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Chapter I

Introduction
Chapter I: Introduction

The plasma cellular membrane together with the cytoskeleton defines the shape of the cell.\textsuperscript{1} The cellular membrane is an essential component in all cells that defines the boundaries between the cytoplasm and the external environment. The cellular membrane consists of a complex mixture of lipids, proteins and carbohydrates that control and regulate the transport of ions and molecules across the membrane to perform complex biological cellular functions. Due to the complexity of cellular membranes, there is great interest in generating simplified membrane models to enable the study of membrane function.\textsuperscript{2} The use of supported bilayers,\textsuperscript{3-8} nanodiscs\textsuperscript{9-16} and liposomes\textsuperscript{17,18} are some of the platforms that have been used as biophysical models.

Giant unilamellar vesicles (GUVs) with sizes between 5-100 µm, have been proposed as a biophysical platform because they are readily observable by optical microscopy and the size and membrane curvature are similar to cells.\textsuperscript{19,20} GUVs are assemblies of natural or synthetic lipids composed of a single lipid bilayer separating the aqueous interior compartment from the exterior (Figure 1). GUVs have been used as a biophysical model system in studies of lipid organization,\textsuperscript{21} membrane-peptide,\textsuperscript{22} and membrane-protein interactions.\textsuperscript{23}

![Figure 1](image.jpg)

**Figure 1.** Confocal image of Giant Unilamellar Vesicle (GUV) composed of DPhPC/DPPC/Cholesterol (2/2/1). The lipid membrane shows phase separation (L\textsubscript{o} and L\textsubscript{d} phases). Black spots are cholesterol rich domains which prefer the L\textsubscript{o} phase. The scale bar is 20 µm.
Chapter I: Introduction

The morphology, production and size distribution of the GUVs depends on the selected formation method and growth conditions. Traditional methods for the preparation of GUVs are gentle hydration and electroformation.\textsuperscript{24, 25} However, the growth of GUVs under physiologically relevant conditions (>300 mOsm/kg) has been challenging for those methods. As an alternative, the formation of GUVs by emulsion methods has been proposed for the preparation of GUVs at relevant salt conditions, but the presence of remaining solvent in the lipid bilayer of the final GUVs and its effect on the biophysical properties of the membrane are still under discussion.\textsuperscript{26, 27}

Recently the preparation of GUVs assisted by agarose gel films has enabled the production of GUVs at physiological conditions.\textsuperscript{28} Moreover, the use of agarose gel films allow the reconstitution of membrane proteins in the lipid bilayer of GUVs. The agarose film produces successfully GUVs with several lipid compositions, however the agarose gel dissolves during the rehydration of the lipids resulting in traces of agarose contaminating the lipid membrane of the GUVs. Furthermore, a detailed study of agarose-GUVs showed that a fraction of GUVs contains encapsulated agarose in the inner volume of the vesicles and the residual agarose across the membrane affects the mechanical properties of the lipid membrane in electrodeformation studies.\textsuperscript{29} In the same study, Lira et. al. proposed a thermal post-treatment of agarose-GUVs to release the encapsulated agarose and recover the vesicle responses to electro deformation. Alternatively, films of crosslinked polyacrylamide (PAA) and synthetic gels of poly(vinyl alcohol) (PVA) have been employed for the preparation of GUVs.\textsuperscript{28, 30} Whilst PAA and PVA polymers are not detected in the lipid bilayer of the GUVs formed through this approach, they afford minimal ability to control the characteristics of the GUVs in terms of the yield, morphology and size distribution.

This thesis presents a hydrogel-based method for the preparation of GUVs at physiological conditions, without contamination of the lipid membrane of GUVs from the hydrogel matrix. Chapter II presents the method used for the preparation of GUVs at physiological conditions, based on the use of a chemically crosslinked Dextran hydrogel film (DexPEG). The maleimide-functionalized Dextran is chemically crosslinked by a dithiolated polyethylene glycol (PEG) and ligated to the glass substrate to avoid dissolution of the polymeric network during the formation of GUVs. GUVs with several lipid compositions were prepared in PBS and HEPES buffers to validate the general applicability of the method. Chapter III describes how the physical chemical properties of the hydrogel network affect GUV formation. Here, the effect
of the hydrogel precursor composition and structure on GUV formation is systematically studied by modifying the degree of substitution of maleimide in the dextran polymer backbone as well as the architecture of the PEG crosslinker. The resulting GUVs for each combination of precursors were characterized in terms of the yield and size distribution by flow cytometry. In addition, GUV formation in real time experiments was imaged to obtain better understanding of the formation process of GUVs in DexPEG hydrogels.

