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Block Copolymer Giant Unilamellar Vesicles for High-Throughput Screening

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Abstract: Bottom-up synthetic cells offer the potential to study cellular processes with reduced complexity. Giant unilamellar vesicles (GUVs) offer precise control over their architecture and can mimic morphological aspects of cells. We propose a block copolymer-based GUV system that can be used for high-throughput screening. Through droplet microfluidic methods, we produce double emulsions that then serve as templates for GUVs with adjustable inner, polymer membrane, and outer composition. Using flow cytometry, we are able to analyze tens of thousands of GUVs in a short amount of time, enabling their use for screening assays.

Keywords: GUV · High-throughput analytics · Microfluidics · Polymer

Lukas Heuberger received a Bachelor's degree in health sciences and technology and a Master's degree in biomedical engineering from ETH Zürich. After an internship at Roche Diagnostics, he joined the group of Prof. Cornelia Palivan at the University of Basel in 2020. His research revolves around the application of polymer giant unilamellar vesicles for high-throughput screening and as artificial cell model systems.

1. Polymer Giant Unilamellar Vesicles

Giant unilamellar vesicles (GUVs) are micrometer (1–100 µm) sized compartments comprising of a double-layer membrane formed by amphiphilic molecules such as lipids, polymers, or peptides.[1] They are excellent model systems for studying biological processes and have been applied to study mass transport across membranes,[2] membrane protein reconstruction,[3–6] and the mechanical properties of biological membranes.[7,8] GUVs can help increase the understanding of cellular processes by reducing cellular complexity and the number of parameters influencing the process.

Thanks to precise control over the architecture of such compartments, their applications and behavior are easily influenced: molecules can be encapsulated in the vesicle lumen, the membrane composition can be changed, molecules can be inserted into the membrane,[8] and the membrane surface can be functionalized with bioactive molecules (Fig. 1).[9]

Lipids are the most commonly used amphiphiles in GUV formation, though lipid GUVs often lack mechanical stability and chemical versatility.[10] Amphiphilic block copolymers present a multitude of advantages over conventional lipids, such as the tunability of block composition, block ratio, and the ability to add specific functionality (Fig. 1).[11] By choosing amphiphilic di- or triblock copolymers such as PDMS-*b*-PMOXA (poly(dimethylsiloxane)-*block*-poly(2-methyloxazoline), Fig. 2b) with different block sizes and compositions, the membrane thickness of polymer vesicles (nanometer-sized polymersomes and GUVs) can be tuned[12] and by employing different end groups,

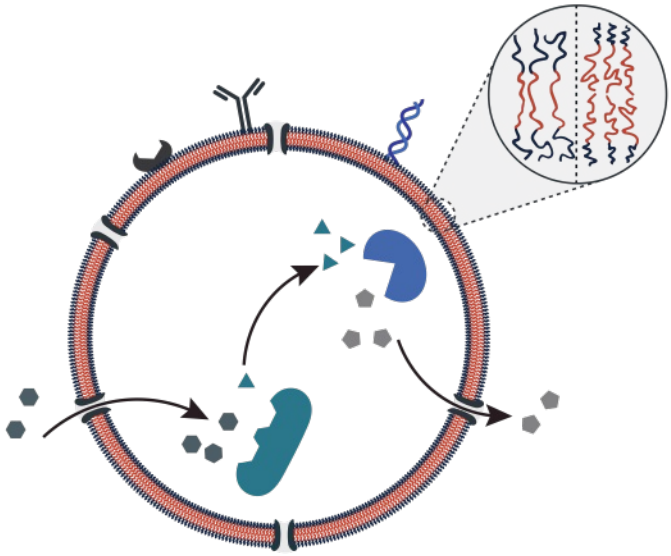

Fig. 1. Schematic representation of a polymer GUV equipped with encapsulated enzymes and membrane pores. Vesicles can be made up of amphiphilic di- or triblock copolymers, forming a membrane with a hydrophobic (red) and hydrophilic region (dark blue) that can be functionalized. GUVs can be permeabilized with pores and used for enzyme cascades or as micro reactors.

