Giant unilamellar vesicles : an efficient membrane biophysical tool and its application in drug delivery studies

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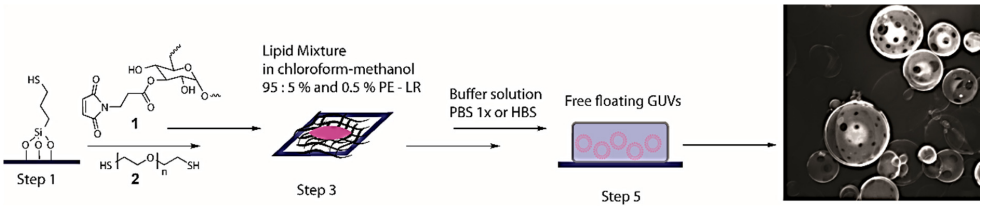
Chapter II

Preparation of size tunable giant vesicles from crosslinked dextran(ethylene glycol) hydrogels



This work is published: Nestor Lopez Mora, Jesper S. Hansen, Yue Gao, Andrew A. Ronald, Roxanne Kieltyka, Noah Malmstadt, and Alexander Kros, *Chem. Commun*. **2014**, 50, 1953-1955.

Abstract

We present a novel chemically crosslinked dextran-poly(ethylene glycol) hydrogel substrate for the preparation of dense vesicle suspensions under physiological ionic strength. These vesicles can be easily diluted for individual study. Modulating the degree of crosslinking within the hydrogel network results in tuning of the vesicle size distribution.

Introduction

The growth of high-quality giant (1-100 µm diameter) unilamellar lipid vesicles (GUVs) under physiologically relevant conditions (>300 mOsm/kg) is generally difficult using most common GUV fabrication methods.[1] For this aim, the most widely used methods are gentle hydration and electroformation.[2, 3] Gentle hydration involves the deposition of a lipid on a glass substrate and swelling of the lipid lamella into vesicles by rehydration in aqueous solutions. To adapt this method to grow vesicles at moderate ionic strength (200 mOsm/kg), it is necessary to include negatively charged lipids and heat the lipids above their phase transition temperatures.[4] Most often, the vesicle yield of this method is variable and occasionally low. However, the addition of non-electrolytic monosaccharides in the dry lipid film promotes lamellar lipid repulsion to increase vesicle yield.[5] Electroformation can provide higher yields and more homogeneous GUVs through the application of an electric field during GUV growth. However, to grow GUVs under high ionic strength conditions, high field frequencies and longer hydration times are required with the main drawback that lipid hydrolysis and peroxidation can occur.[6, 7]

More recently, hydrogel forming polymer substrates have been employed for the preparation of GUVs in order to reach physiological ionic strength conditions. These substrates include agarose gels,[8] polyacrylamide[8] and thin films of poly(vinyl alcohol).[9] While these methods have allowed GUV formation at moderate ionic strengths (~200-280 mOsm/kg), they afford minimal ability to control the characteristics of the GUV in terms of morphology and size distribution.

Here, we form GUVs on a covalently crosslinked hydrogel substrate. We demonstrate that control over crosslink density can alter the size distribution of the GUVs formed. We use dextran polymers crosslinked by poly(ethylene glycol) (PEG) chains using Michael addition to simultaneously prepare the hydrogel (Dex-PEG) and anchor it to a glass surface. Our hypothesis is that an anchored covalent hydrogel cannot be dissolved during the GUV formation process to potentially contaminate the lipid bilayer, which may be a concern with non-covalently crosslinked hydrogels.[8] Moreover, covalent hydrogel matrices enable the possibility for control over GUV size distributions through modulation of crosslinker density and thus, network topology.

Results and Discussion

Dextran (MW= 70 kDa) was modified with N-maleoyl-β-alanine following a previously described protocol to provide polymer **1** to be used for Michael addition with reactive thiols on the PEG polymer and the glass surface.[10-13] The degree of substitution (DS) on the polymer was controlled by the molar ratio of N-maleoyl-β-alanine relative to Dextran and subsequently validated by ^{1}H-NMR. Concurrently, microscope slides were prepared for anchoring of the chemically crosslinked hydrogel directly to the glass surface. A reactive thiol moiety was introduced on the glass slide surface with 3-mercaptopropyl trimethoxysilane. The thiol coated microscope slides were crosslinked to the hydrogel by drop-casting the dextran solution (2 wt %) and PEG dithiol **2** at various molar ratios at 40 °C (**Figure 1**, step 2) until a homogeneous hydrogel film was formed. Following the formation of the hydrogel, the desired lipid mixture was deposited on the hydrogel surface and the solvents were evaporated in a vacuum oven for 30 minutes at 35 °C or at room temperature under a gentle stream of nitrogen gas to prevent lipid oxidation (**Figure 1**, step 3). In a final step, the hydrogel and lipid film on the glass slide were rehydrated in aqueous buffer solutions for 1-2 hours to form free-floating vesicles (**Figure 1**, step 5). In all cases, hydration of the lipid film was performed above the T_m of all the lipids used. To validate the widespread applicability of the Dex-PEG chemically crosslinked network for GUV growth, several buffers, lipid compositions, and crosslinking ratios were tested.

First, the effect of physiological ionic strength conditions on vesicle growth was examined. Hence, two buffers used ubiquitously in biological cell studies were examined for vesicle growth: phosphate buffered saline (PBS) and HEPES potassium chloride saline buffer (HBS). The osmolality of these buffers were experimentally determined by measuring the freezing point depression for both PBS (310 mOsm/kg) and HBS (320 mOsm/kg). In general, qualitative differences were noted between both buffers; HEPES buffer produced slightly greater yields and larger sized vesicles (see *Experimental Section*). However, in both of these buffers high yields of free floating vesicles were easily obtained.

The capacity of the Dex-PEG network to support vesicle growth using a variety of lipid compositions was then explored. Binary mixtures of lipids containing both anionic and neutral lipids and ternary mixtures incorporated cholesterol were examined. The lipid compositions A – H (see details in **Table 1** *Experimental Section*) were designed to examine the effect of three different parameters on vesicle formation; cholesterol (CH) content, negatively charged lipid content (POPG and DOPS), and liquid ordered lipid phases (DPPC). Free floating GUVs using

lipid mixtures (**Figure 1**. A, B and, F up to 25% CH provided a good yield of GUVs with a spherical morphology in PBS and HBS buffers.

This result is in contrast to previous reports where gentle hydration methods showed exclusive formation of tubular morphologies and liposome networks connected by tubes using POPC/CH with 5–30% CH in HEPES.[14] Using the Dex-PEG system, we found numerous spherical GUVs alongside such morphologies.

Another parameter that was explored was the effect of increasing the anionic lipid content in GUV formation. Previously, it was found that lipid mixtures containing more than 10-20% anionic lipids were difficult to grow by gentle hydration methods.[15] Hence, we selected lipid mixtures ranging from 10-50% in POPG (**Figure 1**. C and D). Using our method, we were able to obtain high yields of spherical vesicles under high ionic strength conditions.

Moreover, we examined mixtures with DOPS (10%); a lipid that is known to decrease vesicle yields at high ionic strength conditions. Consistent with this notion we also observed high yields of spherical GUVs with 10% DOPS (**Figure 1** E). Finally, lipid mixtures known to undergo phase separation through phase coexistence of liquid ordered (L_o) and liquid disordered (L_d) phases were tested.[16] We found using the Dex-PEG system, that phase-separated GUVs consisting of 30-50% DPPC can be easily grown at 50 °C (**Figure 2**). Moreover, we can prepare such kinetically trapped GUVs by the Dex-PEG method without the use of additional additives under high ionic strength conditions. Overall, all lipid compositions using the additive free Dex-PEG method to grow GUVs under high ionic strength conditions yielded high quantities of free floating vesicles. A Gaussian size distribution centered between 10-15 μm was found after 1 hour of GUV preparation for all lipid compositions when using an equimolar ratio between the dextran polymer and the crosslinker (see *Experimental Section*). This result suggests a relationship between the method of vesicle growth and size.

