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# Adhesion characteristics and stability assessment of lectin-modified liposomes for site-specific drug delivery

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**Abstract**

Carbohydrate moieties of the cellular glycocalyx have been suggested to play an important role in biological recognition processes during pathologic conditions, such as inflammation and cancer. Herein, we describe lectin-modified liposomes which might have potential for site-specific drug delivery during the therapy of such diseases. Specific interactions of plain (i.e., unmodified) and PEGylated, lectin-grafted liposomes with model membranes were investigated under real-time flow conditions using a quartz crystal microbalance. In addition, the morphology of the liposomal systems was assessed by atomic force microscopy. Plain liposomes exhibited only unspecific adhesion to glycolipid membranes and had a tendency to coalesce. The degree of membrane interaction was significantly increased when plain liposomes were modified with the lectin, Concanavalin A. However, vesicle fusion also markedly increased as a result of lectin modification. Additional PEGylation of liposomes reduced unspecific adhesion phenomena, as well as coalescence. Moreover, our studies enabled us to establish quartz crystal microbalance and atomic force microscopy as powerful and complementary methods to characterize adhesion properties of targeted drug delivery systems.


*Keywords:* Concanavalin A; Quartz crystal microbalance; Atomic force microscopy; Drug targeting; PEGylation; Glycocalyx

## 1. Introduction

Site-directed drug delivery to specific organs or cell types is an attractive means increasing therapeutic effects and reducing unwanted side effect of pharmaceuticals. In this context, the use of liposomes as drug carrier systems has been extensively explored during the last decades [1–3]. Site-specific drug delivery is feasible, because as part of the pathological phenotype of diseases, such as inflammation or cancer, cells often express surface receptors that are not present under physiological conditions [4]. Conjugating appropriate ligands for such a receptor should therefore result in a higher drug concentration at the tissue of interest and thus, increase the efficiency of the therapy [5,6]. Different methods have been described for the conjugation of peptide ligands to liposomal surfaces [7–10]. Lectins are one example, due to their binding specificity to carbohydrate structures of the glycocalyx, which are important in cellular processes, such as differentiation and recognition, inflammation and immune response [11–14]. Concanavalin A (ConA), a plant lectin with binding specificity for mannose and glucose, has been suggested as a model receptor and is one of the most extensively studied members of the lectin family [15–17]. Generally, it is possible to bind the ligand directly to the liposomal membrane using linker-lipids with functionalized end groups or alternatively, the ligand can be bound to the end of a polyethylene glycol (PEG) chain which is attached to a lipid anchor [8]. PEG chains also play a role in the sterical stabilization of liposomes [18–20], STEALTH™

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liposomes being just one example of the successful use of this modification [17,21–25].

Quartz crystal microbalance (QCM) techniques allow characterization of interactions between sensor-supported model membranes or immobilized bioactive molecules with proteins, enzymes, antibodies and complex systems, such as cells, nanoparticles or liposomes. The interaction of liposomes with sensor surfaces and artificial membranes has been previously investigated. In this context, Keller and Kasemo [26] demonstrated that the adhesion and spreading behavior of small unilamellar liposomes was dependent on the chemical composition of the sensor surface. Studies on specific interactions of surface-modified liposomes with supported lipid model membranes using QCM have also been reported by a number of investigators [27–31].

Using ConA-conjugated liposomes simulating carbohydrate-induced cell adhesion, our laboratory previously suggested a combination of the protective effect of PEG-groups with the specific recognition properties of antibodies and lectins in a liposomal formulation [32]. In the present study, we extended these fundamental findings by describing, in time-resolved manner, adhesion, detachment and spreading of liposomal formulations on a glycolipid model membrane under flow conditions using QCM. We evaluated four different liposome designs: (i) plain (i.e., unmodified) DPPC/cholesterol liposomes, (ii) sterically-stabilized (i.e., PEGylated) DPPC/cholesterol liposomes, (iii) ConA-modified DPPC/cholesterol liposome with the lectin directly bound to the surface and (iv) PEGylated ConA-modified DPPC/cholesterol liposomes with the lectin covalently attached to the distal end of PEG-spacers. In addition, the morphology of the QCM sensor surfaces was investigated by atomic force microscopy (AFM).

## 2. Materials and methods

### 2.1. Liposome preparation and modification with lectin

The basic composition of the liposomes was DPPC/cholesterol 2:1 (n/n) with different amounts of activated protein anchor lipids (2.5, 5 and 10 mol% for cyanur-$PEG_{2000}$-PE and cyanur-DSPE, respectively) or $PEG_{2000}$-PE (10 mol %) for the preparation of sterically stabilized liposomes. Large unilamellar vesicles (LUV) were prepared by extrusion of multilamellar vesicles (MLV). Briefly, a mixture of phospholipid and cholesterol in chloroform:methanol (2:1) was dried to a thin film in a rotary evaporator (Büchi, Essen, Germany). This lipid film (15 μmol) was suspended in 1 ml aqueous NaCl (0.15 M) at 60 °C. The resulting MLV were passed seven times through an extruder (Lipex Biomembrane, Vancouver, Canada) fitted with a 200-nm pore polycarbonate membrane.