vesicles can be functionalized or clustered.[13] PDMS-*b*-PMOXA forms polymersomes and GUVs through self-assembly and can be functionalized, *e.g.* with a terminal azide group for biocompatible click chemistry.[6,14] This type of copolymer has been shown to form membranes with a bilayer thickness of 6–21 nm, depending on the block lengths and architecture.[12]

While liposomes are often leaky, risking the loss of their encapsulated cargo, this is less of an issue with amphiphilic polymers: Because of their lower lateral fluidity and higher membrane thickness, PDMS-*b*-PMOXA/PMOXA-*b*-PDMS-*b*-PMOXA vesicles are significantly less permeable than their lipid counterparts.[15]

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These factors also lead to increased stability; polymer vesicles can be stored at room temperature for weeks to months.[16]

Most methods for the formation of polymer GUVs fall into two main domains; bulk techniques such as film rehydration[6] or electroformation,[17] and microfluidic methods.[5] While bulk methods rely on stochastic cargo encapsulation and often produce a polydisperse sample of giant vesicles,[6,17,18] double emulsion-templated microfluidics allows for the high-throughput production of monodisperse GUVs with nearly 100% encapsulation efficiency.[19]

w/o/w (water/oil/water) double emulsions are routinely used with high-throughput screening methodologies to study protein evolution or cell-to-cell interactions.[20] Because GUVs have a size similar to that of double emulsions and cells, they are also suitable for high-throughput analysis using similar methods. There are only very few examples of lipid[21–25] or polymer[6] GUVs being applied in this context. As GUVs are an accurate model for a simplified living system, their production and investigation at high throughput is of interest for the study of cellular processes. Here, we present microfluidic methods to generate stable polymeric GUVs for the high-throughput screening of cellular model systems.

2. GUVs for Screening Applications

2.1 *GUV Formation*

Fabrication of GUVs through microfluidic methods begins with w/o/w double emulsion formation, followed by evaporation of the organic solvent, which results in thin-shelled GUVs.[5,26,27]

A multitude of different microfluidic designs are employed to create double emulsions, such as glass capillaries[28,29] or molded microchannels,[5,27] each with their advantages and drawbacks. Standardized microchips with molded channels can be designed to reduce inter-device differences, resulting in reproducible production of double emulsions.

PDMS is one of the most common materials used for microfluidic chip fabrication.[30] However, a major drawback of PDMS-based microchips is the limited ability to control surface properties like wettability and the resulting absorption of molecules. Furthermore, PDMS has a tendency to swell in the organic solvents that are used to solubilize polymers.[31,32] Silicon or glass microchips serve as a useful alternative to PDMS microfluidic chips as these materials are resistant to organic solvents and through deep-reactive ion etching, precise microchannel structures can be created from a photomask.[30,33]

In our group, we chose a six-way junction design to form double emulsions (Fig. 2a). In this design, three phases are co-flowed to enclose an inner aqueous (IA) phase within a polymer organic (PO) phase. An outer aqueous (OA) phase is used for flow focusing and to break the continuous flow into individual droplets. The block copolymer is added to the organic phase consisting of a 3:2 hexane:chloroform mixture and serves to stabilize the formed double emulsions and subsequently form the GUV bilayer membrane. The resulting double emulsions serve as templates for unilamellar vesicles. By evaporating the organic solvent composing the PO phase, the amphiphiles self-assemble to form the membrane bilayer. Because of the high volatility of the organic solvents, this process takes place within minutes of the double emulsions being exposed to air and results in GUVs with sizes of 30–40 µm depending on the channel size and osmotic pressure.

The contents of the resulting double emulsion can be precisely controlled by adjusting the inner aqueous phase, and biological cargo can be loaded with an encapsulation efficiency of nearly 100%. This process results in the controlled formation of GUVs with a narrow size distribution and controlled inner biomolecular and membrane composition at very high throughput.[27]

When selecting an amphiphilic block copolymer, membrane thickness, vesicle stability, and functionalization potential are essential considerations. Taking these factors into account, we use the diblock copolymer PDMS-*b*-PMOXA, which has been demonstrated to be impermeable to small molecules and ions.[17] To enable access to the GUV inside, vesicles can be selectively permeabilized through pore proteins or pore-forming peptides. Our group has recently demonstrated the incorporation of outer membrane protein F (ompF), a protein pore from *E. coli*, into microfluidic PDMS-*b*-PMOXA GUVs. Using these GUVs, a multi-step enzymatic cascade distributed over several GUVs was established.[27,34]

