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Heat-activated liposome targeting to streptavidin-coated surfaces

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ABSTRACT

There is a great need of improved anticancer drugs and corresponding drug carriers. In particular, liposomal drug carriers with heat-activated release and targeting functions are being developed for combined hyperthermia and chemotherapy treatments of tumors. The aim of this study is to demonstrate the heat-activation of liposome targeting to biotinylated surfaces, in model experiments where streptavidin is used as a pretargeting protein. The design of the heat-activated liposomes is based on liposomes assembled in an asymmetric structure and with a defined phase transition temperature. Asymmetry between the inside and the outside of the liposome membrane was generated through the enzymatic action of phospholipase D, where lipid head groups in the outer membrane leaflet, i.e. exposed to the enzyme, were hydrolyzed. The enzymatically treated and purified liposomes did not bind to streptavidin-modified surfaces. When activation heat was applied, starting from 22 °C, binding of the liposomes occurred once the temperature approached 33 ± 0.5 °C. Moreover, it was observed that the asymmetric structure remained stable for at least 2 weeks. These results show the potential of asymmetric liposomes for the targeted binding to cell membranes in response to (external) temperature stimulus. By using pretargeting proteins, this approach can be further developed for personalized medicine, where tumor-specific antibodies can be selected for the conjugation of pretargeting agents.

1. Introduction

Liposomal systems have been intensively studied for their capacity to deliver therapeutic and diagnostic materials [1]. Currently, most of the liposomal systems on the market have been formulated to reduce the systemic toxicity of the encapsulated drug and to extend the drug circulation time after administration [2,3]. Newer formulations are being designed to increase accumulation of the drug at the desired sites by integrating target recognition molecules on the liposome surfaces [4,5]. Although all of these approaches have potential therapeutic value, further improvements are desired to obtain additional clinical benefits. Liposome systems that exhibit a stimuli-responsive property (e.g., in response to temperature [6], or redox reactions [7]) have been designed to improve cellular uptake, and thus will increase the drug residence time at the target site by controlling the appearance of the targeting molecules. A recently applied variant of targeting in oncology is the so-called pretargeting, where bi-specific protein conjugates are administrated before the delivery of the radioactive therapeutic agents [8,9]. The streptavidin–biotin interaction used in pretargeting strategies can be utilized for the delivery of biotinylated liposomes.

Temperature-responsive liposomes offer the possibility of enhancing the therapeutic efficiency in hyperthermia treatments [10–12]. Stability against leakage is achieved at temperatures where liposomes remain in the gel phase, and the encapsulated materials can be released when the temperature is raised to the phase-transition regime. More pharmaceutical advantages are likely to be obtained if the temperature-responsive liposomes can also change their surface properties upon heat stimulus, in which the targeting function is activated as well as the release. This concept has previously been shown for polymer-based drug carriers [13].

As a tool to develop more advanced liposomes, the study of surface-supported lipid membranes can provide valuable information on how to design temperature-activated liposomal functions. For example, the asymmetry of supported membranes can be studied as a function of temperature [14–17], suggesting that membrane asymmetry can potentially facilitate the positioning-control of ligand-modified lipids. A commonly used strategy for preparing asymmetric supported lipid membranes is the Langmuir–Blodgett (LB) technique [18]. However, it cannot be applied as a protocol to produce asymmetric liposomes in bulk solution. A more general strategy to generate asymmetry is by hydrolyzing lipids in the presence of enzymes such as phospholipase D (PLD). PLD is involved in a variety of cellular functions, one of which is to catalyze the hydrolysis of lipid head groups to form phosphatidate (PA) [19], as shown in Fig. 1A. Hidden surface functions in the asymmetric membranes can be activated by increasing the temperature close to the gel to liquid-crystalline phase-transition temperature (T_m = 41 °C for DPPC lipids) of the asymmetric membrane where lipid flip-flop appears.