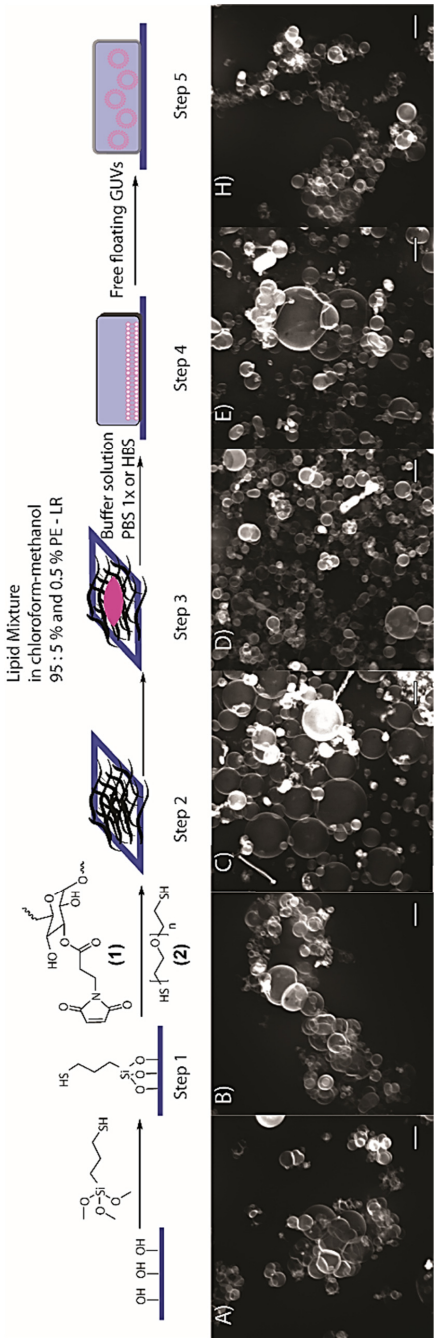


Figure 1. On top GUVs preparation method and on the bottom of the image Z-projections images with high yields of free floating GUVs for various lipid compositions (details for those lipid mixtures are presented in the *Experimental Section*). The scale bars are 10 µm.

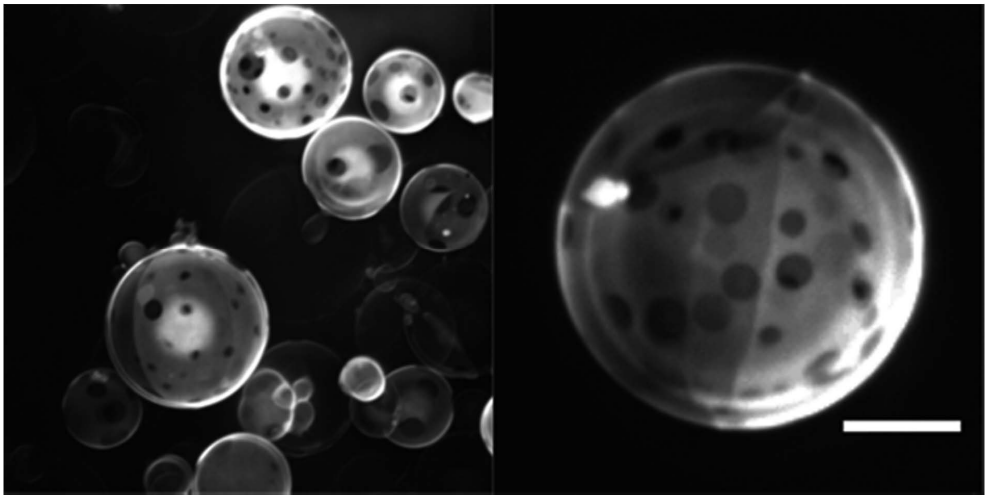


Figure 2. Z-projections of phase separated GUVs with the lipid composition 40% DPhPC / 40% DPPC / 20% CH (mol %) in PBS at physiological ionic strength. The scale bars are 10 µm.

Excitingly, a correlation between crosslink density and vesicle size distribution was observed (**Figure 3**). On average, a population of a hundred GUVs was sized for each experiment. Decreasing the molar ratio of PEG to 75 and 50 % mol with respect to dextran polymer resulted in a concomitant increase in the vesicle size distribution in a single step. Current methods used to gain control over vesicle size require a minimum of two-steps to achieve similar results whereas vesicle size can be tuned within the Dex-PEG system through modifying the composition of the chemical network in a single step.[17] The success of the Dex-PEG method in enabling growth of GUVs based on various lipid compositions under high ionic strength arises from several pertinent chemical features and materials properties.

Based on our data we hypothesize that the driving force for generating free floating GUVs is due to the high swelling behavior of the hydrogel upon hydration. Specifically the water content differs within the hydrogel from 2% in the dry state to 90% in the wet state. The ability for the Dex-PEG hydrogel to imbibe a high percentage of an aqueous solution on the order of less than 1 hour, most likely contributes to interlamellar repulsion that generates the necessary forces to facilitate efficient growth of giant vesicles under physiological ionic strength. In addition, the starting water content of the film plays an important role; vesicles are not formed

without a pre-hydrated film. Moreover, chemical ligation to the glass surface is essential due to the rapid growth of the hydrogel layer on the glass surface upon exposure to buffer. Earlier experiments showed that unligated hydrogels resulted in simultaneous detachment from the glass substrate during vesicle formation. To examine whether hydrogel components were dissociated from the surface or incorporated in vesicles, we synthesized a fluorescently labeled dextran polymer with 1 and 2.5 mol % of methoxycoumarin-3-carboxylic acid (Dex-PEG-C) to be tracked by two-photon fluorescence microscopy. Analysis of free floating GUVs produced from Dex-PEG-C showed no fluorescence either in the membrane or inside the formed GUVs at room temperature (see *Experimental Section*).

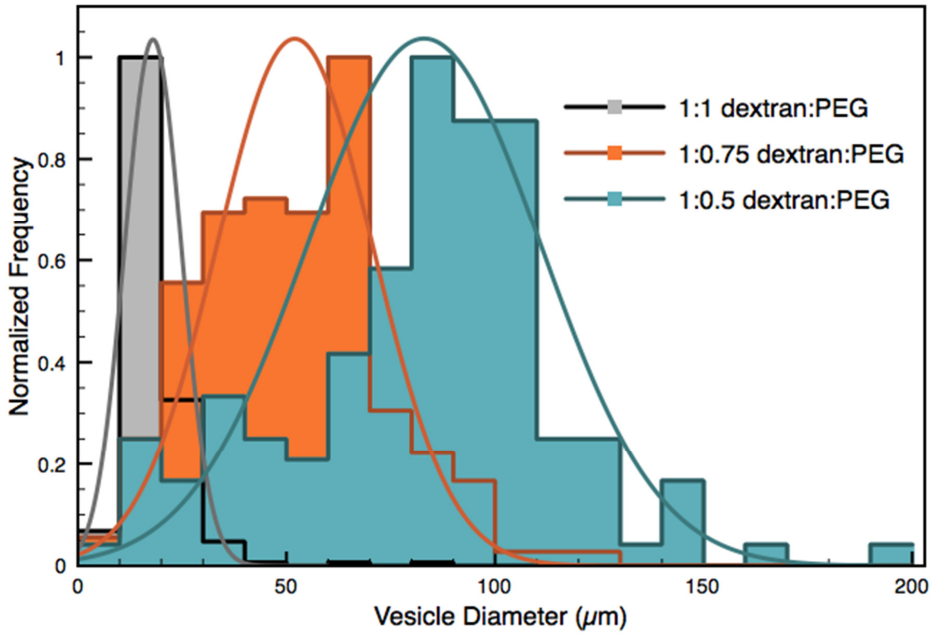


Figure 3. Tuneability of GUVs size distribution (50 % DOPC / 25% DOPE / 25% CH) as a function of crosslink density of PEG relative to dextran in PBS. Average diameters were found to be 18 ± 8 µm (N=215) for 1:1 ratio of dextran-PEG, 52 ± 22 µm (N=171) for a 1:0.75 dextran-PEG and 83 ± 33 µm for a 1: 0.5 dextran:PEG (N=139).

