The handle http://hdl.handle.net/1887/66265 holds various files of this Leiden University dissertation.

Author: Gao, Y.
Title: Design and application of dextran based cross-linked networks
Issue Date: 2018-10-18
CHAPTER 5

CHEMICALLY CROSSLINKED POLYMER NETWORKS ASSIST THE FORMATION OF GIANT UNILAMELLAR VESICLES

Giant Unilamellar Vesicles (GUVs) have become popular as cell membrane model systems for biophysical studies. However, preparation of GUVs is not trivial. The quality, size and yield strongly depend on their preparation method. Here we present a novel method for GUV growth using gentle hydration of a lipid film from a chemically crosslinked dextran–poly(ethylene glycol) hydrogel substrate, wherein the hydrogel was formed by the thiol-Michael addition reaction. Polymer and additive-free GUVs were prepared rapidly under physiological ionic strength conditions in high yield without the need of special equipment. By modulating the physicochemical properties of the hydrogels, the GUV growth can be controlled in terms of yield, size and size distribution. The influence of the molar ratio of thiols and maleimides functional groups, the molecular weight and structure of thiolated poly(ethylene glycol) and the degree of substitution of maleimide modified dextran on GUV formation was studied. Chemical reagents and/or biomacromolecules can be encapsulated during formations and the obtained GUVs can be used for biophysical studies.


Chapter 5

INTRODUCTION

Cell membranes, as the frontier between the cell and its environment, play an important role in cellular phenomena such as mass transfer, energy transformation and signal transduction.\(^1\),\(^2\) Cell membranes are comprised of self-assembled lipid bilayers, hosting a variety of membrane proteins and multi-subunit assemblies. Due to their high degree of sophistication and functionality, cell membranes have long fascinated researchers in Physics, Chemistry and Biology. To study the properties of cell membranes, various simplified biomimetic models of cell membranes have been developed over the past one hundred years.\(^2\),\(^3\)

Giant unilamellar vesicles (GUVs) are widely used model systems for investigating various properties of cell membranes,\(^4\)-\(^6\) because they approximate the size and membrane curvature of living cells and offer the flexibility to visualize and probe biophysical phenomenon of one single vesicle under an optical microscope. GUVs are typically prepared by gentle hydration\(^7\) or the electroformation methods.\(^8\) The gentle hydration method usually involves evaporation of a lipid-containing organic solvent on a glass substrate and subsequent exposure to a buffer solution results in vesicle formation. In order to form vesicles at a moderate ionic strength (200 mOsm/kg), there must be 10-20% negatively charged lipids in the lipid composition and a heating step is required to bring the lipid solution above its phase transition temperature. In some cases, it is also necessary to add monosaccharides in the dry lipid film to promote lamellar repulsion.\(^9\) The main disadvantage of the gentle hydration method is the variable size and relatively low yield of produced vesicles and the gentle hydration method is not suitable for all lipid compositions. On the other hand, the electroformation method is the most widely used vesicle formation method and can provide a high GUV yield with a narrow size distribution.\(^10\) Lipid mixtures are able to swell and grow in non-electrolytic solutions when an electric field is applied. However, to prepare GUVs under high ionic strength conditions, higher electric field frequencies and longer hydration times are required leading to potential lipid hydrolysis and peroxidation.\(^11\),\(^12\)

Recently, researchers have found that the traditional gentle hydration method can be significantly improved using a hydrogel film. Horger \textit{et al.} demonstrated that by applying an agarose gel film on a glass slide, GUVs were formed when a lipid film was hydrated on this substrate under physiological conditions.\(^13\) However, GUV contamination occurred due to dissolution of agarose in the hydration process. Traces of agarose were found both inside and spanning the GUV membrane, which modified the mechanical properties and membrane permeability.\(^14\) Poly(vinyl alcohol) hydrogels were used as an alternative to prevent GUV contamination by the gel components, but a high yield of GUVs was only obtained in presence of sucrose.\(^15\)

To enable GUV formation under physiological conditions, we developed a chemically crosslinked...
hydrogel matrix to be used as a substrate covalently anchored to a glass surface. The hydrogel network consists of dextran and poly(ethylene glycol) (PEG) polymers crosslinked via a thiol-Michael addition reaction and anchored to a thiolated glass surface. Using this method, GUVs can be formed from various lipid compositions under additive-free, physiological ionic strength conditions. Moreover, we studied the influence of the physiochemical properties of the hydrogel with respect to the molar ratio of the thiol and maleimide functional groups, the molecular weight of thiolated PEG and the degree of substitution of both thiolated PEG and Dex-Mal on GUV formation. Dex-PEG hydrogels were characterized by oscillatory rheology, swelling experiments and cryo-SEM imaging. The yield and size distribution of formed GUVs were analyzed by the fluorescence microscopy and fluorescence activated cell sorting (FACS). Based on these results, we developed a chemically crosslinked hydrogel system for GUV growth under physiological conditions without artifacts intercalated or encapsulated within the membrane. Additionally, we found that modulation of the physicochemical properties of the Dex-PEG hydrogel resulted in the potential for control over GUV growth in terms of yield and size.

RESULTS AND DISCUSSION

HYDROGEL SYNTHESIS

Dex-PEG hydrogels were prepared by reacting aqueous solutions of Dex-Mal and thiolated PEG (PEG-SH) using the thiol-Michael addition reaction (Scheme 1). Since no initiator or catalyst is required to facilitate the reaction, contamination of the GUVs or biomolecule-encapsulated GUVs formed using these hydrogel films is prevented.

