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Membrane Protrusion Coarsening and Nanotubulation within Giant Unilamellar Vesicles

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Supporting Information

ABSTRACT: Hydrophobic side groups on a stimuli-responsive polymer, encapsulated within a single giant unilamellar vesicle, enable membrane attachment during compartment formation at elevated temperatures. We thermally modulated the vesicle through implementation of an IR laser via an optical fiber, enabling localized directed heating. Polymer−membrane interactions were monitored using confocal imaging techniques as subsequent membrane protrusions occurred and lipid nanotubes formed in response to the polymer hydrogel contraction. These nanotubes, bridging the vesicle membrane to the contracting hydrogel, were retained on the surface of the polymer compartment, where they were transformed into smaller vesicles in a process reminiscent of cellular endocytosis. This development of a synthetic vesicle system containing a stimuli-responsive polymer could lead to a new platform for studying inter/intramembrane transport through lipid nanotubes.

In an effort to expand our understanding of fundamental biological and biochemical processes, significant effort is being exerted on the modeling and creation of artificial cells. While initially the concept was limited to creating membrane containers with internalized cargo (e.g., hemoglobin used for blood replacement and charcoal used for detoxification[1,2]), more sophisticated systems have recently appeared and improved.[3,4] "Bottom up" and "top down" approaches for generating synthetic cell models have both been demonstrated, with various features designed to reproduce particular components of the living cell.[5−7]

One fundamental but particularly facile bottom-up fabrication method is based on self-assembly of phospholipids to form giant unilamellar vesicles (GUVs). Among all known liposomal structures, GUVs most closely resemble biological cells with respect to size, membrane structure, and internally confined aqueous composition. This particular artificial cell concept has improved significantly in recent years, spawning a variety of novel methodologies with applications in initiation of enzymatic reactions,[8] polymerization of DNA,[9] synthesis of RNA,[10] cell-free protein expression,[11] polymerase chain reaction,[12] microcompartmentalization,[13,14] and thermally gated liposomes for drug delivery.[15] GUVs have previously been used to create advanced nanofluidic platforms, nanotube vesicle networks (NVNs), for the study of enzymatic reactions and to achieve diffusion control for the regulation of chemical compound migration within nanochannels.[8] The lipid nanotubes that interconnect these container vesicles are typically formed when a point force is applied to the membrane surface of a unilamellar liposome.[16] A nanotube can then be transformed into a vesicle by injecting liquid into the tube orifice, enabling the construction of networks.[17]

Lipid nanotubes are also ubiquitous, highly important features of animal cells, both internally in the endoplasmic reticulum and the Golgi network, and between cells as tunneling nanotubes (TNTs).[18−20] Their biological functions include communication between membrane compartments involved in, among others, protein and lipid synthesis, intercellular communication, particle trafficking, and endocytosis.[21,22] The forces required to pull a nanotube from a membrane are on the order of ∼10 pN, a regime attainable by molecular motors,[23] which may hint at a possible mechanism for intracellular tube formation, having intimate involvement in trafficking. In vivo monitoring of tubulation has not been possible to date, but limited examples of motor protein action in vivo and in vitro have been reported.[22,24] Artificial models to approach this unsolved issue are clearly required, in particular when nanotubes are formed in a controlled manner inside a membrane container, where they may also interconnect several internal compartments.[25]

A strongly related fundamental requirement for creating suitable artificial model cells is to engineer micro-[26−29] or nanosized[30] compartments to generate local differences in structure and function, ultimately approaching a differentiated and interconnected internal architecture. We previously demonstrated the introduction of poly(*N*-isopropylacrylamide) (PNIPAAm), in an aqueous solution, to unilamellar vesicles by microelectroinjection in order to obtain a structured cytoplasm-like interior. This polymer has a characteristic sol−gel phase transition at a lower critical solution temperature (LCST) of 32 °C.[31] Below this temperature, the polymer is fully water-soluble as a result of hydrogen bonding between the amide groups and the surrounding water molecules. Above the LCST, hydrophobic interactions dominate, causing the hydrogen bonds to break down, and water is expelled from the vicinity of the polymer chains, leading to rapid phase separation and eventually a significant volume reduction of the resulting hydrogel.[32] When a PNIPAAm-containing vesicle is heated above 32 °C, hydrogel formation typically occurs immediately,