Conclusion

In conclusion, we present a widely applicable method that facilitates the additive-free growth of GUVs under physiological ionic strength conditions based on a variety of lipid compositions. The high swelling capacity of the Dex-PEG promotes the formation of high yields of spherical, free-floating GUVs. Additionally, this method enables the growth of GUVs possessing phase separated domains under physiological conditions. Finally, modulating the crosslink density of the Dex-PEG network provides a handle to tune vesicle size. This Dex-PEG hydrogel system is a powerful method that can be exploited to grow vesicles for applications such as membrane interactions, drug delivery, molecular recognition, lipid raft organization, and membrane fusion studies.[18-21]

Experimental Section

Materials and methods

Cholesterol (CH), 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-(1'-rac-glycerol) (sodium salt) (POPG), 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[methoxy(polyethylene glycol) - 2000] (ammonium salt) (PEG2000-PE), 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(lissamine rhodamine B sulfonyl) (ammonium salt) (PE-LR) were purchased from Avanti Polar Lipids. 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), 4-nitrophenyl disulfide, 4-(dimethylamino) pyridine (DMAP), 1500 and 3400 Da poly (ethylene glycol) dithiol (PEGDT), 7-methoxycoumarin-3-carboxylic acid, and 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid, *N*-(2-Hydroxyethyl) piperazine-*N*′-(2-ethanesulfonic acid) (HEPES) were purchased from Aldrich. Maleic anhydride, *N*,*N*'-Diisopropylcarbodiimide (DIC), *p*-toluene sulfonic acid monohydrate (PTSA) and maleimide were purchased from Fluka. Dextran (M_n = 2 000, 70 000 and, 150 000, Pharmacia Fine Chemicals, Sweden) was dried in the vacuum oven for several days before use. *N*-Maleoyl-*β*-alanine was synthesized according to a reported procedure.[22] 4-(Dimethylamino) pyridinium 4-toluenesulfonate (DPTS) was synthesized from DMAP and PTSA.[23] Dextran Maleimide (Dex-Mal) was synthesized by esterification of the hydroxyl group of the dextran with *N*-Maleoyl-*β*-alanine.[11]

Synthesis of Dex-Mal (**1**)

Dex-Mal (molecule **1**) was synthesized by DIC mediated esterification of the hydroxyl groups of dextran with *N*-Maleoyl-*β*-alanine. Briefly, *N*-Maleoyl-*β*-alanine (1 eq.), DPTS (0.15 eq.) and DIC (1.5 eq.) were dissolved in anhydrous DMSO. The mixture was stirred at room temperature for two hours, followed by adding the DMSO solution of Dextran (2.5 eq.). After overnight stirring at room temperature, the formed *N*, *N*'-dialkylurea was removed by filtration and the crude product was obtained by precipitation in cold isopropanol. The precipitate was dissolved in water and extensively dialyzed against Milli-Q water for two days and subsequently lyophilized. ^{1}H NMR (400 MHz, D2O): δ 3.3-4.0 (m, dextran glucopyranosyl ring protons), 4.9 (s, dextran anomeric proton), 6.8 (s, maleimide). The degree of substitution (DS) of Dex-Mal is defined as the number of maleimide groups per 100 glucopyranose residues of dextran, which was calculated from the ^{1}H NMR spectra based on the protons of maleimide

(δ 6.8) and the anomeric proton (δ 4.9). The DS of Dex-PEG was controlled by the molar ratio between dextran and N-Maleoyl-β-alanine.

Synthesis of Dex-Mal-C.

The covalently fluorescent labeled Dex-Mal (Dex-Mal-C) was synthesized by a similar procedure as Dex-Mal. Briefly, 7-methoxycoumarin-3-carboxylic acid (0.025 eq. or 0.05 eq.) was added together with *N*-Maleoyl-*β*-alanine (1 eq.), DPTS (0.15 eq.) and DIC (1.5 eq.) in anhydrous DMSO. Dextran (2.5 eq.) was also dissolved in anhydrous DMSO and added into the previous mixture. After overnight stirring at room temperature, the product was purified by filtration, precipitation and dialysis. All these steps were carried out in dark to prevent the bleaching of coumarin.

Glass slides functionalization

Cleaning and thiol surface modification (silanization) was performed according to a previously reported method.[24] Glass substrates were standard microscope glass slides (Menzel-Gläser) 76 x 26 mm.

Dex-PEG Hydrogel

Maleimide-modified Dextran (**1**) (2 % weight solution) was crosslinked by using PEG (**2**) in three different ratios (1:1, 1:0.75 and, 1:0.5) at room temperature. Typically, **1** (60 mg) (DS=4) was dissolved in water (2.5 g) and 11.11 mg of molecule **2** (1500 Da) in water (0.5 g) were mixed to provide a hydrogel solution in 1:1 molar ratio. The mixture was shaken in a vortex for 1 minute and immediately used for the substrate preparation.

Dex-PEG coated glass slides

A solution of 2% Dex-PEG (600 µL) was added onto the prepared thiol functionalized microscope glass slides. A homogenous polymeric film was formed after 30–45 minutes at 40 °C. The Dex-PEG coated microscope slides were stored for further use.

Lipid Mixtures

Each of the lipid mixtures used was made by mixing 14 mM lipid solutions of the individual various lipids together to obtain the indicated molar ratios and yield a final mixture of a 14 mM lipid solution (see **Table 1**). Additionally 1,2–dioleoyl–*sn*–glycerol–3–phosphoethanolamine–

N-(lissamine rhodamine B sulfonyl) (ammonium salt) (PE-LR) was added to each lipid mixture in 0.5 molar % for the purpose of gathering fluorescence images. All lipid mixtures were stored at -20 °C until they were used.

GUVs formation

The desired lipids (10 μL) were drop-casted, using a micropipette, onto the Dex-PEG coated slides. Subsequently, the deposited lipid layer was dried for 30 minutes in a vacuum oven at 35°C. The GUV growth chamber was made by placing a 15 mm (OD) glass O-Ring on top of the hydrogel and sealed with high vacuum silicon grease. Finally, the lipid film was swelled using the desired immersion media (300 μL). GUVs were growth in two different immersion media: PBS and HEPES Potassium Chloride saline (HBS) buffers. All lipid compositions (see **Table 1**) were tested in PBS and HBS growth buffers. The vesicle diameter is the average of measuring between 50 to 100 different GUVs in PBS buffer. GUVs smaller than 1 μm cannot be resolved due to they are below the resolution limit of optical microscopy. Lipid mixtures (A–I) were prepared in 95 chloroform : 5 methanol % volume at 14 mM. PE-LR was used as fluorescent probe in 0.5 % mol (A–E, H–I) or 0.1 % mol (F, G) concentration. Results for all different tested lipid compositions are presented in **Figures A1, A3, A5, A7, A9, A11, A12, A13,** and **A15**. At the left side results for GUVs growth in PBS and right side in HBS. The compositions of those buffers are shown in **Table 2** (PBS) and **Table 3** (HBS). All images were taken from free floating vesicles after waiting 30 minutes in order to allow them to sink in the bottom of the observation chamber. Imaging of GUVs was made using 20x (top image) and 63x (bottom image) objectives in fluorescence microscopy mode (see **Annex**). Comparing qualitatively the sizes of GUVs in those two different immersion media, the diameter size of GUVs was bigger when the immersion media was HBS than in PBS. Additionally, quantitative size distribution analysis was performed by counting GUVs that were grown in PBS buffer during one hour (**Figures A2, A4, A6, A8, A10** and **A14**).

Osmomolality

Osmomolality was determined from the freezing point depression using an Osmometer Roebling Type 13. The Osmometer was calibrated using 100 mOsm/kg NaCl standard solution.

Overview of lipid compositions and GUVs diameters

Table 1. Lipid compositions tested for GUVs growth on Dex-PEG at physiological ionic strength (>300 mOsm/kg).

	Lipid composition (% mol)	PBS	HBS	Diameter (µm)	Figure
A	90% POPC / 10% CH	√	√	12.62 ± 5.89	A1
B	80% POPC / 20% CH	√	√	9.38 ± 5.04	A3
C	90% POPC / 10% POPG	√	√	12.19 ± 4.75	A5
D	50% POPC / 50% POPG	√	√	14.45 ± 7.51	A7
E	90% POPC / 10% DOPS	√	√	9.80 ± 3.50	A9
F	50% DOPC / 50% DPPC	√	√	N.D.	A11
G	33.3% DOPC / 33.3% DPPC / 33.3% CH	√	√	N.D.	A12
H	50% DOPC / 25% DOPE / 25% CH	√	√	11.62 ± 5.35	A13
I	50% DOPC / 20% DOPE / 5% PEG2000-PE / 25% CH	√	√	N.D.	A15

N.D. represents not determined.