Oscillatory time sweeps were used to probe the rate of network formation and its final mechanical properties, while amplitude and frequency sweeps were used to confirm the formation of a chemically crosslinked network. Typically, solutions of 3.5% (w/v) Dex70k-Mal5.5 (Mw=70 kDa, DS[Mal]=5.5) and PEG2k-2SH (Mw=2 kDa, 2 thiol end groups) were loaded on the rheometer plate and the storage modulus (G') and the loss modulus (G'') were followed as a function of time. Within a few seconds, G' increased dramatically and became higher than G'', indicative of the formation of a viscoelastic material. Once a plateau in G' was attained, amplitude and angular frequency sweeps were conducted. G' and G'' remained nearly constant with G' being two orders of magnitude greater than G'' in both amplitude and frequency sweeps, consistent with the formation of a chemically crosslinked network (Figure 1).

In order to grow GUVs from these hydrogel films, a solution of Dex70k-Mal and thiolated PEG was drop-casted on a thiolated glass slide at 40 °C until a thin hydrogel layer was formed through a Michael addition reaction between the polymers and the glass surface. By visual inspection,
different combinations of polymer precursors resulted in distinct appearances of hydrogel films formed on the glass slides. Dex-PEG gels prepared from Dex70k-Mal with DS[Mal] of 2~4 resulted in transparent and homogeneous layers on glass surfaces. In contrast, Dex70k-Mal with a DS[Mal] greater than 5 led to the formation of an inhomogeneous film. We found that the surface roughness affected the GUV yield and size (*vide infra*). Moreover, the hydrogel coated glass slides
were examined for their capacity to swell upon rehydration by their immersion in PBS buffer at room temperature. The swollen weight of hydrogels prepared by various DS[Mal] of Dex70k-Mal attached onto glass slides was measured over time. The weight of all swollen samples reached a plateau within an hour, suggestive of rapid swelling kinetics (Figure S1).

**Figure 1.** Oscillatory rheology measurements of Dex-PEG hydrogels composed of 3.5% (w/v) Dex70k-Mal5.5 crosslinked with PEG2k-2SH at 25 °C. (A) Time sweep using 1% strain at 1 rad/s. (B) Amplitude sweep from 0.1 to 100 rad/s with 1% strain. (C) Angular frequency measurement from 0.1% to 100% strain at 1 rad/s.

**GIANT VESICLE PREPARATION USING CROSSLINKED Dex-PEG FILMS**

To evaluate the potential of the chemically crosslinked hydrogel for assisting GUV growth, several buffers and lipid compositions were tested for this purpose. 1% (w/v) Dex70k-Mal4 (M\text{w}=70 \text{ kDa}, DS[Mal]=4) and PEG3.4k-2SH (M\text{w}=3.4 \text{ kDa}, 2 thiol end groups) were mixed together to produce the gel layer for the following studies.

Vesicle production in buffer systems of physiological ionic strength was first examined. The osmolality of phosphate buffered saline (PBS) and HEPES buffered saline (HBS) was 310 mOsm/kg and 320 mOsm/kg, respectively, which are on par with buffers found and used in
biology and physiological fluids (300 mOsm/kg). For both buffers, free floating vesicles were obtained in high yield without any extra reagents or special requirements regarding the lipid composition.

The capacity of Dex-PEG gel to promote GUV formation with various lipid compositions was subsequently assayed. Lipid compositions with different percentages of cholesterol (CH), negatively charged lipids (POPG and DOPS) and liquid ordered lipid phases (DPPC) were deposited on gel-coated glass slides (See Table 1) to yield vesicles as described earlier. In presence of CH (Table 1: a, b and h), free floating GUVs with a spherical morphology (Figure 2: A, B and F) were prepared in good yields in contrast to previous reports\textsuperscript{17} where the use of the gentle hydration method showed exclusive formation of tubular morphologies (POPC with 5–30% CH in HEPES). The content of anionic lipids was increased up to 50% using the Dex-PEG hydrogel method, which is in contrast to the gentle hydration methods where this value is kept below 10% to promote growth (Table 1: c, d and e). More specifically, in presence of relatively high percentage of POPG or DOPS, spherical vesicles were successfully formed in high yield by our method (Figure 2: C, D and E). When using lipids that form liquid ordered lipid phases, it is known that

Figure 2. Formation of GUVs from Dex-PEG hydrogel coated glass slides with various lipid compositions (scale bar: 10 μm). (A) 90% POPC : 10% CH, (B) 80% POPC : 20% CH, (C) 90% POPC : 10% POPG, (D) 50% POPC : 50% POPG, (E) 90% POPC : 10% DOPS and (F) 50% DOPC : 25% DOPE : 25% CH. Reproduced from ref. [16].
lipid mixtures can undergo phase separation through phase coexistence of liquid ordered (L\textsubscript{o}) and liquid disordered (L\textsubscript{d}) phases.\textsuperscript{18} Thus far, phase separated GUVs have only been formed under non-physiological conditions (KCl, 50 mM).\textsuperscript{19} On our hydrogel matrices, phase-separated GUVs consisting of 30-50% DPPC were easily grown at 50 °C (Figure S2). Hence, we demonstrated that the Dex-PEG gel-assisted hydration method can successfully produce GUVs from different types of lipids showing its potential to enable their facile growth.