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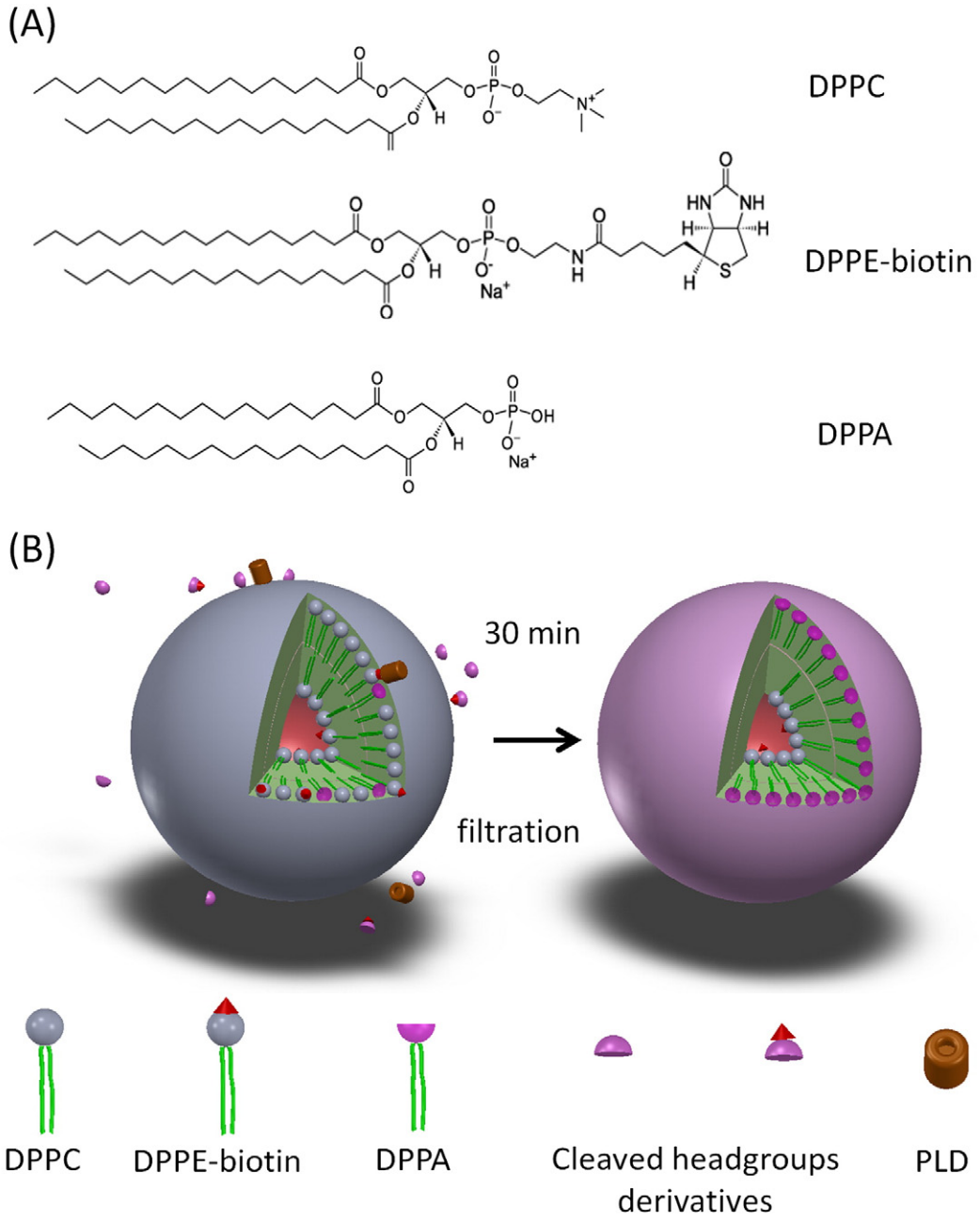

Fig. 1. (A) Chemical structures of the lipids used in the present study. (B) Schematic representation of the preparation of the asymmetric liposomes.

In a previous study, we showed temperature-activated flip-flop of biotinylated lipids originally hidden in the lower leaflet of a supported lipid membrane prepared using sequential deposition of the lipid leaflets in situ [16]. Here, we take advantage of PLD to prepare asymmetric liposomes through hydrolysis of lipid head groups at the outer membrane leaflet while leaving the functionality of the lipids in the hidden leaflet, assuming that the asymmetry is maintained below the phase transition temperature.

