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Chapter VIII

Summary and Outlook
Summary

In this thesis Giant Unilamellar Vesicles (GUVs) were used as a minimal model system of the cellular membrane. GUVs are spherical assemblies of natural or synthetic lipids composed of a single lipid bilayer which separates the aqueous interior compartment from the exterior. The main advantage of this membrane model is that GUVs can be readily observed by optical microscopy. The giant size (5-100 µm) allows to image morphological changes in the lipid bilayer of GUVs. This minimal model of the plasma cellular membrane has been broadly used for studying mechanical properties of membranes, lipid organization, interactions with proteins and peptides and more recently as a tool for creating a minimal cell in a bottom-up approach. Several protocols have been proposed to form GUVs, being the most common electroformation and gentle hydration methods for the formation of GUVs at low salt concentrations. However, the study of biological interactions requires the use of biologically relevant salt concentrations. To tackle this drawback from traditional preparation methods, alternative protocols such as water in oil emulsion, solvent exchange and gel films have been proposed. However, traces of impurities coming from the preparation method have been detected in the lipid bilayer as it has been deeply discussed in this work (Chapter I and II).

This thesis presents a new method, based on a chemically crosslinked hydrogel as a substrate for the growth of GUVs at relevant biological salt conditions without the transfer of impurities coming from the substrate (Chapter I). We studied the physical chemical properties of this hydrogel network and we characterized the final GUVs in terms of yield and size (Chapter II). Moreover the GUVs were used as a biophysical platform for membrane studies at high salt concentrations (Chapters III and IV). Finally, the application of GUVs as a cell mimicking platform for studying drug delivery (Chapter V) and membrane fusion processes (Chapters VI and VII) were investigated.

This work covers some biophysical aspects and applications of the GUV model applied at relevant salt concentrations and also shows that there is room for other studies. In the final chapter (Chapter VIII) preliminary results were obtained for filling gaps in the understanding of the GUV model and future research lines are proposed.
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Outlook

Hydrogel substrates for GUV formation

The use of DexPEG hydrogel substrates for facilitating the growth of GUVs at high ionic strength conditions is presented and validated for several lipid compositions in Chapter II; however the preparation of those substrates is time consuming and requires a basic training in chemistry to perform the esterification of Dextran and maleimide (Dex-Mal), purification of the product and preparation of hydrogel functionalized glass substrates (DexPEG). In Chapter III an attempt to form a polyethylene glycol (PEG) hydrogel film by dissolving the commercially available 4 arms PEG thiol and mixing with catalytic quantities of H$_2$O$_2$ did not produce a suitable hydrogel film for the formation of GUVs. Moreover, thermal characterization (DSC) of the hybrid PEG hydrogel and DOPE lipid showed a strong interaction between lipids and PEG scaffold, while the interaction between lipids and DexPEG scaffold was weaker. Thus the use of other polysaccharides besides Dextran in the formation of hydrogel films is a subject of deeper research.

The high swelling efficiency of the DexPEG hydrogel films promotes the formation of GUVs at relevant salt conditions (Chapter III). In addition to DexPEG hydrogels, photocrosslinked Dextran-methacrylate hydrogels might be another option to produce Dextran-based hydrogel films for the vesicle growth. The Dextran backbone and the high swelling ratios of Dextran-methacrylate hydrogels might produce similar results to those in DexPEG hydrogels. However, the ability of those hydrogels in the production of GUVs has not been tested as yet. The formation of inhomogeneities in the hydrogel structure due to unreacted methacrylate groups might be a possible drawback of Dextran-methacrylate hydrogels if the UV irradiation is variable through the thickness of the hydrogel during photocrosslinking. The formation of inhomogeneities might affect directly the control of the size distribution in the final GUV population.

Encapsulation of bio-molecules

The formation of size controlled GUVs and efficient encapsulation of biologically active compounds has only been achieved successfully by microfluidic jetting until now. In this thesis, DexPEG hydrogels were used in GUV formation and efficient encapsulation of small water soluble molecules such as lucigenin (Chapter V and VI) and carboxyfluorescein (Chapter VII). Furthermore, fluorescently labelled DNA was encapsulated into GUVs by
hydration of the hybrid DexPEG-lipid film with a solution (100 mM NaCl, 10 mM TES, 1 mM EDTA, 1 mM CaCl$_2$) containing fluorescently labelled DNA (5 µM). Bright field and fluorescence microscopy of DNA loaded GUVs are presented in Figure 1. It has been shown that DNA loaded GUVs represent a minimal model for studying the self-replication of DNA and the self-reproduction of the lipid compartment.$^{18}$ Thus, the use of DexPEG hydrogel substrates for the encapsulation of biomolecular cargo inside lipid compartments might have biotechnological applications and could be potentially a tool in synthetic biology for the creation of minimal cell models.$^9$

Figure 1. Encapsulation of biomolecular cargo into GUVs with the lipid composition DOPC:DOPE:Cholesterol (2:1:1). A) Bright Field microscopy and B) Fluorescence microscopy. The scale bar is 30 µm.