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

<table>
<thead>
<tr>
<th>No.</th>
<th>Lipid composition (% mol)</th>
<th>Buffer system</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>PBS</td>
</tr>
<tr>
<td>a</td>
<td>90% POPC / 10% CH</td>
<td>√</td>
</tr>
<tr>
<td>b</td>
<td>80% POPC / 20% CH</td>
<td>√</td>
</tr>
<tr>
<td>c</td>
<td>90% POPC / 10% POPG</td>
<td>√</td>
</tr>
<tr>
<td>d</td>
<td>50% POPC / 50% POPG</td>
<td>√</td>
</tr>
<tr>
<td>e</td>
<td>90% POPC / 10% DOPS</td>
<td>√</td>
</tr>
<tr>
<td>f</td>
<td>50% DOPC / 25% DOPE / 25% CH</td>
<td>√</td>
</tr>
<tr>
<td>g</td>
<td>50% DOPC / 50% DPPC</td>
<td>√</td>
</tr>
<tr>
<td>h</td>
<td>33.3% DOPC / 33.3% DPPC / 33.3% CH</td>
<td>√</td>
</tr>
<tr>
<td>i</td>
<td>50% DOPC / 20% DOPE / 5% PEG2000-PE / 25% CH</td>
<td>√</td>
</tr>
</tbody>
</table>

**INFLUENCE OF THE HYDROGEL PHYSICOCHEMICAL PROPERTIES ON GUV PRODUCTION**

Since the pioneering work by Horger et al. on the gel-assisted hydration method for GUV growth,\textsuperscript{13} few research groups have studied and improved this advanced technique with various types of gels.\textsuperscript{14, 15} The mechanism of giant vesicle formation via this method, especially the role of the hydrogel films, is however still unclear. According to Horger et al., the swelling of the hydrogel film is a key step in the formation of the lipid lamellae, the growth of liposomes and their fusion.\textsuperscript{13} Because the degree of hydrogel swelling strongly depend on the crosslinking density (ρ\textsubscript{x}) of the network,\textsuperscript{20} we hypothesized that the production of GUVs could be controlled by the polymeric structure of Dex-PEG hydrogels. Starting from the initially developed Dex-PEG gel film composition for GUV formation (hydrogel prepared from 1% Dex70k-Mal4 and PEG3.4k-2SH
Chapter 5

at equimolar thiol to maleimide), we modulated the physicochemical properties of Dex-PEG hydrogels using three parameters: the molar ratio of thiol to maleimide (MR[SH:Mal]), the molecular weight and architecture of thiolated PEG, and the degree of substitution of the maleimide modified dextran (DS[Mal]). The obtained GUVs were analyzed by fluorescence microscopy and the fluorescence-activated cell sorting (FACS). To gain insight into the effect of these three factors on GUV production, we further examined the difference in the network properties of the hydrogels using oscillatory rheology, swelling experiments and cryo-SEM imaging.

**Thiol-Maleimide Molar Ratio**

To study the effect of the molar ratio of thiol to maleimide groups (MR[SH:Mal]), Dex70k-Mal4 was crosslinked with PEG2k-2SH at a molar ratio of thiol to maleimide of 0.5, 0.75, 1 and 1.25. In these experiments, the molar concentration of maleimides was kept at 8 mM while the molar concentration of thiols was varied.

The gelation kinetics of Dex-PEG hydrogels (3.5%, w/v) with 4 different SH:Mal ratios were followed by oscillatory rheology using time sweep measurements. Significant differences in gel formation kinetics and plateau moduli were observed (Figure 3A). When the molar ratio of SH:Mal was decreased from 1 to 0.75, the Dex-PEG hydrogel formed more slowly reaching a lower plateau modulus (260 Pa). When the molar ratio of SH:Mal was further decreased to 0.5, the gelation time of Dex-PEG hydrogels was two times slower in comparison to the 1:1 sample with final storage modulus one order of magnitude lower (20 Pa). When the molar ratio of SH:Mal was increased to 1.25, the gelation of Dex-PEG hydrogels proceeded faster than other three samples, however the final storage modulus did not exceed samples prepared with MR[SH:Mal] of 1. The formation of viscoelastic materials by all samples was confirmed by amplitude and angular frequency sweeps.

The equilibrium mass swelling ratio ($q$) of different hydrogel systems with different molar ratios of thiol to maleimide were compared. As shown in Figure 3B, the value of $q$ increased when the hydrogel formulation departed from a 1:1 stoichiometry. For Dex-PEG hydrogels prepared with a large excess of maleimide groups (0.5 molar ratio) the greatest amount of swelling is observed with a two-fold increase in $q$.

Combining the results of the rheological and swelling measurements, modulation of the molar ratio of thiol to maleimide groups can result in significant changes in hydrogel physicochemical properties in terms of mechanical strength and swelling behavior. These properties are influenced by the crosslinking density ($\rho_c$) of the network. When one of the functional groups is in excess a network with fewer crosslinks is obtained, resulting in lower storage moduli and higher equilibrium swelling ratios. This trend is on par with a previously reported polymeric hydrogel system synthesized by the azide-alkyne click reaction. The stoichiometric imbalance of functional groups
in the Dex-PEG system may promote the formation of closed loops, which is formed by thiols on the same PEG chain of the PEG-SH crosslinker reacted to maleimides on the same Dex-Mal chain. These closed loops, called primary cycles, do not connect to the crosslinked network but only dangle within it, decreasing the hydrogel crosslinking density resulting in spatial inhomogeneity of hydrogel networks.\textsuperscript{22}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure3.png}
\caption{The effect of the thiol to maleimide molar ratio in the Dex-PEG hydrogel composed of 3.5\% (w/v) Dex70k-Mal4 and PEG2k-2SH on (A) the gelation kinetics and mechanical strength, (B) the equilibrium swelling ratio. (C) The size distribution of generated GUV’s (lipid composition: 50\% DOPC : 25\% DOPE : 25\% CH, wherein Dex-PEG hydrogel films for the hydration process were prepared by 1\% (w/v) Dex70k-Mal4 and PEG2k-2SH with a molar ratio of thiol to maleimide at 0.75, 0.5, 1 and 1.25). Reproduced from ref. \cite{16, 23}.}
\end{figure}

Hydrogel films were prepared from 1\% (w/v) Dex70k-Mal4 and PEG2k-2SH with a molar ratio of thiol to maleimide of 0.5, 0.75 and 1 and were attached to glass slides for GUV formation. With a molar ratio of thiol to maleimide of 1, a very narrow size distribution of GUVs were obtained with an average size of 15 \(\mu \text{m}\). When the hydrogel film departed from a 1:1 stoichiometry, the GUV size distribution broadened (Figure 3C) and the value of the average peak size turned greater. This observation confirms that the size distribution and the diameter of obtained GUVs by gel-assisted hydration method is related to the polymeric structure of Dex-PEG hydrogels. On the
other hand, decreasing the molar ratio of thiol to maleimide of the gel network led to the formation of a more inhomogeneous network with the formation of GUVs (50 – 100 µm) that were larger and more polydisperse in size.