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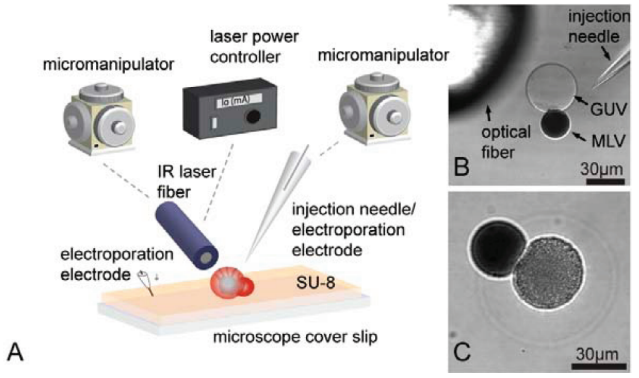

Figure 1. (A) Schematic representation of the components used in the experimental setup. A deposited GUV connected to a multilamellar vesicle (MLV) is shown attached to the SU-8-coated coverslip. (B) Transmission image from the confocal microscope of the optical fiber aligned toward a GUV containing injected polymer solution and connected to an MLV. (C) MLV−GUV with a contracted PNIPAAm−VFc microcompartment, which is centered in the image.

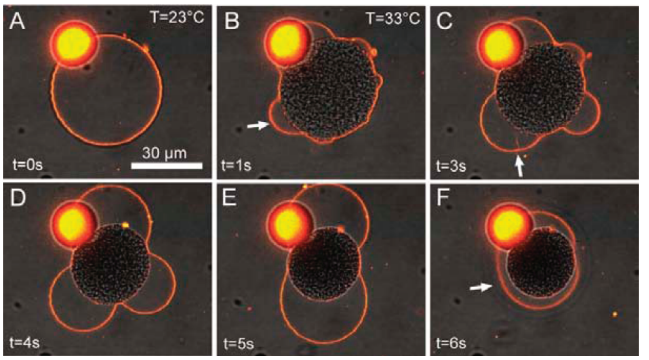

Figure 2. Behavior of the PNIPAAm−VFc system containing 2.0 wt % VFc after injection of the polymer and subsequent heating. Images displayed are composite overlays of fluorescence and transmission. (A) Vesicle with injected polymer at room temperature. (B−F) Changes in the vesicle when the temperature is increased to 33 °C and polymer responds by contracting. As the polymer contracts, the vesicle is deformed as a result of interactions with the membrane. The white arrows point to observed shape distortions that indicate additional membrane−polymer interactions. All images have the same resolution but vary in *Z* focal plane. The apparent vesicle size change is due to the optical sectioning nature of confocal microscopy, as the image plane was chosen to illustrate best the polymer compartment, which was lower than the GUV midpoint.

followed at slightly higher temperatures by contraction to a more compact gel compartment.[33]

Here we report on the dynamic formation of lipid nanotubes within GUVs, during contraction of an internalized thermoresponsive polymer material, through unusual polymer−membrane interactions that result in anchoring of the polymer to the micrometer-sized liposomes. The material, a water-soluble copolymer of *N*-isopropylacrylamide (NIPAAm) and vinylferrocene (VFc), was initially selected for its increased hydrophobicity in order to create highly localized and homogeneous hydrogel compartments.

