps are attributed to electroporation.

Gel-filled vesicles demonstrate electrical behavior comparable to that of unfilled vesicles undergoing electroporation [27]. Fig. 6 shows the current through a vesicle after subtraction of current resulting from a constant 410 kΩ leakage resistance that was calculated from midpoints of the lower voltage steps. This leakage current is larger than that previously reported for unfilled vesicles by a factor of nearly 10. Larger leakage currents can be expected for more rigid vesicles that do not seal as easily to the edge of the orifice. However, between the higher voltage steps, we observe abrupt increases in current of comparable magnitude. These are indicative of either electroporation or of deformation of leakage paths. An argument against the latter is that more rigid vesicles would be less prone to such deformation, so in that case, a change so large and so similar to that seen for gel-free vesicles would not be observed. Furthermore, while we were not able to use dyes with the gel formulation, our previous work demonstrated a decrease in fluorescence intensity of dye-filled vesicles concurrent with this electrical behavior [27]. This evidence suggests that the inclusion of gel in the vesicle does not compromise the ability of a vesicle to undergo electroporation, a mechanism by which it can be loaded and unloaded. Because they can be trapped for loading and unloading much more reliably than ordinary vesicles, they constitute a much more valuable approach.

4. Conclusion

Phospholipid vesicles filled with poly(allylammonium acetate) crosslinked into a gel with poly(ethylene glycol) diglycidyl ether exhibit a sufficient resistance to deformation to make them able to reliably withstand forces applied to them during manipulation in a microfluidic environment, including trapping and electroporation. This vesicle formulation is simple and scalable, and also potentially compatible with on-chip formation without requiring integration of complicated or expensive components. The rigidity of these vesicles should permit on-chip filtration or sorting, which could provide a path to improved uniformity in size and mechanical properties. Further improvements will be necessary before a full-fledged technology for the manipulation of picoliter containers can be widely applied, but if phospholipid vesicles are to be a major component of that, their reinforcement by internal gelation is likely to be a necessary step.

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