**PEG molecular weight and architecture**

To study the effect of crosslinker architecture and size, five different thiolated PEG (PEG-SH) crosslinkers (Scheme 1B and Table 2) were reacted with 3.5% (w/v) Dex70k-Mal4 at a 1:1 molar ratio of thiol to maleimide in PBS buffer. By oscillatory rheology, more rapid gelation kinetics and a higher mechanical strength were observed for hydrogels prepared from thiolated PEG with a higher molecular weight (Figure 4A). By increasing the $M_w$ of the linear PEG-dithiols from 2 kDa to 10 kDa (i.e. PEG2k-2SH and PEG10k-2SH), the storage modulus of the Dex-PEG hydrogels increased one order of magnitude. Hydrogels prepared from PEG10k-SH ($M_w$=10 kDa) with a non-linear architecture and an increased number of functional groups provided a more complicated trend. Compared to PEG10k-2SH ($M_w$=10 kDa, 2 thiol-ending groups), PEG10k-4SH ($M_w$=10 kDa, 4 thiol ending groups) increased the gelation rate and improved the mechanical strength of the obtained Dex-PEG hydrogel. However, the use of PEG10k-8SH ($M_w$=10 kDa, 8 thiol-ending groups) resulted in a lower storage modulus in comparison to PEG10k-4SH.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Molecular weight</th>
<th>SH per molecule</th>
</tr>
</thead>
<tbody>
<tr>
<td>PEG2k-2SH</td>
<td>2 kDa</td>
<td>2</td>
</tr>
<tr>
<td>PEG3.4k-2SH</td>
<td>3.4 kDa</td>
<td>2</td>
</tr>
<tr>
<td>PEG10k-2SH</td>
<td>10 kDa</td>
<td>2</td>
</tr>
<tr>
<td>PEG10k-4SH</td>
<td>10 kDa</td>
<td>4</td>
</tr>
<tr>
<td>PEG10k-8SH</td>
<td>10 kDa</td>
<td>8</td>
</tr>
</tbody>
</table>

This observation was in accordance with results of the viscosity measurement performed on mixtures composed of 0.5% (w/v) Dex70k-Mal2.5 and various thiolated PEG (Figure S3). Crosslinked polymer samples containing the 4-arm PEG10k-4SH showed the highest dynamic viscosity, while similar values were obtained from the 8-arm PEG10k-8SH and the linear PEG-2SH. Equilibrium swelling measurements displayed a similar trend to the oscillatory rheology data after reaction of Dex70k-Mal4 with various thiolated PEG (Figure 4B). By increasing the molecular weight of the linear PEG-2SH molecules (i.e. PEG2k-2SH, PEG3.4k-2SH and PEG10k-2SH), the equilibrium mass swelling ratio ($q$) of obtained hydrogels decreased. When the molecular weight of
the linear PEG-2SH polymer increased four-fold, $q$ decreased 54%. Conversely, when the molecular weight of PEG-SH was maintained at 10 kDa, $q$ increased with its modulation of its architecture. In the case of the 8-arm PEG10k-8SH, a 68% increase of $q$ was recorded in comparison to the linear PEG variant (PEG10k-2SH).

Figure 4. Effect of molecular weight and architecture of various thiolated PEGs on (A) the gelation process and the mechanical strength and (B) the equilibrium swelling ratio of Dex-PEG hydrogels. Hydrogels were prepared by reacting 3.5% (w/v) Dex70k-Mal4 with various thiolated PEG at a MR[SH:Mal] of 1 in PBS. Thiolated PEGs used to prepare Dex-PEG hydrogels: PEG2k-2SH (black), PEG3.4k-2SH (red), PEG10k-2SH (blue), PEG10k-4SH (yellow) and PEG10k-8SH (purple). Reproduced from ref. [23].

Cryo-SEM images of the Dex-PEG hydrogel films prepared using the various types of PEG-SH crosslinkers provided insight into their microstructure (Figure 5). For linear PEG-2SH with the lowest molecular weight (PEG2k-2SH), the porous structure was discontinuous. On the other hand, hydrogels prepared with PEG3.4k-2SH, PEG10k-4SH or PEG10k-8SH presented a more homogeneous microstructure having interconnected pores similar to honeycombs.