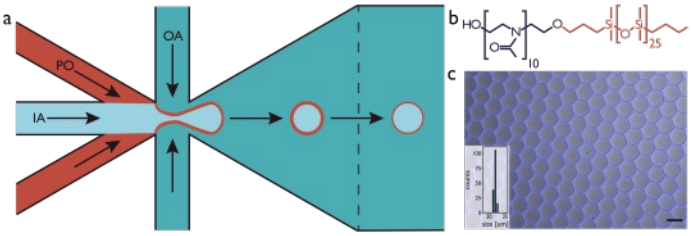

Fig. 2. Microfluidic process of creating GUVs. a) Schematic representation of the working principle of GUV production using double emulsion microfluidics. Inner aqueous (IA) is surrounded by a polymer organic (PO) phase that is subsequently pinched off by an outer aqueous (OA) phase. The resulting double emulsion can then be dewetted to form a GUV. b) Chemical structure of the block copolymer $PDMS_{25}$-*b*-$PMOXA_{10}$. c) CLSM micrographs of GUVs stained with the boron-dipyrromethene dye BODIPY 630/650, insert representing a histogram of 160 measured GUVs. Scale bar corresponds to 30 µm.

Here, we selected a diblock copolymer with a short block length ($PDMS_{25}$-*b*-$PMOXA_{10}$, Fig. 2b) mixed with an azide-functionalized version of the same copolymer ($PDMS_{22}$-*b*-$PMOXA_8$-OEG_3-N_3) for subsequent surface functionalization. Strain-promoted alkyne–azide cycloaddition with the azide-bearing copolymer enables facile GUV membrane functionalization at mild conditions.[35]

Using these polymers, we produced monodisperse GUVs with a diameter of 31.8 ± 0.5 µm with typical production rates on the order of 1 kHz (Fig. 2c).

2.2 *Screening GUVs at High Throughput*

As a proof of concept, we have screened fluorescent PDMS-*b*-PMOXA GUVs with flow cytometry. For this, the green fluorescent dye calcein was encapsulated into GUVs by adding it to the IA phase. Further, GUV membranes were functionalized with an azide-reactive far-red fluorescent dibenzocyclooctyne (DBCO)-Cy5 dye.

The aforementioned fluorescent GUVs and non-fluorescent control GUVs were produced and then dewetted for several hours to ensure the complete partitioning of the organic phase. They were washed twice with outer aqueous phase to remove unbound dye, organic phase, and imploded GUVs. Subsequently, individual GUVs were imaged using a confocal laser scanning microscope (CLSM) (Fig. 3a) and a set of 50'000 GUVs was analyzed with a flow cytometer (Fig. 3b). GUVs with different fluorescent dye combinations can be seen in Fig. 3a. The resulting cytograms were gated to remove debris and aggregating GUVs. The observed Cy5 and calcein fluorescence of ~47'000 GUVs indicate that fluorescence in GUVs can be reliably detected using flow cytometry. It can be seen that both the encapsulated calcein and Cy5 membrane functionalization increase the fluorescence signal by >10-fold. Using these methods, GUVs could be separated based on their internal fluorescent. These findings are in agreement with the fluorescence intensity of the GUVs seen in Fig. 3a. While gating removes ~3–4% of the GUVs, this can be