With the aim to demonstrate heat-activation of liposome binding to a target model surface, the present protocol is first developed using supported lipid membranes as a model system, and then applied to liposomes. The supported biotinylated membranes are also used as a biomimetic platform for the investigation of the temperature dependence of liposome binding. Streptavidin binds highly specifically and with high affinity to biotinylated lipids, and can be used as a pretargeting agent to label the targeted surfaces, here by attracting liposomes when biotin-lipids flop to the surface.

The protocol development using supported membranes as well as the binding of targeted liposome were monitored by the quartz crystal microbalance with dissipation (QCM-D) technique.

2. Materials and methods

2.1. Materials

Chemicals were purchased from commercial suppliers and used without further purification unless otherwise stated. 1, 2-Dipalmitoyl-sn-glycero-3-phosphatidylcholine (DPPC), 1, 2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine-N-(biotinyl) (DPPE-biotin), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), and 1, 2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-N-(biotinyl) (DOPE-biotin) were purchased from Avanti Polar Lipids Inc., USA. Phospholipase D (PLD) and streptavidin were purchased from Sigma.

A Milli-Q water purification system (Millipore, France) was used to purify water to a minimum resistivity of 18.2 MΩ·cm. Tris-buffered saline containing 10 mM Tris, and 100 mM sodium chloride (NaCl) was prepared in water. The pH of the Tris buffer was adjusted to 8.0 by adding 0.1 M HCl solution, and the buffer was filtered through 0.2 μm filters and degassed prior to use. Streptavidin was dissolved in Tris buffer (50 μg/mL) and PLD was dissolved in water (1 mg/mL) and further diluted in Tris buffer, of which the pH had been lowered to 5.5 by addition of HCl.

2.2. Liposome preparation and characterization

DPPC/DPPE-biotin liposomes were prepared by the following procedure: 1 mg of DPPC and 10 μg of DPPE-biotin dissolved in methanol were added to a round-bottomed flask. The solvent was evaporated under a gentle flow of nitrogen in a fume hood, after which residual methanol was removed under reduced pressure. The subsequent rehydration and extrusion were performed at 55 °C, i.e., well above the T_m for DPPC (T_m = 41 °C). The lipid film was rehydrated by mixing with 1 mL Tris buffer, and the suspension was kept in an oven for at least 1 h with occasional vortex until lipids were fully dissolved. The solution was then extruded through polycarbonate membranes with pore sizes of 50 nm (Whatman, UK) 51 times in a temperature controlled mini-extruder (Avanti Polar Lipids, USA). POPC/DOPE-biotin liposomes were prepared following the same protocol, but at an ambient temperature.

The asymmetric liposomes were prepared by incubating 0.1 mg extruded DPPC/DPPE-biotin liposomes (symmetric) with 25 units (100 μL) PLD in 380 μL Tris solution at pH 5.5 at ambient temperature for 30 min. The suspension was then filtered through an Amicon Ultra −0.5 mL centrifugal filter (100 K) (Merck Millipore, IRL) at 14,000*g* for 3 min, followed by 4 times washing with Tris buffer (pH 8) to separate the liposomes from PLD, the cleaved biotin derivative and choline. The asymmetric liposomes were collected by spinning the filter reversely at 1000*g* for 2 min. The filtered liposome solution was diluted to a concentration of 0.02 mg/mL for further measurements. The recovery of the liposome was calculated by measuring the concentration of liposomes and PLD using a UV/visible spectrophotometer (JENWAY, UK). The absorbance at 400 nm and 280 nm were used to determine the concentration of liposomes and proteins respectively.

Both symmetric and asymmetric liposomes were characterized by dynamic light scattering (DLS) (Malvern, UK) with respect to size and zeta potential. The polydispersity index (PDI) was used to estimate the size distribution of the liposomes. Measurements were carried out at room temperature using liposome solutions that were diluted in Tris buffer to 0.02 mg/mL. For liposome zeta potential measurements, liposome solutions were diluted in water to a concentration of 0.02 mg/mL and the measurements were repeated 5 times at 22 °C.