We investigated a series of three PNIPAAm−VFc copolymers with different ferrocene contents [3.0, 2.0, and 0.1% (w/w)] whose physicochemical properties have been reported previously.[34,35] The ferrocene content of each polymer was verified by flame atomic absorption spectroscopy. Aqueous solutions of PNIPAAm−VFc with a concentration of 30 mg/mL were injected into a GUV. When it was heated to 33 °C (below the heat capacity increase threshold of unilamellar vesicles[36]) using an IR laser by means of an optical fiber, the polymer began to contract, forming a single dense hydrogel compartment (Figure 1). In contrast to unmodified PNIPAAm, where the hydrogel formation did not significantly affect the vesicular membrane [Figures S3 and S4 in the Supporting Information (SI)], we were clearly able to observe interactions of the new copolymer with the double bilayer, forming membrane protrusions. Several of these membrane protrusions occurred, and they merged together and increased in volume as the hydrogel compartment contracted. Their development in PNIPAAm−VFc with 2.0% VFc content is depicted as a sequence in Figure 2. This protrusion formation and the associated coarsening phenomenon are based on strong interactions and anchoring of the polymer with the phospholipid membrane.

During investigations into the polymer action on the membrane, we observed peculiar membrane distortions and the appearance of protrusions (arrows in Figure 2) and discovered that numerous lipid nanotubes were pulled from the vesicle membrane (Figure S1). The nanotubes were connected with the double bilayer at one end and the internal hydrogel compartment on the other. The nanotubes were visualized by prestaining the vesicle with FM1-43, a fluorescent membrane dye, allowing for ease of monitoring (Figure 3). To label only the outer membrane leaflet of the GUV, the membrane dye was introduced into the vesicle after formation.[37] Observation of fluorescence in the nanotubes indicated that the membrane material anchored to the contracting polymer was being pulled from both leaflets and likely formed open tubes. A control experiment was performed without membrane dye to confirm that the formation of protrusions was not the result of interactions of the polymer with dye molecules. Additional controls were performed with unmodified PNIPAAm and poly(ethylene glycol) to demonstrate that the effect is not an unspecific polymer interaction (Figures S3−S5).

Lipid nanotubes are nonequilibrium structures with high membrane curvature and a high surface free energy, which increases with the tube length.[38] In our study, the nanotubes appear to decrease in length to minimize their energy by migrating along the GUV membrane toward the next hydrogel−membrane attachment point. This is visible in the sequence shown in Figure 3, indicating that the nanotubes play a key role in the coalescence of individual protrusions, driving the coarsening dynamics. This is especially apparent in the case where no direct membrane attachment points are available and tube shortening is thus not possible (Figure 3J). The nanotubes are temporarily immobilized and hold the membrane at a fixed distance, which also facilitates imaging and estimation of the tube density.

Elucidation of the mechanism for anchoring the polymer to the membrane is a key step in understanding the nanotube formation mechanics, and a broader discussion is highlighted in the SI. We hypothesize that membrane attachment is not directly related to the ferrocene groups but rather to a general increase in the hydrophobicity of the polymer. A model based on the Kramers potential[39] describing polymer reorganization to cross a boundary was considered, suggesting that the polymer may transition to a site within the vesicle leaflets while strongly interacting with both. This interaction is sufficiently strong to maintain attachment as the polymer contracts.

Protrusion formation on the vesicular membrane requires significant reorganization of lipid material in the system we describe,