The experimental results described above show the molecular weight and the architecture of the thiolated PEG precursor also strongly influence the effective crosslinking density of the network. As mentioned earlier, the formation of inhomogeneities in the network is strongly related to the presence of primary cycles and other non-idealities formed during the gelation process precluding effective crosslinks from forming. Moreover, according to a study by Elliott, the formation of primary cycles in the polymeric network can be lessened by increasing the size of the crosslinking agent and the precursor concentration.\textsuperscript{24} Similarly, Metters reported that decreasing the crosslinker concentration and functionality generated more non-idealities in the network.\textsuperscript{22} It became evident that we also encountered a similar phenomenon at low concentrations of precursors when preparing the thin hydrogel films for GUV formation. The steric accessibility of the functional groups and kinetics of the thiol-maleimide addition reaction play a significant role in formation of the network. After one end of the linear PEG-2SH is chemically ligated to Dex-Mal, the other reactive group will react with the closest maleimide unit. For a linear PEG-2SH of a relatively low
molecular weight, such as PEG2k-2SH, the second thiol group is more likely to react with a maleimide group on the same Dex-Mal chain or remain unreacted due to its short chain length, thus generating primary cycles and other inhomogeneities in the network. The effect of the polymeric architecture of thiolated PEG on hydrogel properties can be explained by the same hypothesis. In comparison to the linear PEG10k-2SH and the 4-arm PEG10k-4SH, the size of PEG10k-8SH is more compact, thus the accessibility of reactive thiols is restricted. Therefore, it is reasonable that hydrogels prepared with PEG10k-8SH display lower crosslinking density and, thus weaker mechanical properties and higher equilibrium swelling ratios than that of hydrogels prepared with PEG10k-2SH and PEG10k-4SH. In summary, polymeric networks of Dex-PEG hydrogels show strong dependence on the molecular weight and the architecture of the reactive PEG-SH polymers and, therefore the design of the hydrogel system is important in order to maximize GUV growth. Based on the effect of the molecular weight and architecture of PEG-SH on the formation of the hydrogel networks, films of various samples were prepared to study their influence on GUV formation. Two different lipid compositions, 75 mol% POPC : 20 mol% cholesterol : 5 mol% PEG2000-PE and 80 mol% POPC : 20 mol% cholesterol, were used to prepare GUVs and the yields of GUVs were quantified by FACS (Figure 6A). It was found that

Figure 5. Cryo-SEM images of Dex-PEG hydrogels prepared by 1% (w/v) Dex70k-Mal4 crosslinked to various PEG-SH. (A) PEG2k-2SH, (B) PEG3.4k-2SH, (C) PEG10k-4SH and (D) PEG10k-8SH. Scale bar: 1 µm. Reproduced from ref. [23].
hydrogel films made from 1% Dex70Mal4 and PEG10k-4SH resulted in the lowest yield of GUVs for both lipid compositions. Considering PEG10k-4SH preformed a much lower equilibrium mass swelling ratio than other types of thiolated PEG, this result indicates that the swelling of Dex-PEG hydrogel film is crucial to the formation of GUVs during the hydration of the lipid and hydrogel film. In contrast, although hydrogels made weak mechanical property, relatively high yields of GUVs were obtained. Thus a higher equilibrium mass swelling ratio facilitates GUV production.

Figure 6. Yields of GUVs produced on Dex-PEG hydrogel films composed of (A) 1% (w/v) Dex70k-Mal4 and various thiolated PEG (PEG2k-2SH, PEG3.4k-2SH, PEG10k-4SH and PEG10k-8SH) at an equimolar ratio of thiol to maleimide; (B) 1% (w/v) Dex70k-Mal with different DS[Mal] (Dex70k-Mal2.5, Dex70k-Mal4, Dex70k-Mal5.5 and Dex70k-Mal13) and PEG3.4k-2SH at an equimolar ratio of thiol to maleimide. Line patterned bars represent GUVs with a lipid composition of 75 mol% POPC : 20 mol% cholesterol : 5 mol% PEG2000-PE. Cross-hatched bars represent GUVs with a lipid composition of 80 mol% POPC : 20 mol% cholesterol. Reproduced from ref. [16, 23].

**Modulation of the Degree of Substitution of the Dex-Mal Polymer**

The effect of the degree of substitution of maleimide (DS[Mal]) of the Dex-Mal polymer on the physiochemical properties of Dex-PEG gels was examined using PEG2k-2SH crosslinked to Dex70k-Mal with various degree of substitution (DS[Mal]=4, 5.5 and 9) at a stoichiometric ratio of thiols to maleimides in PBS. It should be noticed that the set-up of hydrogel systems prepared from Dex-Mal with different DS[Mal] is crucial to the resulting trend, which will be discussed below in detail.

3.5% (w/v) Dex70k-Mal4, Dex70k-Mal5.5 and Dex70k-Mal9 were crosslinked to PEG2k-2SH respectively and measured by oscillatory rheology. Dex70k-Mal with higher DS[Mal] displayed faster gelation process and resulted in stronger hydrogels (Figure 7A). The oscillation time sweep curves seems to indicate a positive correlation between the DS[Mal] and the final storage modulus.
of the corresponding hydrogel. However, the influence of the DS[Mal] of Dex-Mal polymers cannot be assessed due to different functional group concentrations of these hydrogel systems. In fact, when the concentration of Dex-Mal in all three hydrogel systems (DS[Mal]=4, 5.5 and 9) was kept at 3.5% (w/v), different numbers of maleimide groups per dextran chain led to various concentrations of maleimide groups in each system. The observed trend (Figure 7A) is probable caused by the functional group concentration instead of the architecture of the Dex-Mal polymer. To verify this, hydrogels composed of 2% (w/v) Dex70k-Mal9 crosslinked to PEG2k-2SH were prepared and measured by oscillatory rheology (Figure 7B). As shown in Figure 7B, the gelation was slowed down and the final storage modulus of 2% gels was one order of magnitude smaller than that of 3.5% gels. Also, high similarity in oscillation time sweep curves was observed between the Dex-PEG gel prepared from 2% Dex70k-Mal9 and the Dex-PEG gel prepared from 3.5% Dex70k-Mal4. These results showed that the observed trend in Figure 7A may be dominated by
the concentration of maleimide, making it difficult to assess the influence of DS[Mal] on the resulting hydrogel properties.