2.3. Quartz crystal microbalance with dissipation (QCM-D) measurements

The preparation of supported asymmetric lipid membranes and the subsequent transmembrane lipid exchange (flip-flop), as well as the binding of heat-activated asymmetric liposomes to immobilized streptavidin layers, were monitored by QCM-D and repeated at least 4 times for each case. Prior to mounting, the QCM-D crystals (SiO_2-coated sensors obtained from Q-Sense AB, Sweden) were rinsed with ethanol and water, and then cleaned in a UV-ozone cleaner for 30 min. QCM-D measurements were performed at several harmonics (3, 5, 7, 9, 11, and 13) using a QCM-D E4 system (Q-Sense AB, Sweden). The frequency and dissipation shifts were plotted using the Q-tools software (Q-Sense AB, Sweden). The presented frequency shifts obtained at different overtones were normalized by dividing each frequency shift by the corresponding overtone number.

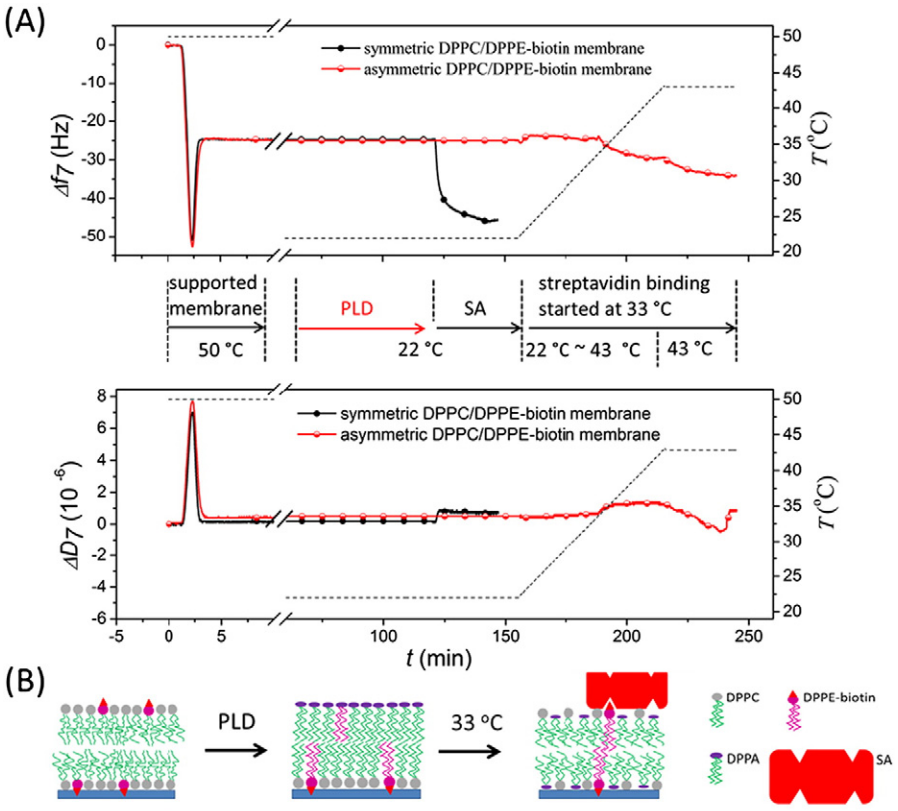

Fig. 2. (A) Streptavidin binding to supported symmetric and asymmetric DPPC/DPPE-biotin membranes as detected by QCM-D. (B) Schematic representation of the formation of asymmetric membrane and the heat-induced biotin lipid flopping and binding to streptavidin.

Table 1
Average size, size distribution and zeta potential of the symmetric, asymmetric, and heat-treated asymmetric liposomes.

Storage time		Symmetric liposomes	Asymmetric liposomes	Heat-treated Asymmetric liposomes
+0 days	Zeta potential (μV)	−3 ± 1	−44 ± 6	−30 ± 7
	Average diameter (nm)	92 ± 1	126 ± 3	134 ± 7
	PDI	0.031 ± 0.005	0.103 ± 0.010	0.121 ± 0.011
+7 days	Zeta potential (μV)	−4 ± 2	−49 ± 5	−32 ± 6
	Average diameter (nm)	93 ± 2	126 ± 3	136 ± 8
	PDI	0.031 ± 0.005	0.108 ± 0.006	0.120 ± 0.003
+14 days	Zeta potential (μV)	−5 ± 1	−51 ± 7	−33 ± 4
	Average diameter (nm)	93 ± 1	129 ± 3	136 ± 1
	PDI	0.031 ± 0.005	0.108 ± 0.006	0.113 ± 0.011