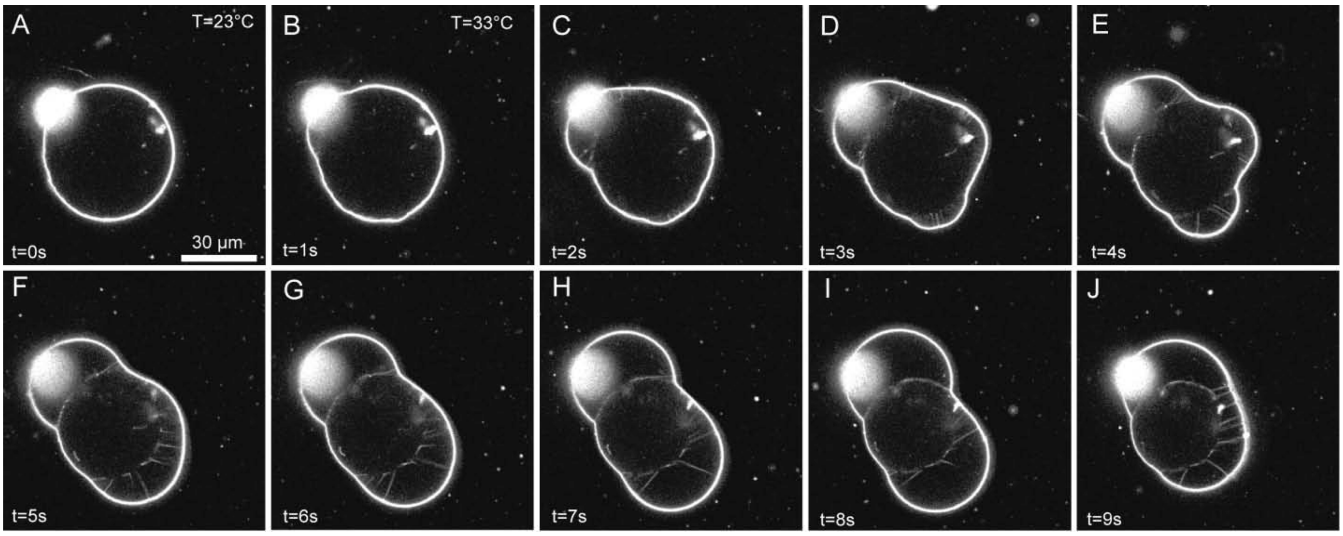

Figure 3. Behavior of the vesicle system with PNIPAAm−VFe containing 3.0 wt % VFe. (A) Natural state of the GUV at room temperature prior to any temperature increase. (B−J) Confocal image sequence showing the coarsening phenomena above the LCST (33 °C) and the combination of membrane protrusions.

a GUV connected with a multilamellar vesicle (MLV), whereas shape transformations are mediated by the presence of the MLV, which acts as a lipid source. As the hydrogel compartment is formed and begins to contract, the GUV membrane, in response to the forces being applied, draws material from the MLV to minimize tension and adjust its surface area accordingly.[40] When the MLV was removed as the lipid reservoir, no protrusions were observed (Figure S6).

Interestingly, all three polymers exhibited the same behavior, indicating that the absolute content of VFc, which ranged from 3.0 to 0.1 wt %, does not have any significant variance in its influence on the lipid bilayer but contributes to its overall hydrophobicity. It can be expected that all or some of the fluid contents of the tube, which originates from the vesicle exterior, will also remain inside the vesicle. This constitutes evidence for endocytosis-like behavior of our polymer-internalized GUV. The lipid material, which is effectively extracted from the GUV's membrane, remains confined to the surface of the microcompartment and can coalesce and fuse to form smaller vesicles on the surface (Figure S2). We see strong potential for an effective model system that can aid in investigations of endocytotic mechanisms as well as endosome formation and maturation. Endosomes have been reported to reach 500 nm in diameter when mature, which is roughly comparable to the size of the small vesicles observed in Figure S2C.[41]

In summary, the introduction of hydrophobic side groups to a GUV-internalized, thermally responsive polymer aids in the reversible formation of lipid nanotubes within the vesicle. PNIPAAm−VFc, like other poly(*N*-isopropylacrylamides), exhibits a coil-to-globule transition and contracts to a dense and compact hydrogel compartment when heated above the LCST. The ferrocene-modified variant maintains strong membrane interactions during compartment formation, which leads to the formation of vesicle protrusions. These membrane protrusions combine rapidly in a stepwise coarsening process to revert to a single spherical vesicle. During progressive separation of the contracting gel compartment from the bilayer membrane, numerous lipid nanotubes are pulled into the container. They spontaneously undergo length reduction driven by the minimization of their surface free energy, which contributes to the dynamics of the shape changes the vesicle undergoes. The tubes are retained on the surface of the hydrogel compartment, where they are transformed into smaller vesicles in a process reminiscent of cellular endocytosis. The relatively mild treatment of the GUVs, only requiring heating within the physiological range, suggests that pathways to nanotube formation within cells, which involve polymer−membrane interactions,[23] may occur by a similar transitional behavior.

■ ASSOCIATED CONTENT

Supporting Information. Experimental setup and details, chemical information, supporting figures, and a further description of the membrane interaction. This material is available free of charge via the Internet at http://pubs.acs.org.

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