Therefore the concentration of PEG2k-2SH was maintained to 1.2% for the preparation of 1%Dex-PEG hydrogels using Dex70k-Mal4, Dex70k-Mal5.5 and Dex70k-Mal9 respectively at an equimolar ratio of thiol to maleimide. The concentration of Dex-Mal in each hydrogel system was not the same, Dex70k-Mal4 (5.1%), Dex70k-Mal5.5 (3.5%) and Dex70k-Mal9 (1%). Figure 7C shows that hydrogels prepared from Dex70k-Mal with a higher DS[Mal] displayed a slower gelation process resulting in a weaker hydrogel. When the DS[Mal] of Dex-Mal increased from 4 to 9, the gelation process was retarded and the final storage modulus of resulting hydrogels decreased by one order of magnitude. The swelling experiments also revealed that the equilibrium mass swelling ratio \( \psi \) of hydrogels doubled when the DS[Mal] of Dex-Mal increased from 4 to 9 (Figure 7D). As the viscoelastic property of dextran solution is nearly independent of dextran concentration at such a low concentration of dextran (below 5%), any obvious change in the oscillatory rheology and swelling experiments is due to the DS[Mal] rather than the concentration of Dex-Mal. Thus these results indicate that hydrogels prepared from Dex-Mal with higher DS[Mal] have a lower effective crosslinking density.

Cryo-SEM images of Dex-PEG hydrogels prepared by 1% Dex70k-Mal with various DS[Mal] (from 4 to 13) and PEG3.4k-2SH displayed differences in the hydrogel microstructure (Figure 8). For the sample prepared from Dex70k-Mal4 and Dex70k-Mal5.5, multiple layers of continuous pores were observed. The pore size seemed to increase with the DS[Mal] of the applied Dex-Mal. For the sample prepared from Dex70k-Mal4 an average pore size around 200 nm was observed. For the sample prepared from Dex70k-Mal5.5, an average pore size around 1 µm was observed. However, a further increase of the DS[Mal] to 9 led to the formation of inhomogeneous network (Figure 8C). When Dex70k-Mal13 was used, the sample presented a poorly connected structure lacking well defined pores (Figure 8D).

Taken together, too high DS[Mal] of Dex-Mal may reduce the effective crosslinking density of prepared hydrogels. For example, when the DS[Mal] of Dex-Mal increases from 4 to 9, the amount of maleimide groups on each dextran polymer chain increases from 17 to 39 (Table 3), thus too many maleimide functional groups are substituted on the same polymer chain. In the case of applying Dex70k-Mal9 \( \langle \text{DS[Mal]} \rangle = 9 \), once one of these maleimide groups on the dextran chain reacts to one thiol group of the thiolated PEG, the accessibility of other maleimide groups on the same dextran chain decreases. For each maleimide group on the same dextran chain, the probability to be reacted to different thiolated PEG also decreases. Thus using Dex-Mal with a higher DS[Mal] for Dex-PEG hydrogel preparation may lead to a drop of the number of effective crosslinks. Furthermore, the hydrophobicity of Dex-Mal may increase with the DS[Mal], resulting a more
Figure 8. Cryo-SEM images of Dex-PEG hydrogels prepared from 1% (w/v) (A) Dex70k-Mal4, (B) Dex70k-Mal5.5, (C) Dex70k-Mal9 and (D) Dex70k-Mal13 crosslinked to PEG3.4k-2SH. An equimolar ratio of thiol to maleimide was maintained in all samples. Scale bar: 1 µm. Reproduced from ref. [23].

Table 3. List of prepared Dex-Mal polymers with increasing degree of substitution.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Number of maleimide groups per 100 glucopyranose units (DS[Mal])</th>
<th>Number of maleimide groups per dextran chain</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dex70k-Mal2.5</td>
<td>2.5</td>
<td>10.8</td>
</tr>
<tr>
<td>Dex70k-Mal4</td>
<td>4</td>
<td>17.3</td>
</tr>
<tr>
<td>Dex70k-Mal5.5</td>
<td>5.5</td>
<td>23.8</td>
</tr>
<tr>
<td>Dex70k-Mal9</td>
<td>9</td>
<td>38.9</td>
</tr>
<tr>
<td>Dex70k-Mal13</td>
<td>13</td>
<td>54.9</td>
</tr>
</tbody>
</table>

compact conformation of the Dex-Mal polymer in the solution. Such compact conformation may hinder part of the maleimide groups to react with the thiolated PEG.

The influence of the DS[Mal] of Dex-Mal on the yield of GUV production was investigated using Dex-PEG hydrogel films prepared from various Dex70k-Mal (Dex70k-Mal2.5, Dex70k-Mal4, Dex70k-Mal5.5 and Dex70k-Mal13) and PEG3.4k-2SH at an equimolar ratio of thiol to maleimide.
Chapter 5

It was found that the hydrogel films prepared from Dex70Mal4 resulted in the lowest yield of GUVs while the hydrogel films prepared from Dex70Mal13 resulted in the highest yield of GUVs. Such results are similar to the observation discussed in the above section related to the type of thiolated PEG. It seems that hydrogel films having relatively high equilibrium mass swelling ratios assist the formation of GUVs more efficiently during the hydration.

In summary, the generation of GUVs depends on the physicochemical properties of the hydrogel films in the gel-assisted hydration method. Swelling properties of the hydrogel film, such as the equilibrium swelling ratio, influence on the yield of GUVs. As the swelling process of the hydrogel film influences the hydration of the lipid, interactions between lipids and the hydrophilic environment of the hydrogel film is also important for the GUV formation.

**CONCLUSION**

We developed a chemically crosslinked Dex-PEG hydrogel to improve the GUV growth method using gentle hydration. Polymer and additive-free GUVs can be made readily under physiological ionic strength conditions in high yield without any special equipment. This approach makes it possible to prepare GUVs in PBS from various lipid compositions showing important advantages for certain lipid compositions that are otherwise difficult for vesicle formation.

Dex-PEG hydrogels are prepared by mixing two functionalized hydrophilic polymers in aqueous buffer. Compared to physical gels, the gelation of the covalent Dex-PEG hydrogel system proceeds rapidly to form stable hydrogels with a controllable structure. The GUVs production was systematically investigated by controlling the molar ratio of the thiol and maleimide functional groups, the molecular weight of thiolated PEG and the architecture of both thiolated PEG and Dex-Mal. We found that to produce GUVs in high yield, it is essential for Dex-PEG gel films to have a relatively high equilibrium swelling ratio. These hydrogel films may promote the interaction between the lipid components and hydrophilic polymeric network, which is likely the key factor for successful GUV formation.