The experiment for the study of supported asymmetric membranes was carried out as follows: DPPC/DPPE-biotin liposomes (0.2 mg/mL) were adsorbed onto SiO_2 QCM-D crystals at 50 °C and formed supported DPPC/DPPE-biotin membranes. After stabilization of the QCM-D signals at −25 Hz and $< 0.5 \times 10^{-6}$, which are characteristic values for a supported lipid membrane [20,21], the temperature was decreased to 22 °C. PLD was then introduced, and after the flow had been stopped the membranes were incubated with PLD for 1 h. The hydrolysis was interrupted by removal of the enzyme by rinsing with Tris buffer. The temperature was then increased to 43 °C and maintained for 1 h to allow the lipids to redistribute between the two leaflets. Before the heat-activation, streptavidin (50 μg/mL) solution was injected to probe the presence of biotinylated lipids at the outer membrane surface. The flow was then stopped and the temperature was increased following a linear gradient. The streptavidin solution left above the sensors was used to demonstrate the reappearance of the biotinylated lipid on the upper leaflet.

The experiment was performed as follows for the investigation of heat-activated asymmetric liposome adsorption on streptavidin layers: two parallel experiments were carried out in two sensors where POPC/DOPE-biotin liposomes (diluted just before injection to a total lipid concentration of 0.2 mg/mL in Tris buffer) were adsorbed onto SiO_2 QCM-D crystals at 22 °C to form supported lipid membranes with biotin. Streptavidin was immobilized onto the membrane, followed by rinsing with Tris buffer. Symmetric liposomes (0.1 mg/mL) were introduced to the immobilized streptavidin layers. At the same time, the filtered asymmetric liposomes (0.1 mg/mL) were added to the other sensor, then the flow was stopped after 10 min. The temperature was again increased to 43 °C and maintained for 1 h. The temperature-induced effects on the QCM-D signal shifts were removed by subtracting the signals from reference measurements performed using the same blank crystals exposed to buffer under the same conditions (as described in [22] and [23]).

In order to evaluate the stability of the asymmetric liposomes, the experiments were repeated after 7 days and 14 days.

3. Results and discussion

3.1. Development of the protocol: flip-flop in supported asymmetric lipid membranes

As a first step towards the preparation of asymmetric liposomes, we investigated whether asymmetric lipid membranes could be prepared on silica-coated QCM-D sensors where the head groups of lipids in the upper leaflet of symmetric membranes were hydrolyzed into phosphatidate through the action of PLD.

Our strategy to form a supported lipid membrane with an asymmetric structure is shown in Fig. 2. The spontaneous rupture of DPPC/DPPE-biotin liposomes at 50 °C is followed by well-established pathway as shown in more detail in the left part of Fig. 2(A), frequency decreased (mass increase) and dissipation increased during the adsorption of intact liposomes, reaching a minimum in frequency (maximum in mass) and maximum in dissipation. A subsequent rise in frequency and a decrease in dissipation then occurred, when the layer of supported liposomes transformed into a supported membrane. The spontaneous liposome rupture started when the critical coverage of liposomes on the silica surface had been reached [20,24]. When the signals had reached the expected values [20] ($\Delta f = -25$ Hz and $\Delta D = 0.2 \times 10^{-6}$ after 5 min in Fig. 1), the temperature was decreased to 22 °C (far below the T_m of the membrane, thus lipids were in the gel phase) in order to "freeze" the transmembrane motions (flip-flop) of the lipids. Hydrolysis of the lipids in the upper leaflet was initiated by the addition of PLD at 60 min, see in Fig. 2 (no signal shifts indicated that no PLD was bound to the surface), and was maintained for 1 h. It is suggested by the QCM-D curves that the membrane remained intact. The change in mass on the sensor surface (hydrolyzed choline and biotinylated ethanolamine, molecular weights are 104 Da and 305 Da respectively) was too small to be detected.