**Compartmentalization in GUVs**

One of the advantages of GUVs over liposomes is that they can be visualized, because of their “giant size”, by optical microscopy. If those GUVs can be compartmentalized, they become a model system that can be studied as an individual entity. Thus, more insights of the cellular structure and function can be researched with the direct visualization of individual compartmentalized GUVs as a cellular synthetic model. The formation of compartmentalized GUVs is challenging so far and attempts for encapsulating particles inside GUVs have been done with the use of sophisticated equipment.$^{17}$ Here, the use of the DexPEG scaffold allowed the encapsulation of polystyrene-poly(ethylene oxide) (PS300-PEG44-OMe) polymersomes$^{19,20}$ (diameter=500 nm) in the lumen of GUVs. Polymersomes were embedded
into the hydrogel scaffold, firstly mixing Dextran precursor and polymersomes dispersion and finally by the addition of the PEG cross-linker to form the hydrogel (Figure 2A). The rehydration of hybrid DexPEG-lipids films with the polymersomes dispersion does not encapsulate the polymersomes into GUVs. This might be due to the growth mechanism of GUVs on the DexPEG scaffold (Chapter III). It might be possible that polymersomes are encapsulated when GUVs take on the honeycomb DexPEG network during swelling.

Fluorescence imaging of polymersome-loaded GUVs is presented in Figure 2B. The encapsulation of the polymersomes was observed by fluorescence microscopy in all GUVs. Inspection of individual GUVs showed few GUVs with a high concentration of polymersomes (super-filled GUVs). This is in contrast with the inner concentration of most of the GUVs and the external solution. This deviation from the average concentration was recently observed in liposome studies. In those studies proteins and dextrans were encapsulated into liposomes and a small fraction of liposomes were found super-filled of cargo. Understanding the origin of super-filled GUVs, might give new clues about the origin of primitive cells. Herein, we propose the use of the DexPEG-polymersomes hybrid scaffold to study the formation of super-filled GUVs. Our hypothesis is that electrostatic interactions might play an important role in the formation of super-filled GUVs. Therefore GUVs with different lipid composition and colloidal particles with different charges and sizes should be considered in the formation of super-filled GUVs on DexPEG hydrogels.

Figure 2. A) Preparation of the hybrid-DexPEG substrate for polymersomes encapsulation in GUVs. (1) Dext-Mal backbone, (2) PEG dithiol crosslinker and (3) Polymersomes. B) Encapsulation of polymersomes in conventional GUVs and in the center of the micrograph a polymersomes-super-filled GUV. The scale bar is 100 µm.
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Membrane fusion and GUVs as bioreactors

The use of GUVs and LUVs in membrane fusion studies allowed the imaging of docking, mixing of membranes and mixing of inner contents (Chapter VII). However, a severe limitation of the content mixing assay is the huge dilution factor ($10^6$) once the cargo of the LUVs is released into GUVs. The use of an enzymatic reaction, where small quantities of substrate loaded in the inner volume of LUVs are delivered by membrane fusion to GUVs might improve the assay. The system proposed by Kahya et. al., consisting of β-Galactosidase entrapped into LUVs and fluorescein conjugated Galactopyranoside in GUVs might tackle the dilution drawback of our content mixing assay between GUVs and LUVs triggered by membrane fusion.

Compartmentalization creates separation of biomolecules and processes between different compartments of the cell. Compartmentalized GUVs containing enzymatic polymersomes in the lumen of GUVs resemble the structure of the cell and its organelles. This “minimal cell” model might be used to simulate some of the cellular reaction pathways that take place in multiple and successive compartments of eukaryotic cells. Cascade reactions are a good example of reactions that can be performed in polymersomes-loaded GUVs. The Figure 3 shows a schematic representation of a cascade reaction between LUVs containing NAD$^+$ and compartmentalized GUVs with polymersomes containing G6P and Resorufin (nanoreactors). The substrate NAD$^+$ is delivered to GUVs by membrane fusion between LUVs and the lipid membrane of GUVs. Once the substrate is delivered to the lumen of the GUV, it diffuses to the interior of the nanoreactors triggering an enzymatic reaction which is detected by the fluorescence of Resorufin in polymersomes. The importance of this model relies in the effort to provide insight over evolutionary molecules and processes in the origin of life.