Buffer composition

Table 2. Phosphate buffered saline (PBS) composition in 1 liter water.

Reagent	Final concentration	Mass
NaCl	150 mM	8.77 g
K_2HPO4	15 mM	2.61 g
KH_2PO4	5 mM	0.68 g

Table 3. HEPES buffered saline (HBS) composition in 1 liter water.

Reagent	Final concentration	Mass
KCl	150 mM	11.18 g
HEPES	20 mM	5.2 g

Characterization GUVs

Fluorescence Microscopy of GUVs

A Zeiss axiovert-200 inverted microscope equipped with Chroma TRITC and DAPI BP 445/50 fluorescence filter sets was used. Images were recorded with a black and white CCD camera (AxioCam NRm). GUVs were grown from lipid mixture doped with 0.5 mol % PE-LR on Dex-PEG-C hydrogel film. To examine if there is association between Dex-PEG-C and GUVs, two different experiments were performed on Dex-PEG-C hydrogel film. Firstly, GUVs were grown without any fluorescent probe on the lipid mixture. Imaging of non-fluorescent GUVs was performed using phase contrast at 20 x magnification (**Figure 4A**) and phase contrast at 63 x magnification (**Figure 4B**). For imaging in epifluorescence mode, a DAPI bandpass filter centered at 450 nm was used to determine the fluorescence of free floating GUVs. The result is presented in **Figure 4C** where non- fluorescence due to coumarin was found either in the membrane or inside GUVs.

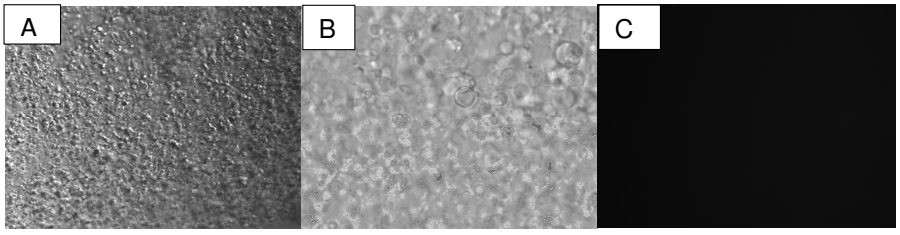


Figure 4. A) Phase contrast microscopy 20 x magnification; **B**) Phase contrast microscopy 63 x magnification and, **C**) Epifluorescence microscopy using a DAPI bandpass filter centered at 450 nm.

A single GUV was imaged in phase contrast microscopy (**Figure 5A**) and in epifluorescence microscopy using a TRITC filter (**Figure 5B**); however when the filter was changed to DAPI bandpass filter there was no membrane fluorescence of this GUV (**Figure 5C**). Even when the lipid mixture penetrates around 15 µm into the hydrogel network (*vide infra*), the association between GUVs and Dex-PEG gel was not detected via fluorescence in the membrane or inside the GUVs. The high swelling efficiency of the hydrogel (90 %) promotes the lipid inter-lamellar repulsion, without doping the lipid lamella with none-electrolytic monosaccharides, producing high yields of GUVs.

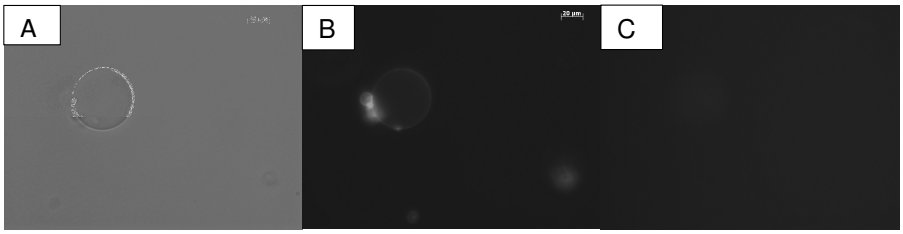


Figure 5. A) Phase contrast microscopy 20 x magnification; **B**) Epifluorescence using a TRITC filter and, **C**) Epifluorescence using a DAPI bandpass filter.

Confocal Imaging of GUVs

A TI-Eclipse inverted microscope (Nikon, Japan) equipped with a 16-bit Cascade II 512 EMCCD camera (Photometrics, USA) was used. Spinning-disc confocal microscopy was performed using a CSUX confocal head (Yokogawa, Japan). Illumination was provided by a 50 mW solid-state laser at 561 nm (Coherent Inc., Germany). Fluorescence was imaged through a bandpass filter centred at 595 nm. Epifluorescence was performed with a 40× objective and confocal microscopy was carried out using a 60× NA1.43 Plan-Apo Nikon oil-immersion objective. Size distribution analysis was made by using Analyze Particles tool in ImageJ software.

Characterization of Dex-PEG substrate

Contact Angle

The characterization of functionalized microscope glass slides before and after silanization was made by drop shape analysis. To measure the contact angle, drop shape analysis was made by using LBADSA plugin in ImageJ software. Results for chemically cleaned and modified surfaces (**Figure 6**) showed an angle of 10 ± 1° before, 67 ± 4° after silanization and 26± 7° for Dex-PEG film.

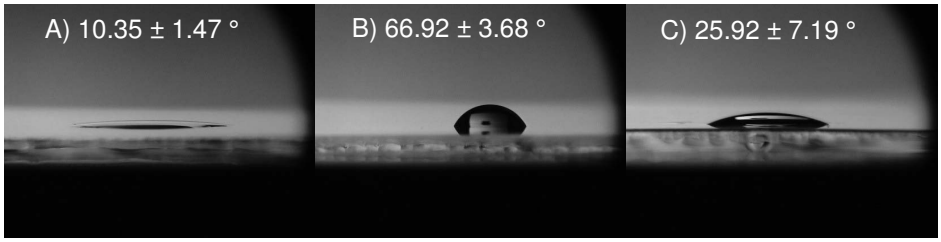


Figure 6. Contact angle measurements for **A**) Microscope glass slide after cleaning, **B**) Microscope glass slide after silanization and, **C**) Microscope glass slide after Dex-PEG hydrogel coating.

Water Uptake

The water uptake of the Dex-PEG hydrogel film was determined by weight increase. Dex-PEG functionalized microscope glass slides were immersed in 1x PBS and the weight was taken every hour. The water uptake content against time is plotted in **Figure 7**. These results indicate that the hydrogel film in the wet state takes up to 90 % PBS buffer during swelling after one hour and then stays constant. The remaining water content in the dry Dex-PEG film was determined by weight lost. We found, after drying the hydrogel film at 110°C overnight, a water content of 1.9±0.14 % in the dry state. This high swelling efficiency produces the forces normal to lipid bilayers that promote the growing of GUVs, without addition of any non-electrolytic monosaccharaides to promote lamellar repulsion.

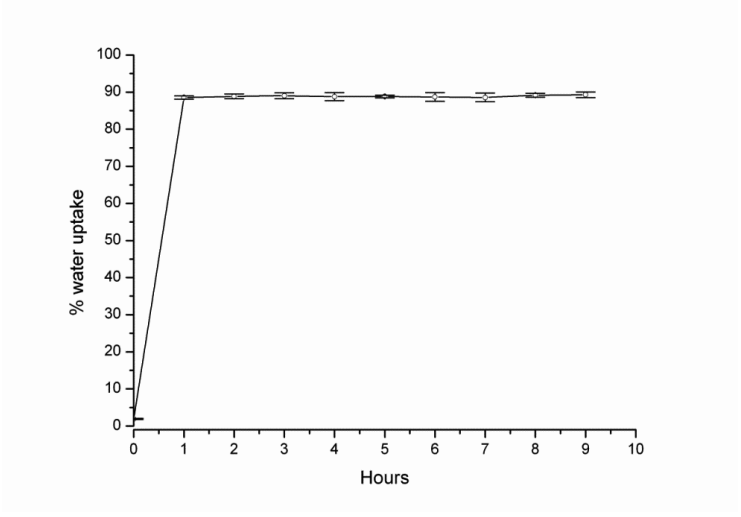


Figure 7. Water uptake in a Dex-PEG hydrogel film after 9 hours of swelling in 1x PBS buffer.