The heat-activated asymmetric membrane surface function (i.e., to induce membrane affinity to streptavidin) was demonstrated by the binding of streptavidin to biotinylated lipids. Those were preserved in the lower leaflet and then flopped-up to the upper one during heating by applying a linear gradient from 22 °C to 43 °C for 1 h. Saturated adsorption of streptavidin on symmetric membranes (not exposed to PLD) was observed (120 min in Fig. 2). For the asymmetric membrane at ambient temperature, the QCM-D signals did not respond to the addition of streptavidin at the same time. It showed that biotinylated lipid head groups had been removed on the PLD-treated membrane surfaces. In contrast, distinct streptavidin binding was detected during the temperature increase (190 min, 33 °C). The stabilized signals of streptavidin bound to the 'activated' membrane corresponded to half of a saturated layer of streptavidin, similarly to our previously published observation [16]. The lowering of the dissipation signals after 220 min was likely caused by difficulties related to the reference subtraction, but could also indicate rearrangements in the layer on the surface.

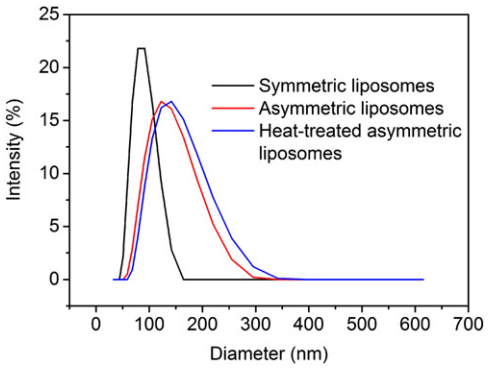

Fig. 3. Size distributions of the symmetric, asymmetric, and heat-treated asymmetric liposomes (0 days).

In summary, data presented in Fig. 2 demonstrates that asymmetry in supported DPPC/DPPE-biotin membranes can be generated and preserved at ambient temperature. In the next step, the protocol developed using the supported model membranes was applied for the preparation of asymmetric liposomes.

3.2. Preparation and characterization of the asymmetric liposomes

Asymmetric DPPC/DPPE-biotin liposomes were prepared by incubating DPPC/DPPE-biotin liposomes with PLD in a Tris solution at pH 5.5 for 30 min. The purification of the liposomes was done with an Amicon filter. PLD was detected based on the absorbance at 280 nm in the filtrate for the first 4 times of centrifugation. The concentration of PLD decreased until undetectable for the last washing. Asymmetric liposomes were prepared with a yield of 78%, and no PLD could be detected in the recovered solution.

No precipitation was observed in the suspensions due to the electric repulsion between the hydrolyzed highly negatively charged PA lipids presented on the outer leaflet of the asymmetric liposomes. The surface of the asymmetric, PLD-treated liposomes had a lower zeta potential compared to the symmetric liposomes, as shown in Table 1. An increase in the zeta potential of the heat-treated asymmetric liposomes indicated that the number of negatively charged PA lipids decreased in the outer leaflet. PA lipids diffused inwards to the inner leaflet, and neutral PC lipids flipped outwards. The change in surface properties of the asymmetric liposomes after the heat treatment is a proof of the redistribution of lipids, the zeta potential of the liposomes increased due to lipid exchange between the neutral inner and negatively charged outer leaflets.