In conclusion, these preliminary results demonstrate that DexPEG hydrogels are flexible scaffolds for preparing and encapsulating of a broad range of cargo in GUVs. The design of DexPEG hydrogels represents an advantage over other methods for preparing GUVs with several lipid compositions, defined size distribution and high ionic strength conditions. In addition to the use of GUVs as a membrane model, the encapsulation of biomolecular cargo and/or polymersomes makes GUVs a good candidate for the construction of more complex lipid-based systems. We envisage the application of the DexPEG method as a novel tool in the construction of “minimal cellular” models for studying origin and evolution of cellular life with
potential applications in synthetic biology and biotechnology. Furthermore, the incorporation of the membrane fusion system in the construction of synthetic cells increases the applicability of GUVs in the study of cellular functions and drug delivery.

Figure 3. Schematic representation of membrane fusion between compartmentalized GUVs and LUVs. The release of NAD$^+$ by membrane fusion triggers the fluorescence of Resorufin in the interior of GUVs that can be detected by fluorescence microscopy.
Samenvatting

In dit proefschrift zijn Giant Unilamellar Vesicles (GUVs; reusachtige enkelwandige blaasjes) gebruikt als minimaal modelsysteem voor het membraan van een cel. GUVs zijn bolvormige structuren van natuurlijke of synthetische lipiden waarin de lipiden een enkele lipidendubbellaag vormen die het waterige binnenvolume van het buitenvolume scheidt. Het belangrijkste voordeel van dit membraanmodel is dat GUVs eenvoudig waargenomen kunnen worden met een optische microscoop. Door de reusachtige afmeting (5-100 µm) zijn morphologische veranderingen in de lipidendubbel laag van de GUVs zichtbaar. Het GUV model wordt veel gebruikt voor het bestuderen van de mechanische eigenschappen van membranen, lipidenorganisatie, interactie met eiwitten, en pepti de, en recentelijk ook voor het creëren van een eenvoudige cel in een “bottom-up” aanpak. Verschillende protocollen voor het vormen van GUVs zijn voorgesteld en hiervan worden electroformatie en langzame hydratie methodes het meest toegepast. Deze methodes werken alleen bij lage zoutconcentraties, maar de studie van biologische interreacties vereist hogere zoutconcentraties, gelijk aan het niveau in levende biologische systemen. Om dit nadeel van de traditionele methoden te omzeilen zijn alternatieve protocollen ontwikkeld die gebruik maken van bijvoorbeeld water in-olie-emulsies, oplosmiddeluitwisseling en dunne hydrogelfilms. Bij gebruik van deze methodes zijn echter sporen van onzuiverheden waargenomen in de lipidendubbellaag waardoor de (bio)fysische eigenschappen veranderen, zoals uitvoerig besproken in dit werk (Hoofdstuk I en II).

Dit proefschrift presenteert een nieuwe methode met een chemisch gecrosslinkte hydrogel als substraat voor de groei van GUVs in relevante biologische zoutconcentraties, zonder overdracht van onzuiverheden uit het substraat (Hoofdstuk I). De chemisch-fysische eigenschappen van de hydrogelnetwerken werden bestudeerd en de gevormde GUVs gekarakteriseerd in termen van opbrengst en grootte (Hoofdstuk II). Deze GUVs zijn vervolgens gebruikt als platform voor biofysische membraanstudies bij hoge zoutconcentraties (Hoofdstukken III en IV) en als een modelsysteem voor cellen om medicijnafgifte (Hoofdstuk V) en membraanfusieprocessen (Hoofdstukken VI en VII) te bestuderen. Dit werk gaat over de biofysische aspecten en toepassingen van het GUV model bij relevante zoutconcentraties, maar het toont ook aan dat er ruimte is voor andere studies. In het laatste hoofdstuk (Hoofdstuk VIII) worden de voorlopige resultaten betreffende het invullen van lacunes in de kennis van GUVs als model systeem gepresenteerd. Ook worden toekomstige onderzoekslijnen voorgesteld.
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