Fluorescence spectroscopy of DexPEG

A FS920 Fluorometer (Edinburgh Instruments) equipped with a DTMS-300X excitation monochrometer and a peltier-controlled thermostatic cell was used. A quartz cuvette with a 1 cm path length was used. The excitation and emission slit widths were 1 nm. Scans were performed with steps of 0.5 nm, with a sampling time of 0.5 s per wavelength. Dex-Mal was fluorescently labeled (1 and 2.5 % mol) with 7-methoxycoumarin-3-carboxylic acid as fluorescent probe to produce Dex-Mal-C (labeling of molecule **1**). On the right side of **Figure 8** the absorption spectra of Dex-Mal-C (2 % weight solution) is presented. The emission spectra of coumarin had a displacement from 402 nm to 410 nm (maximum peak) after labeling (Dex-Mal-C) due to chemical modification.

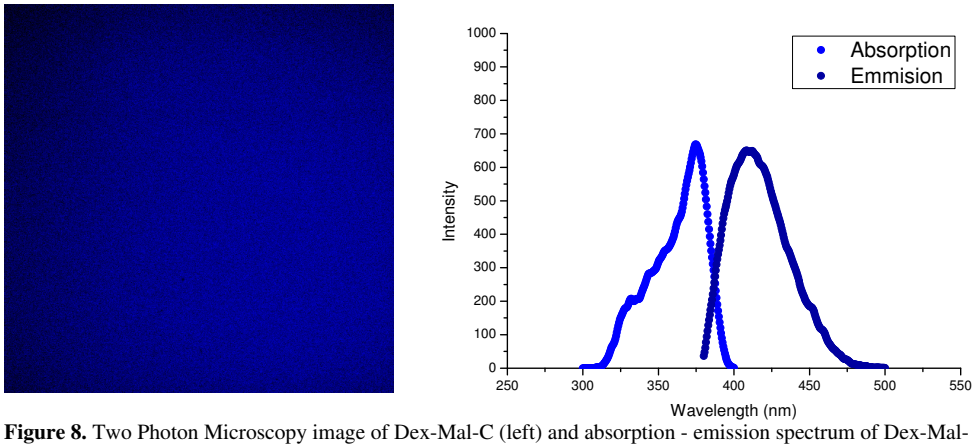


Figure 8. Two Photon Microscopy image of Dex-Mal-C (left) and absorption - emission spectrum of Dex-Mal-C (right) both of them at 2.5 mol % labeling.

Two photon microscopy

A Bio-Rad 2100MP confocal and multi-photon microscope with inverted TE 2000U Nikon microscope was used. Fluorescence was imaged using a 20x Nikon objective (Plan Apo, NA 0.75). Illumination was provided by a 5-W Tsunami laser (Spectra Physics, Mountain View, CA, USA) with a range of 700 – 900 nm. The images were collected using Lasersharp 4.0 software by Biorad. Two Photon microscopy (**Figure 8** left) was used to visualize Dex-PEG-C hydrogel film in a microscope glass slide. The background signal corresponding to none labeled Dex-PEG hydrogel film in a glass slide was subtracted from the signal of Dex-PEG-C

image to remove the noise associated to measurement. Similar results were obtained for Dex-PEG-C (1 % mol) labeling. Two photon confocal microscopy was performed to determine the thickness of the hydrogel film in the microscope glass slide. Sectioning of 1 µm was done from the highest to the lowest fluorescence intensity layer. The resulting images are presented in **Figure 9** after the background has been subtracted from those measurements. Those images correspond from left to right to the highest fluorescence, the middle fluorescence and non-fluorescence images in the confocal sectioning. The estimated thickness of the film is around 26 µm going from the highest to none fluorescence point.

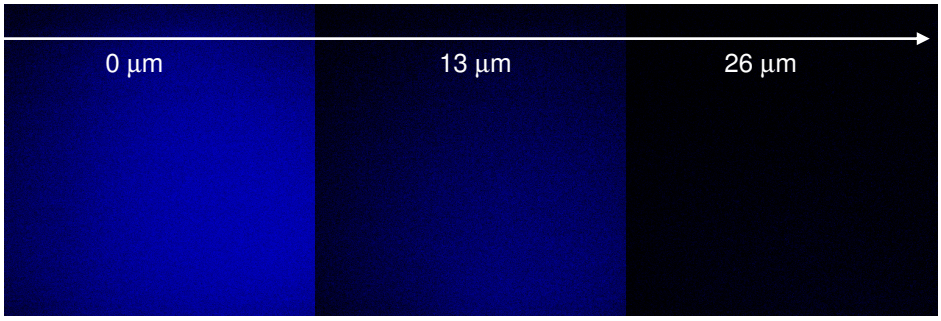


Figure 9. Two photon confocal microscopy images of Dex-PEG-C hydrogel film.

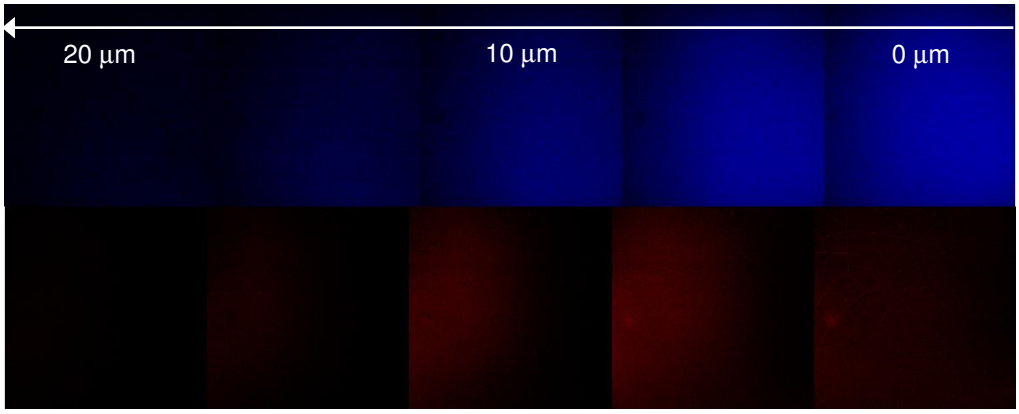


Figure 10. Two Photon Confocal Microscopy of an hybrid film composed of Dex-PEG-C hydrogel and lipid mixture doped with 0.5 mol % PE-LR before immersion in buffer. On top, channel for coumarin and on bottom channel for rhodamine. Each sequence of images corresponds to 5 µm layer sectioning.

Lipid mixture doped with 0.5 % PE-LR was place on top of a Dex-PEG-C hydrogel film and 1 µm sectioning was made following the previously described procedure. The fluorescence signal was detected by using two photon confocal microscopy in two different channels, one for coumarin and another one for rhodamine. These sequence of images is presented in **Figure 10** from highest to lowest intensity layer (right to left). The thickness of the film was measured in 25 µm by doing 1 µm confocal sectioning. The image shows how the doped lipid mixture penetrates the hydrogel network until 15 µm.

References

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Annex

Chapter II

Microscopy imaging and size distribution for lipid mixtures presented in Table 1.

*Lipid mixture **A** (90 % POPC / 10 % CH)*

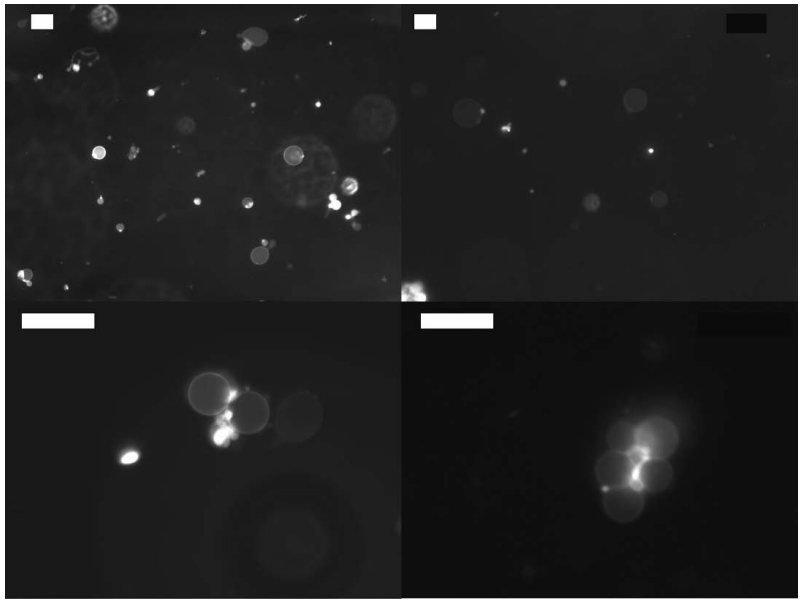

Figure A1. Free floating GUVs formed from POPC/CH (90:10 % mol) in PBS left column and HBS right column. On top 20x magnification and, bottom 63x magnification. The scale bars are 50 μm.