We have explored the Dex-PEG hydrogel film-assisted hydration method for encapsulation of chemical reagents, biomacromolecules and even polymersomes. This simple and versatile approach has facilitated the visualization and quantification of transmembrane activities and the study of membrane proteins. We anticipate that this method can be useful to research areas such as biomimetic chemistry, biomembrane physics and artificial cell mimics.
EXPERIMENTAL

MATERIALS

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), maleic anhydride, p-toluene sulfonic acid monohydrate (PTSA) 4-nitrophenyl disulfide, 4-(dimethylamino) pyridine (DMAP), dextran (70 kDa), N,N'-diisopropylcarbodiimide (DIC), dimethyl sulfoxide (DMSO), poly(ethylene glycol) dithiols (M<sub>w</sub>=3.4 kDa) were purchased from Sigma-Aldrich. Poly(ethylene glycol) dithiols (M<sub>w</sub>=2 kDa, M<sub>w</sub>=10 kDa) were purchased from NANOCS (USA), 4-arm poly(ethylene glycol) thiols and 8-arm poly(ethylene glycol) thiols (M<sub>w</sub>=10 kDa) were purchased from Jenkem Technology (USA). Phosphate buffered saline (PBS) was composed of 150 mM NaCl, 15 mM K<sub>2</sub>HPO<sub>4</sub> and 5 mM KH<sub>2</sub>PO<sub>4</sub>. HEPES buffered saline (HBS) was composed of 150 mM KCl and 20 mM HEPES.

Dextran was dried in a vacuum oven (30 °C) and DMSO was dried over 4Å molecular sieves before use. 3-maleimidopropionic acid and 4-(dimethylamino)pyridinium 4-toluenesulphonate (DPTS) were synthesized as previously reported.26, 27 Dialysis membranes (MWCO 3.5-5 kDa) were obtained from Spectrum Laboratories Inc.

SYNTHESIS OF Dex-Mal

Dex-Mal was synthesized by DIC mediated esterification of the hydroxyl groups of dextran with N-maleoyl-β-alanine. Briefly, N-maleoyl-β-alanine (313 mg, 1 eq.), DPTS (86.8 mg, 0.15 eq.) and DIC (434.9 µL, 1.5 eq.) were dissolved in anhydrous DMSO (15 mL). The mixture was stirred at room temperature for two hours, followed by the addition of a dextran solution in DMSO (15 mL). After stirring overnight 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 dialyzed against Milli-Q water five times over two days and subsequently lyophilized. 1H NMR (400 MHz, D<sub>2</sub>O): δ 3.3-4.0 (m, dextran glucopyranosyl ring protons), 4.9 (s, dextran anomeric proton), 6.8 (s, maleimide).

The degree of substitution of Dex-Mal (DS[Mal]) is defined as the number of maleimide groups per 100 glucopyranose residues of dextran, which was calculated from the 1H NMR spectra based on the protons of the maleimides (δ 6.8) and the anomeric proton (δ 4.9). The DS[Mal] of Dex-
Mal was controlled by the molar ratio between dextran and N-Maleoyl-β-alanine.

**DEX-PEG HYDROGELS PREPARATION**

Dex-Mal and thiolated PEG were dissolved in PBS individually and then mixed together using a pipette to prepare the hydrogel samples. Compositions of the Dex-PEG hydrogel are shown below (Table 4).

**Table 4. Composition of Dex-PEG hydrogels.**

<table>
<thead>
<tr>
<th>Gel conc. (w/v)</th>
<th>Dex70-Mal</th>
<th>Thiolated PEG</th>
<th>Molar ratio</th>
<th>Buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>DS [Mal]</td>
<td>Weight (mg)</td>
<td>Type</td>
<td>Mw</td>
<td>Weight (mg)</td>
</tr>
<tr>
<td>1.0%</td>
<td>4</td>
<td>10.0</td>
<td>linear</td>
<td>3400</td>
</tr>
<tr>
<td>3.5%</td>
<td>4</td>
<td>35.6</td>
<td>linear</td>
<td>2000</td>
</tr>
<tr>
<td>3.5%</td>
<td>4</td>
<td>35.6</td>
<td>linear</td>
<td>2000</td>
</tr>
<tr>
<td>3.5%</td>
<td>4</td>
<td>35.6</td>
<td>linear</td>
<td>2000</td>
</tr>
<tr>
<td>3.5%</td>
<td>4</td>
<td>35.6</td>
<td>linear</td>
<td>2000</td>
</tr>
<tr>
<td>3.5%</td>
<td>4</td>
<td>35.6</td>
<td>linear</td>
<td>2000</td>
</tr>
<tr>
<td>3.5%</td>
<td>4</td>
<td>35.6</td>
<td>linear</td>
<td>3400</td>
</tr>
<tr>
<td>3.5%</td>
<td>4</td>
<td>35.6</td>
<td>linear</td>
<td>10000</td>
</tr>
<tr>
<td>3.5%</td>
<td>4</td>
<td>35.6</td>
<td>4-arm</td>
<td>10000</td>
</tr>
<tr>
<td>3.5%</td>
<td>4</td>
<td>35.6</td>
<td>8-arm</td>
<td>10000</td>
</tr>
<tr>
<td>3.5%</td>
<td>5.5</td>
<td>35.6</td>
<td>linear</td>
<td>2000</td>
</tr>
<tr>
<td>2.0%</td>
<td>9</td>
<td>21.1</td>
<td>linear</td>
<td>2000</td>
</tr>
<tr>
<td>5.0%</td>
<td>4</td>
<td>52.0</td>
<td>linear</td>
<td>2000</td>
</tr>
</tbody>
</table>