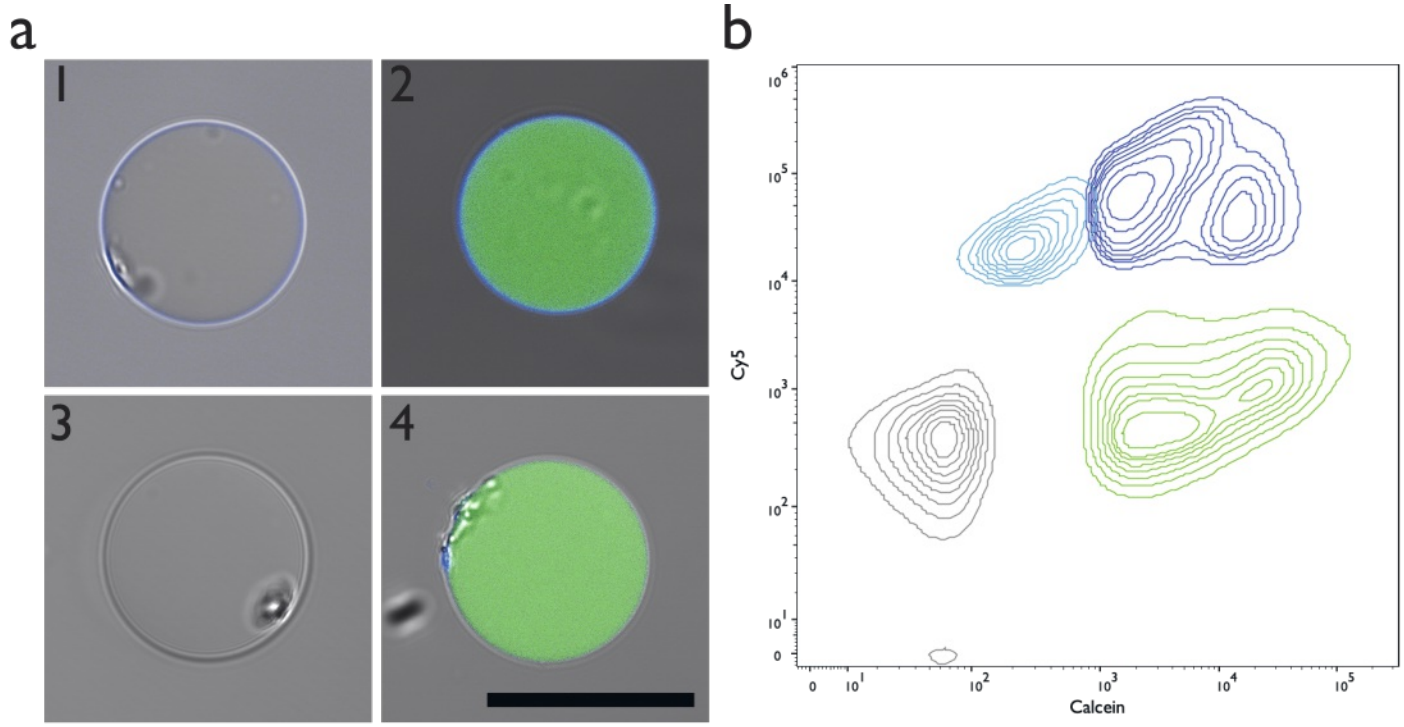

Fig. 3. a) CLSM micrographs of PDMS-*b*-PMOXA-OEG-N_3 GUVs stained with Cy5 *via* a copper-free click reaction (1+2), encapsulated calcein (2+4) or without fluorescent dye (3). Scale bar corresponds to 40 µm. b) Flow cytometry analysis of the GUV population from (a). GUVs were gated to remove debris and aggregated vesicles.

easily overcome by simply increasing sample sizes, since GUVs can be made at high throughput.

Taken together, our results demonstrate the applicability of flow cytometry for the high-throughput screening of GUVs. While similar studies can be done with double emulsions, the main advantage of using GUVs for high-throughput screening lies in the potential for selective permeabilization and functionalization of the vesicles. By adding or removing substrates, the inner composition of GUVs can be continuously changed to influence encapsulated cargo such as enzymes, organelles, bacteria, or eukaryotic cells.

4. Conclusion and Outlook

Tight control over both lumen and membrane composition of GUVs is necessary for their application as cell mimics or high-throughput screening vesicles. Microfluidic double emulsions serve as ideal templates for the creation of monodisperse GUVs at high throughput. Thanks to their size being comparable to cells, GUVs can be screened using flow cytometry. Using these techniques, tens of thousands of vesicles can be analyzed in a short amount of time, making GUVs a convenient alternative to w/o/w double emulsions. By enabling access to the GUV lumen via pores in the polymer membrane, organelles or small organisms can be encapsulated and exposed to different environmental conditions, which ultimately can contribute to the creation of a high-throughput method of screening organism networks.

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