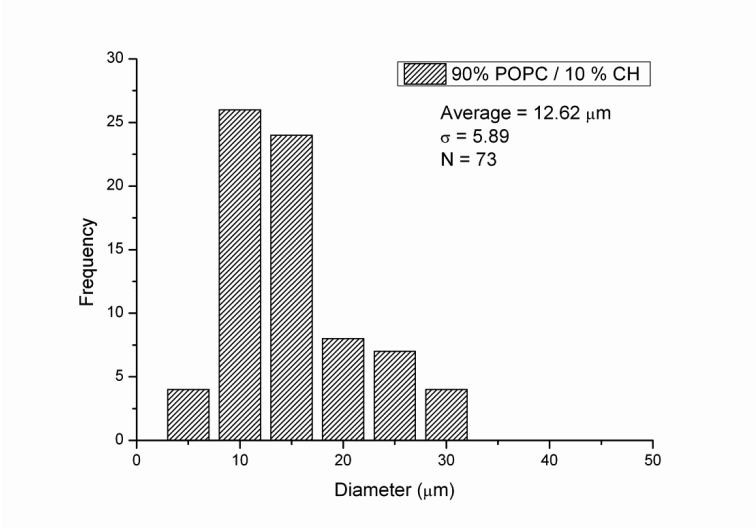

Figure A2. Size distribution of GUVs with lipid composition **A** (90% POPC / 10% CH) in PBS.

*Lipid mixture **B** (80 % POPC / 20 % CH)*

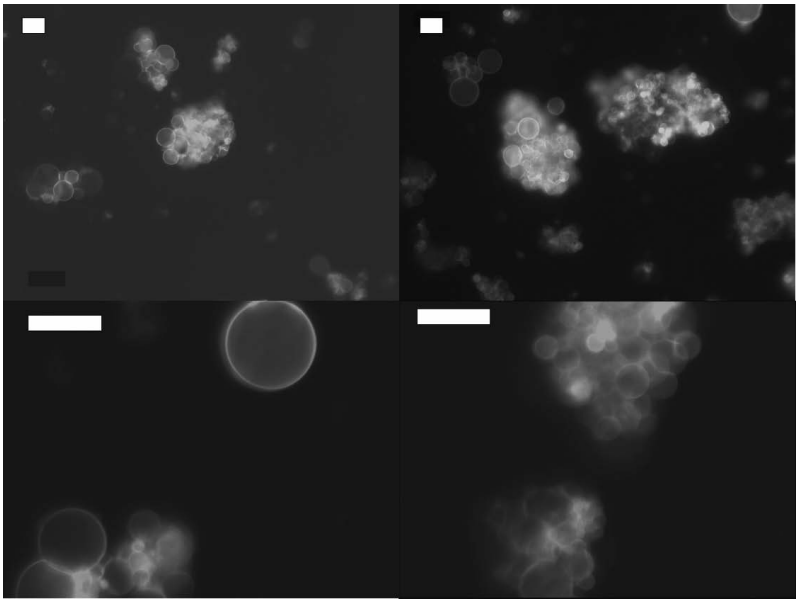

Figure A3. Free floating GUVs formed from POPC/CH (80:20 % mol) in PBS left column and HBS right column. On top 20x magnification and, bottom 63x magnification. The scale bars are 50 μm.

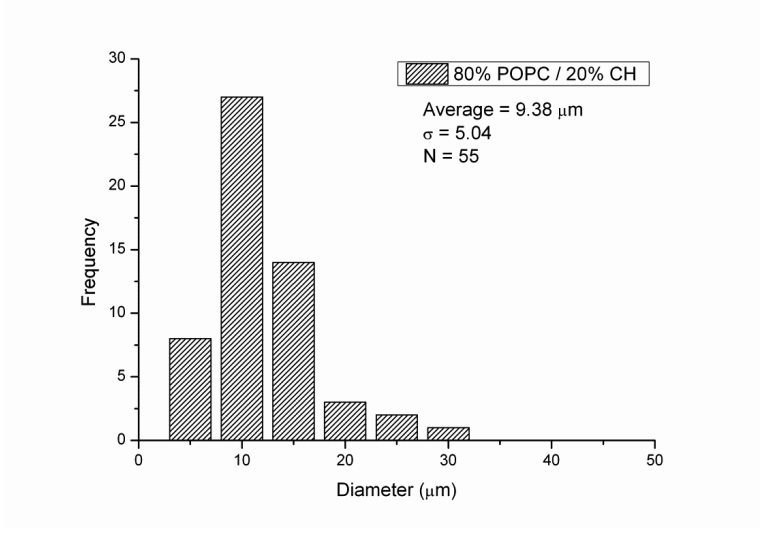

Figure A4. Size distribution of GUVs with lipid composition **B** (80% POPC / 20% CH) in PBS.

*Lipid mixture **C** (90 % POPC / 10 % POPG)*

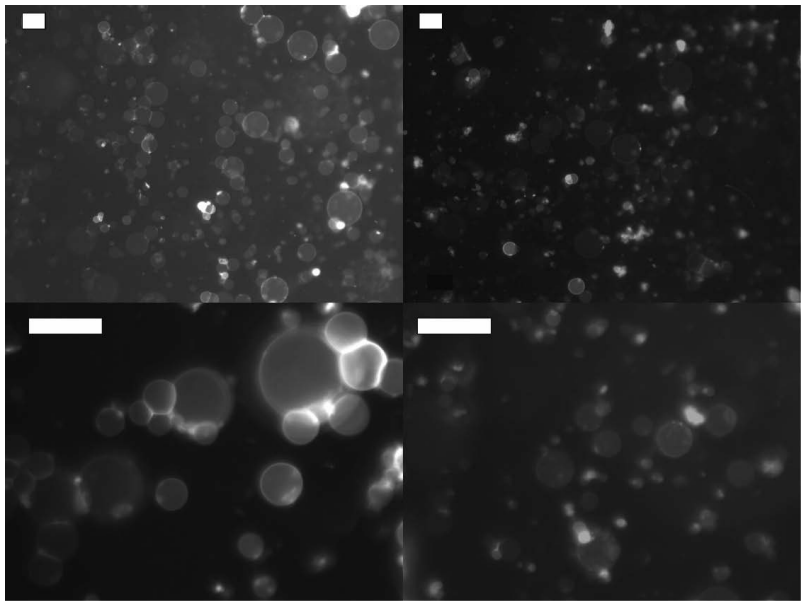

Figure A5. Free floating GUVs formed from POPC/POPG (90:10 % mol) in PBS left column and HBS right column. On top 20x magnification and, bottom 63x magnification. The scale bars are 50 μm.

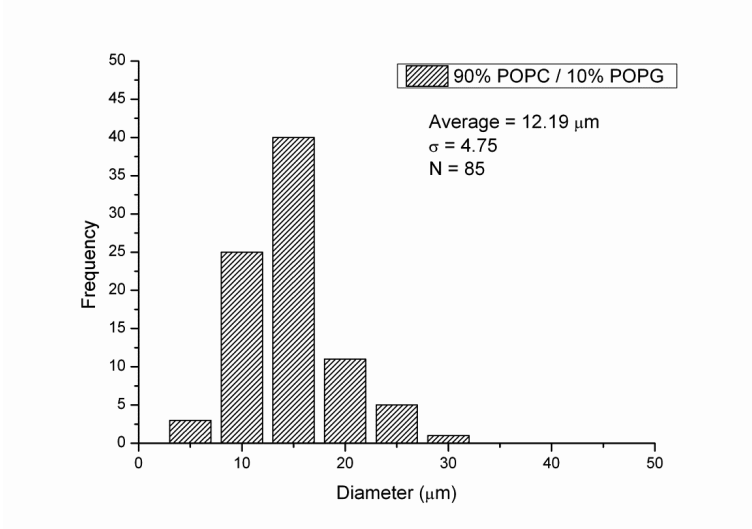

Figure A6. Size distribution of GUVs with lipid composition C (90% POPC / 10% POPG) in PBS.