**GEL-ASSISTED GUV FORMATION**

The surface of a microscope glass slide was functionalized with 3-mercaptopropyl trimethoxysilane according to a previously reported method\(^28\) (Figure 9, step 1) and sequentially drop-casted by a mixture of the Dex-Mal solution and thiolated PEG at 40 °C (Figure 9, step 2) until a homogeneous hydrogel film was formed and anchored to the glass surface. The molar ratio of maleimides to thiols was 1:1 in all samples unless otherwise stated. After the hydrogel layer was fully gelated, a lipid solution (14 mM, 10 µL) was deposited on the top of the hydrogel film. The lipid and hydrogel film was dried in a vacuum oven for 30 minutes at 35 °C or under a gentle stream of nitrogen gas.
Chapter 5

which prevented lipid oxidation at room temperature (Figure 9, step 3). In the hydration step, a liquid chamber was made by placing a 15 mm (OD) glass O-Ring on top of the hydrogel film and sealed with high vacuum silicon grease. PBS (or HBS) buffer solution (400 μL) was added to the chamber and the film (Figure 9, step 4) was allowed to swell for 1-2 hours at room temperature resulting in free-floating vesicles (Figure 9, step 5). Swelling of the film was performed above the Tm of all lipids. For samples containing solely unsaturated lipids, this swelling was performed at room temperature. For mixtures containing saturated lipids, swelling was performed at 50 °C. The formed free floating GUVs were transferred into an eppendorf tube for further analysis.

Figure 9. Procedure of the preparation of GUVs from Dex-PEG hydrogel coated glass slide. Reproduced from ref. [16].

The contact angle of the original, thiolated and gel-coated glass slide surfaces were measured by using the LBADSA plugin in ImageJ software. The osmolality of buffer solutions were determined from the freezing point depression using an Osmometer Roebling Type 13. The osmometer was calibrated using 100 mOsm/kg NaCl standard solution. The confocal imaging of the GUV growth was performed by a TI-Eclipse inverted microscope (Nikon, Japan) equipped with a 16-bit Cascade II 512 EMCCD camera (Photometrics, USA). Spinning-disc confocal microscopy was executed 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 centered at 595 nm. Epifluorescence was measured with a 40× objective and confocal microscopy was carried out using a 60× NA1.43 Plan-Apo Nikon oil-immersion objective. The size and yields of the obtained GUVs were visualized by a Zeiss axiovert-200 inverted microscope equipped with a Chroma TRITC and DAPI BP 445/50 fluorescence filter sets. Images were recorded with a black and white CCD camera (AxioCam NRm).

OSCILLATORY RHEOLOGY
The mechanical properties of the Dex-PEG hydrogels were measured on a DHR-2 rheometer (TA
Instruments) at 25 °C using a parallel plate-plate geometry (25 mm diameter). Solutions of Dex-Mal and PEG-SH (150 µL of each) were loaded on the bottom plate and the geometry was lowered to the gap distance of 600 µm. Time sweep measurements were performed to follow gelation process at a frequency of 1 rad s\(^{-1}\) with 1% strain. The linear viscoelastic regime (LVE) was determined for each sample using an amplitude sweep measurement at 1 rad s\(^{-1}\) from 0.1% to 100% strain. Frequency sweep measurements were conducted from 100 to 0.1 rad s\(^{-1}\) with 1% strain in the linear viscoelastic regime.

**SWELLING TEST**

Hydrogels (200 µL) were prepared in PBS buffer at room temperature (22 °C) according to the gelation protocol in glass vials. 2 mL PBS buffer (0.01% NaN\(_3\), pH=7.4) was added on top of each hydrogel at room temperature. At various time points, the buffer was removed from the top of hydrogels, then each swollen hydrogel was weighed and fresh buffer was put on top afterwards. When the weight of swollen hydrogel reached a plateau, the equilibrium mass swelling ratio (\(q\)) was calculated using the equation below:

\[
q = \frac{W_s}{W_d}
\]

Where \(W_s\) is the equilibrium swollen weight and \(W_d\) the dry weight of the hydrogel. Each set of hydrogels were measured in triplicate.

**CRYO-SCANNING ELECTRON MICROSCOPY (CRYO-SEM)**

The morphologies of the hydrogels were imaged using a JEOL 6330 Cryo Field Emission Scanning Electron Microscope from the General Instrumentation Facility at Radboud University (Nijmegen, The Netherlands). 5 µL of Dex-PEG hydrogel was injected into a hollow cylindrical sample holder and immediately flash frozen in liquid nitrogen. The sample was inserted in the cold-stage of the SEM cryo-preparation chamber and cleaved to make a horizontal fracture plane. Water was sublimated during 15 minutes and the fracture plane was coated with a thin gold-palladium layer and subsequently the sample was transferred into the SEM chamber, where it remained frozen during the imaging.
REFERENCES


Figure S1. Equilibrium swelling ratios of hydrogel-coated glass slides prepared with various compositions of 1% (w/v) Dex-PEG. (A) Dex70k-Mal4, (B) Dex70k -Mal9, and (C) Dex70k -Mal13 crosslinked with thiolated PEG (□: PEG2k-2SH. ○: PEG3.4k-2SH. Δ: PEG10k-4SH. ×: PEG10k-8SH) respectively.
Figure S2. Confocal microscopy Z-projections showing phase separated GUV’s (lipid composition: 40% DPhPC : 40% DPPC : 20% CH, mol%) in PBS at physiological ionic strength (Scale bar: 10 µm). Images adopted from ref. [16].

Figure S3. Comparison of the dynamic viscosity of 0.5% (w/v) Dex70k-Mal2.5 crosslinked to PEG prepared by various thiolated PEG: PEG2k-2SH, PEG3.4k-2SH, PEG10k-4SH and PEG10k-8SH.