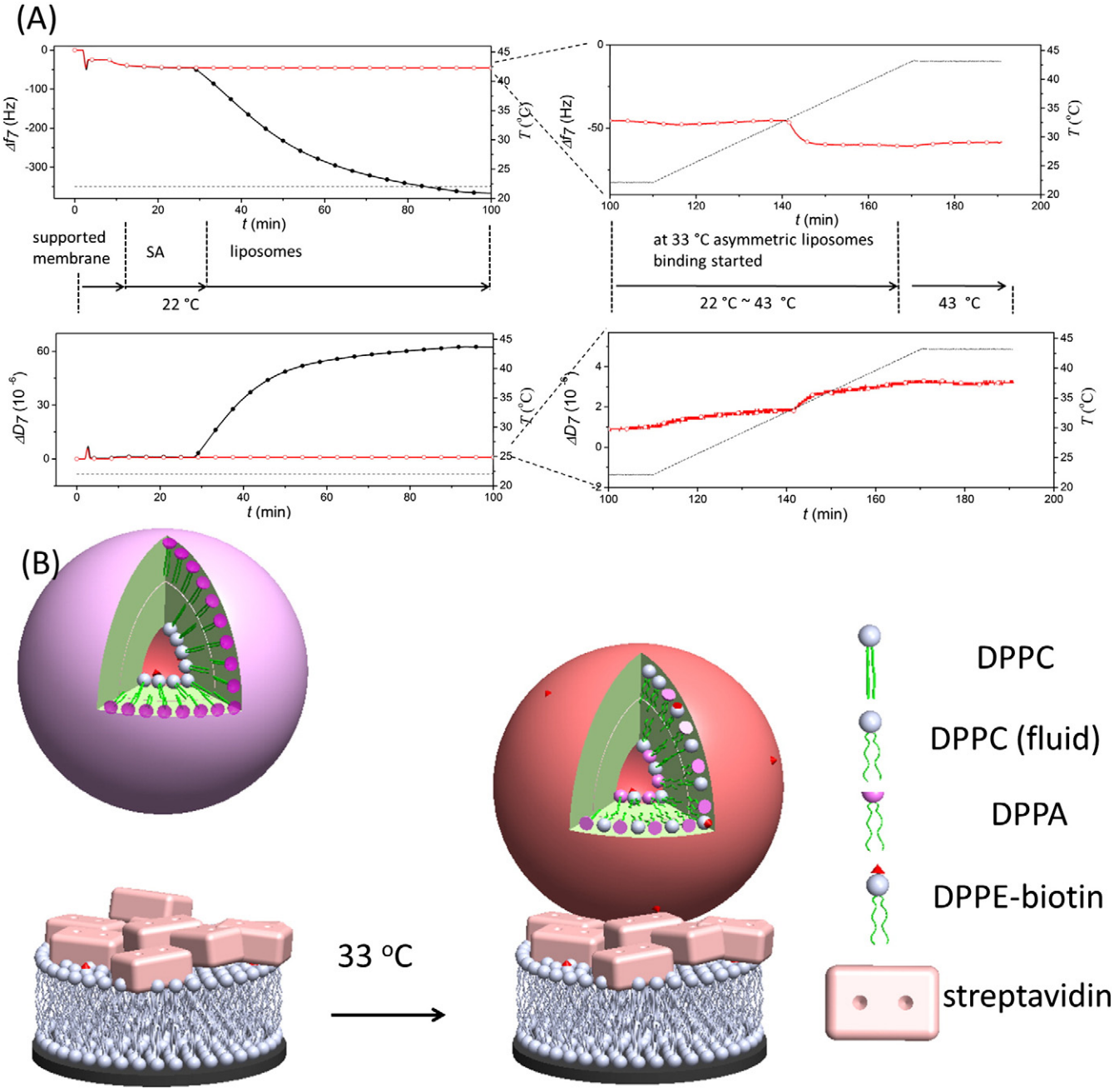

Fig. 4. (A) Binding of symmetric and asymmetric DPPC/DPPE-biotin liposomes to a streptavidin-modified surface as detected by QCM-D. (B) Schematic representation of the heat-induced biotin-lipid flopping to the surface of asymmetric liposomes and binding to streptavidin. Note that the size of the liposome is not to scale.

The stability of the asymmetric liposomes was demonstrated by monitoring of the liposome size and size distribution over time, as shown in Table 1. Symmetric liposomes maintained their structural characteristics through storage at 4 °C for more than 14 days. The asymmetric liposomes remained intact after the filtration and the heat treatment. However, the size as well as size distribution of the asymmetric liposomes was larger compared to that of the symmetric ones, as shown in Fig. 3. We assumed that this was caused by the fusion of liposomes during PLD hydrolysis or aggregation during centrifugation. The heat-treated asymmetric liposomes did not change significantly during the 2-week-experiment.