*Lipid mixture **D** (50 % POPC / 50 % POPG*

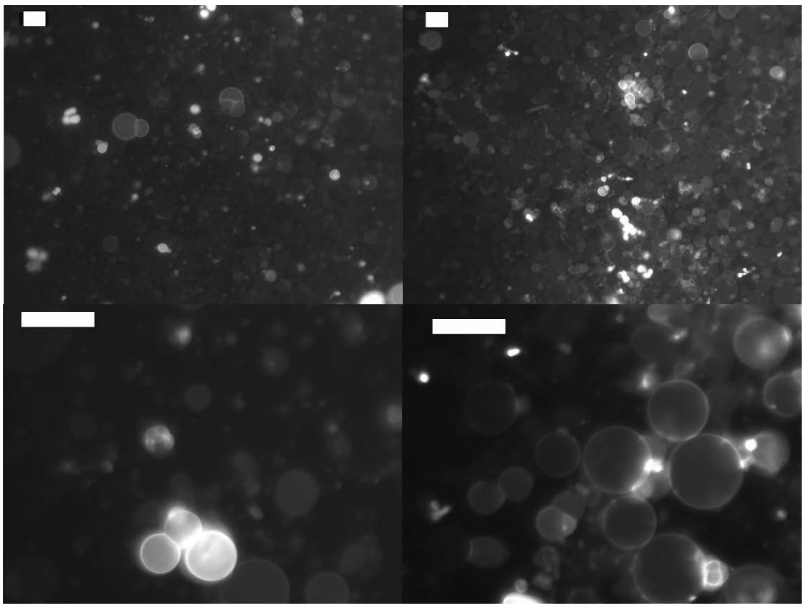

Figure A7. Free floating GUVs formed from POPC/POPG (50:50 %mol) in PBS left column and HBS right column. On top 20x magnification and, bottom 63x magnification. The scale bars are 50 µm.

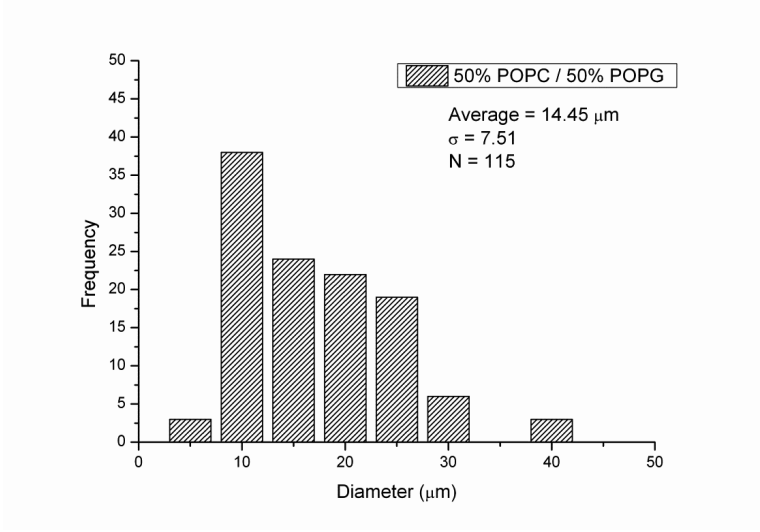

Figure A8. Size distribution of GUVs with lipid composition **D** (50% POPC / 50% POPG) in PBS.

*Lipid mixture **E** (90 % POPC / 10 % DOPS*

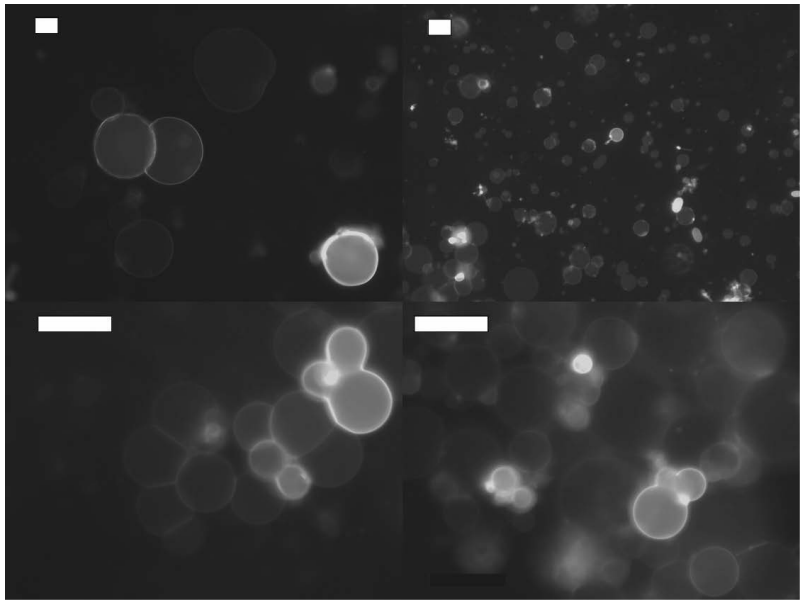

Figure A9. Free floating GUVs formed from POPC/DOPS (90:10 % mol) in PBS left column and HBS right column. On top 20x magnification and, bottom 63x magnification. The scale bars are 50 µm.

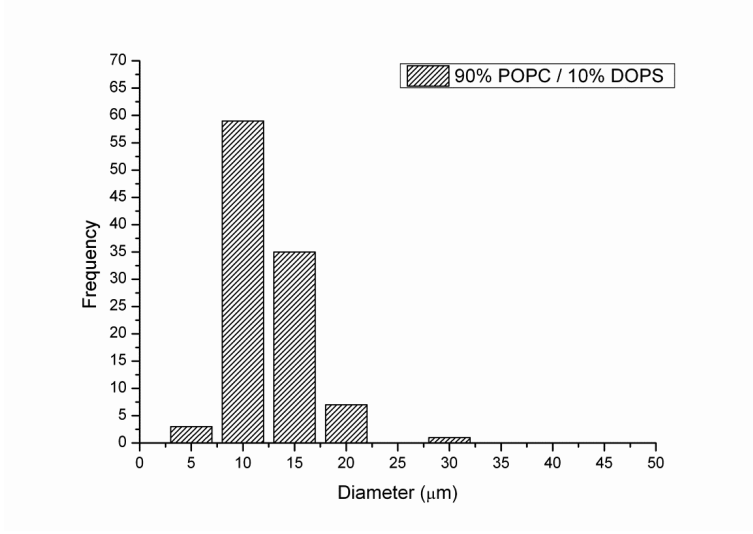

Figure A10. Size distribution of GUVs with lipid composition **E** (90% POPC / 10% DOPS) in PBS.

*Lipid mixture **F** (50 % DOPC / 50 % DPPC)*

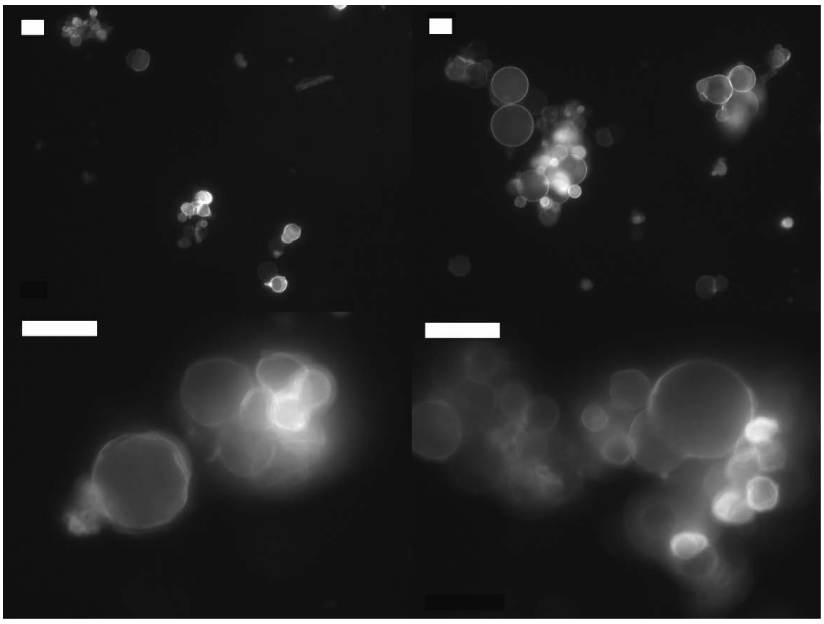

Figure A11. Free floating GUVs formed from DOPC/DPPC (50:50 % mol) in PBS left column and HBS right column. On top 20x magnification and, bottom 63x magnification. The scale bars are 50 µm.