Next in this thesis, the production of GUVs by this method is applied to cases where high ionic strength conditions are a prerequisite for biophysical studies in the GUV membrane model. **Chapter IV** discusses the use of GUVs as biophysical membrane models for the imaging of red to blue light upconversion, driven by triplet-triplet annihilation. Here, GUVs growth in the presence of high concentrations of sodium sulphite (300 mM), included in the buffer as an oxygen scavenger, allowed the imaging of light upconversion within the lipid membrane of GUVs.

**Chapter V** describes the transport of ions across the lipid membrane of GUVs through membrane incorporation of a synthetic transmembrane chloride transporter. Giant vesicles with the anion transporter located in the lipid bilayer were formed in a solution of a chloride-sensitive fluorophore and NaNO₃ (225 mM). The external addition of NaCl triggers the transport of chloride and the exchange with nitrate through the lipid membrane of GUVs. The quenching of the encapsulated fluorophore in the GUVs allowed the visualization of ion transport and the evaluation of the activity of the chloride transporter. The potential use of this synthetic transporter is limited by the lipophilicity of the molecule which difficult the deliverability of the transporter. Therefore in **Chapter VI** this delivery problem was solved by targeted membrane fusion. Membrane fusion is triggered by two complementary coiled-coil forming peptides K₄ [(KIAALKE)₄] and E₄ [(EIAALEK)₄], which are located in different membranes. The formation of a dimeric coiled-coil by these peptides brings the two opposing membranes into close proximity, thereby inducing efficient membrane fusion and the targeted delivery of the synthetic transporter. Large unilamellar vesicles (LUVs) were used as drug delivery system with the transporter pre-incorporated in the lipid membrane and with the insertion of the complementary peptide amphiphiles on the membrane, the lipophilic transporter was delivered to the membrane of GUVs by targeted membrane fusion. GUVs as a biophysical model in relevant physiological conditions, validated that the transporter is efficiently transferred and active in the membrane of GUVs after membrane fusion. Next, the
same methodology was successfully applied for the delivery of the transporter to live cells by membrane fusion.

The formation of GUVs on DexPEG substrates at physiological ionic strength conditions allowed monitoring the membrane fusion process by fluorescence microscopy. Membrane fusion between GUVs and Large Unilamellar Vesicles (LUVs) driven by the complementary K₄ and E₄ coiled-coil peptide amphiphiles was further studied in the Chapter VII. Membrane fusion was assayed by lipid and content mixing between GUVs and LUVs. The visualization of the fusion process in GUVs gave new insights about the incorporation of the lysine rich lipopeptide K₄ [(KIAALKE)₄] on the lipid membrane of GUVs. Fluorescence correlation spectroscopy (FCS) experiments in GUVs supported the aggregation of lipopeptide in the membrane of GUVs founded by fluorescence microscopy. The use of a co-surfactant (Tween 20) improved the incorporation of this peptide amphiphile and the membrane fusion between GUVs and LUVs.

Finally in the Chapter VIII, new research lines are proposed and supported by preliminary experiments. This chapter shows that there is plenty of room for further applications of the GUV biophysical model and the study of membrane - molecule interactions at physiologically relevant ionic strength conditions. Moreover, the possibility of forming multi-compartmentalized GUVs by the DexPEG method; opens a novel window of future applications in the “bottom – up” approach of creating an artificial cell in the field of Synthetic Biology.

The use of GUVs as a biophysical model is leading to a better understanding of membrane related processes because they are easily produced, readily observable by microscopy techniques, and their similarity with cellular membranes. Thus, fluorescence-based methods and the synthesis of new fluorescent indicators together with the use of GUVs at relevant ionic strength conditions are a powerful tool for studying membrane fusion, viral infection, signalling and drug targeting in the coming years.
# Chapter I: Introduction

## References