3.3. Heat-activation of asymmetric liposomes: targeting to streptavidin layer

The heat-induced transition in asymmetry of the asymmetric liposomes was verified by the binding of the liposomes onto streptavidin-modified surfaces, as visualized in Fig. 4. The supported POPC/DOPE-Biotin lipid bilayers did not contribute to the signal shifts. The melting temperature of the supported membrane was −2 °C which was well below the room temperature, and the membrane remained in the liquid phase when temperature raised. Upon heat-activation, liposomes transformed from the gel phase to the liquid-crystalline phase where biotinylated lipids that were originally 'frozen' in the inner leaflet of the asymmetric liposomes flopped outwards and PA lipids in the outer leaflet flipped inwards. As the ligands (biotin) reappeared on the surface of the asymmetric liposomes, binding to the streptavidin-modified surface could be observed. As described in Section 2.2, streptavidin layers were immobilized to the sensor surfaces at t = 25 min. Symmetric liposomes and asymmetric liposomes were then introduced to the surface of streptavidin layers. From the frequency decrease and significantly increased dissipation at t = 30 min, we can observe that symmetric DPPC/DPPE-biotin liposomes started to bind to streptavidin layers. On the contrary, asymmetric liposomes exhibited no signal shift due to their inert surface. For the asymmetric liposomes, the flows were stopped at 40 min. At t = 110 min, the temperature was raised at a rate of 0.35 °C/min and at 141 min (33 °C) the liposomes began to attach to streptavidin layer as evident from the decreased frequency. The onset temperature of binding (33 ± 0.5 °C) did not change with respect to prolonged storage period (7 days and 14 days). However, lipid flip-flop appears earlier than the melting temperature T_m = 41 °C for DPPC. It can be hypothesized that the changes in mechanical property (soften) at around 33 °C (pre-melting in the process of phase transition [16,25]) will facilitate flip-flop, which corresponds well with previous results [22]. In addition, the heat-activation of lipid flip-flop was verified by studying the stability of the asymmetric liposomes against streptavidin when heat was applied in bulk. The size of mixed asymmetric liposomes (0.02 mg/mL) and streptavidin (0.1 mg/mL) solution was measured by DLS, and aggregation was observed when the mixture was incubated at 43 °C for 30 min in a water bath.

In summary, with the aim to show heat-activated binding of liposomes to a target surface, we prepared asymmetric supported lipid membranes in-situ and asymmetric liposomes in bulk solution. The asymmetry in the model membranes, both when supported on a solid surface and in liposomes, was well-preserved at room temperature where lipids were in their gel state. Heat-activated liposome targeting to the streptavidin-modified surfaces was identified at 33 ± 0.5 °C. The activation temperature was repeatedly examined after the purification of the liposomes and after storage in room temperature for 7 days. Stability was also verified by uniform characteristics of the asymmetric liposomes during the storage period. The described asymmetric liposomal system offers an interesting approach for the development of targeted liposome delivery under heat stimulus. This method has the potential to improve diagnostic imaging and drug delivery, where lipid membranes can alter their surface affinity to targeted sites in response to temperature changes under hyperthermic conditions. However, due to the disturbance in lipid diffusion at the pre-phase transition at around 33 °C, the transition temperature is lower than the expected temperature for hyperthermia application (39 °C to 42 °C). In order to improve the targeting performance, a higher transition temperature is therefore required. This can be achieved by incorporation of lipids with higher phase-transition temperature into the liposome. The formation of the pretargeting layer of protein on supported membranes and further attachment of the liposomes are monitored by QCM-D in real time, which can be developed as an in-vitro platform for the investigation of targeted delivery of drug carriers. Further modifications are interesting to study, where the biotin functional group is replaced by another targeting ligand.

4. Conclusions

In conclusion, we report on the use of asymmetric model membranes as a useful tool for the development of liposomal drug carriers with temperature-activated surface properties. Asymmetric liposomes were prepared based on selective phospholipase hydrolysis of lipid head groups in the outer leaflet of symmetric liposomes. The asymmetry of the liposomes was maintained in the gel phase. Flopping of the biotinylated lipids from the inner to the outer leaflet of the asymmetric liposomes was induced by raising the temperature close to the gel to liquid-crystalline phase-transition temperature of the lipid membrane, as demonstrated by controlled binding of asymmetric liposomes to target surfaces in experiments with defined temperature gradients.

Transparency document

The Transparency document associated with this article can be found, in the online version.

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