*Lipid mixture **G** (33.3 % DOPC / 33.3 % DPPC / 33.3)*

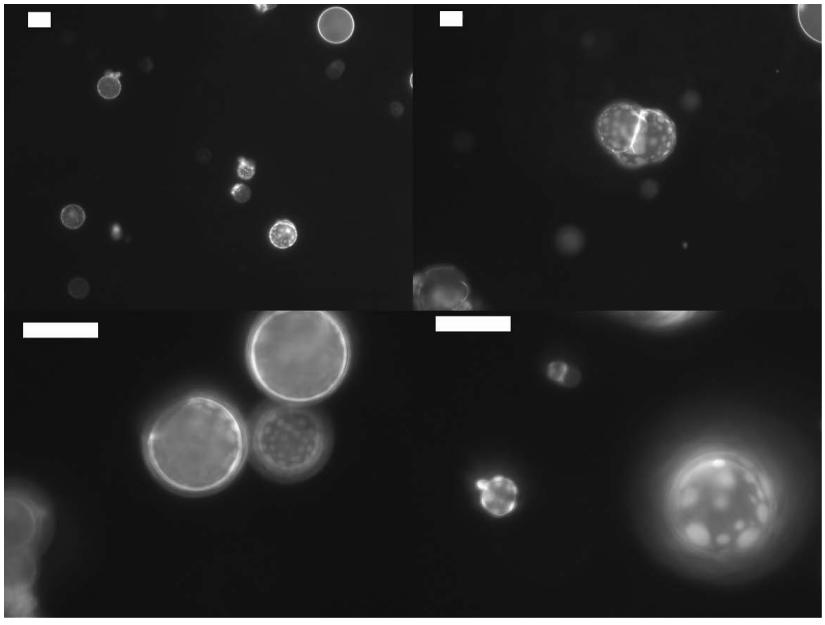

Figure A12. Free floating GUVs formed from DOPC/DPPC/CH (33.3:33.3:33.3 % mol) in PBS left column and HBS right column. On top 20x magnification and, bottom 63x magnification. The scale bars are 50 µm.

*Lipid mixture **H** (50 % DOPC / 25 % DOPE / 25 % CH)*

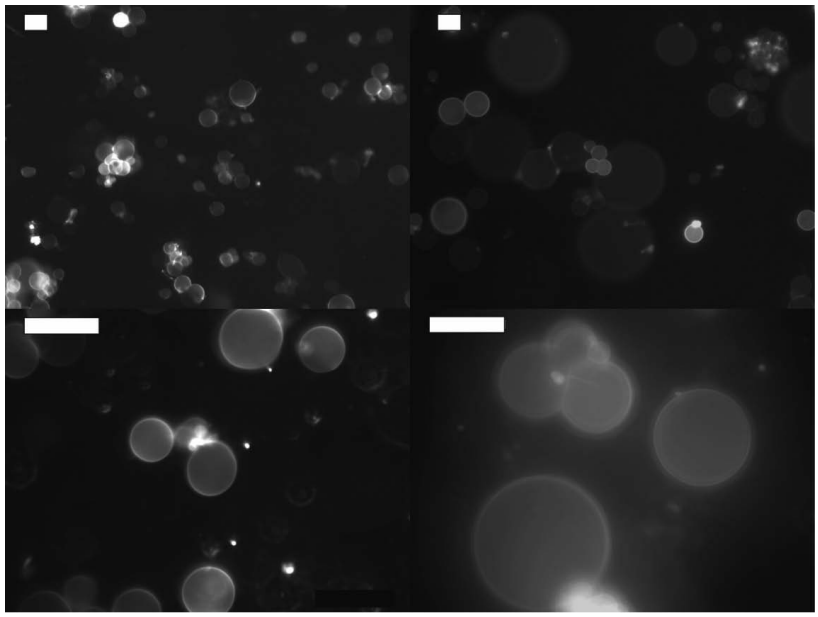

Figure A13. Free floating GUVs formed from DOPC/DOPE/CH (50 : 25 : 25 % mol) in PBS (left column) and HBS (right column). On top 20x magnification and, bottom 63x magnification. The scale bars are 50 µm.

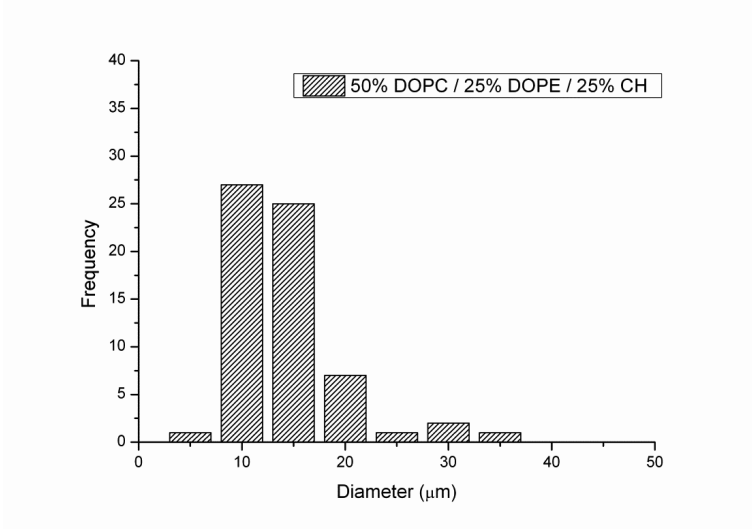

Figure A14. Size distribution of GUVs with lipid composition **H** (50% DOPC / 25% DOPE / 25% CH) in PBS.

*Lipid mixture **I** (50 % DOPC / 20 % DOPE / 5 % PEG2000-PE / 25 % CH)*

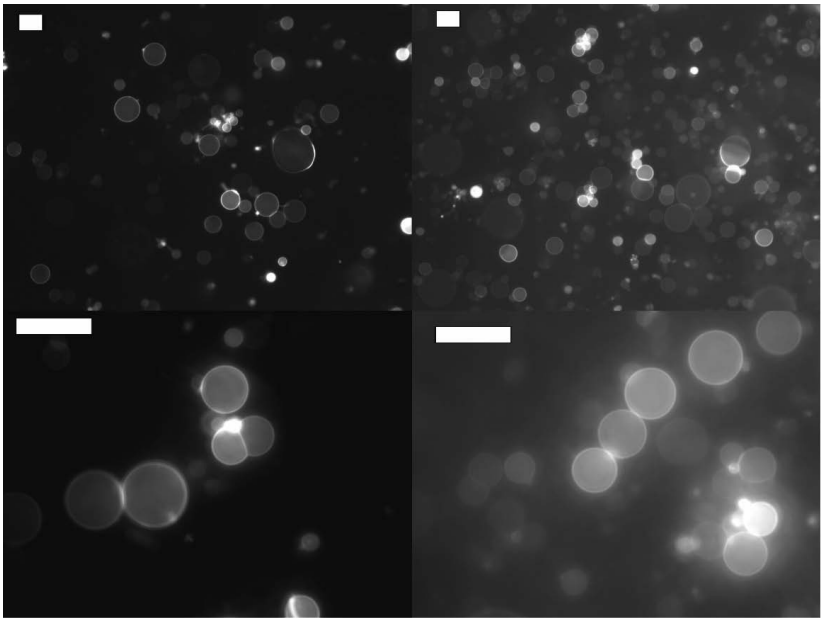

Figure A15. Free floating GUVs formed from DOPC / DOPE / PEG2000-PE / CH (50:20:5:25 % mol) in PBS left column and HBS right column. On top 20x magnification and, bottom 63x magnification. The scale bars are 50 µm.