ConA (Sigma, Seelze, Germany) was covalently coupled to the liposomes via amine-reactive cyanur-groups, either directly to the liposomal surface using cyanuric chloride-activated DSPE (cyanur-DSPE) or to the distal ends of PEG-spacers using activated cyanur-$PEG_{2000}$-PE according to a method previously published by our lab [10]. The activated linker lipids were added to the DPPC/cholesterol mixture, subsequently to a chemical modification and separation step (by thin layer chromatography). To bind the protein, 15 μmol of vesicle suspension were adjusted to pH 8.5 (using 0.01 N borate buffer), then 92 nmol ConA (in 1 ml 0.1 M sodium borate solution) were added and the mixture was incubated for 6 h at room temperature. The lectin-modified liposomes were separated from unbound lectins by gel permeation chromatography on Sepharose 4B columns (Pharmacia, Uppsala, Sweden) with 0.1 M sodium borate buffer as eluent. The coupling efficacy was assessed using a bicinchoninic acid (BCA) protein assay kit (Perbio Science, Bonn, Germany). The final lectin concentration was found to be always between 40 and 120 molecules per vesicle (~200 nm in diameter), depending on the lipid composition (data not shown).

Four different types of liposomes were prepared: plain (i.e., unmodified) liposomes; PEGylated liposomes; non-PEGylated, lectin-functionalized liposomes (i.e., ConA directly bound to the liposome) and PEGylated, lectin-modified liposomes (i.e., ConA bound to the PEG-linker). Fig. 1 shows schematics of the different formulation designs.

### 2.2. Preparation of the glycolipid bilayer

The glycolipid used in this study, 1-*O*-(3,6,9,12,15,18,21,24-octatetracontyl)-α-D-mannopyranoside (α-man $(OE)_8$), with 8 oxyethylene (OE) spacer units between the alkyl chain and carbohydrate head group was synthesized according to methods previously described by Ogawa and Wilhelm, respectively [33,34]. The chemical structure of the glycolipid is shown in Fig. 2.

Preparation and characterization of glycolipid bilayers used in this study has been described in detail elsewhere [31]. Briefly, monolayer investigations were carried out using a Langmuir film balance (Riegler&Kirstein, Potsdam, Germany) with a rectangular thermostatically-controlled PTFE trough (area 100 $cm^2$, 10 mm depth). The surface pressure was measured using the Wilhelmy plate method. The lipid-mixture of DSPC and α-man $(OE)_8$ (10:1 n/n) was dissolved in freshly-distilled chloroform:methanol 2:1 and added to PBS (pH 7.4) at a final concentration of 1 mM. Films were compressed with a constant speed of 0.15 $cm^2$/s (corresponding to $0.6–2.5\times10^{-3}$ $nm^2$/s per molecule depending on the degree of compression) to a surface pressure of 30 mN/m and were then transferred at constant pressure to a thiolated (1-hexadecanethiol) quartz sensor surface by Langmuir–Blodgett-technique via vertical dipping through the compressed monolayer. The transfer ratio was ≥0.95. All experiments were performed at 25 °C. The quality and morphology of the model membrane was characterized by epifluorescence microscopy (Olympus BX2, Planegg, Germany) using 1-hexadecanoyl-2-(*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)aminohexanoyl)-*sn*-glycero-3-phosphocholine (NBD-PC; Sigma) as fluorescence probe. NBD-PC was added to the lipid mixtures at a concentration of 1 mol%. The dye preferentially dissolved in the liquid-expanded phase (data not shown).

### 2.3. Conditioning of the sensor surfaces

Prior to the hydrophobic coating of the quartz plates, a cleaning procedure was carried out. The quartz crystals were immersed in piranha solution [27,28] for 15 min, followed by extensive washing with MilliQ water. This procedure was repeated until no further frequency changes were detected. After cleaning, a hydrophobic basic layer was prepared by self assembly of 1-hexadecanthiole from a chloroform solution ($10^{-4}$ mol/l, 2 h, 25 °C). The frequency shift achived by a tightly packed 1-hexadecanthiole coating on one side of the quartz was found to be 46 Hz (at 10 MHz).

### 2.4. Quartz crystal microbalance (QCM)

A 10 MHz AT-cut quartz-crystal plate (1.766 $cm^2$, Quarzkeramik Stockdorf, Germany) with gold disc electrodes (0.283 $cm^2$) sputtered onto both sides was used as support layer for the α-man $(OE)_8$/phospholipid Langmuir–Blodgett film. The resonance frequency was generated by an oscillator and the frequency of the vibrating quartz was recorded by a microcomputer system (Ifak, Magdeburg, Germany). The relationship between frequency change ($\Delta f$) and mass change ($\Delta m$) is given either by the Sauerbrey equation (Eq. (1)) [35], when measuring in the gas phase or by the equation according to Kanazawa and Gordon (Eq. (2)), when measuring in liquids [36]:

$$\Delta f = -\frac{2f_0^2 \cdot \Delta m}{A\sqrt{\rho_q \mu_q}} \quad (1)$$

$$\Delta f = -f_0^{2/3}(\eta_{fl}\rho_{fl}/\pi\mu_Q\rho_Q)^{1/2} \quad (2)$$

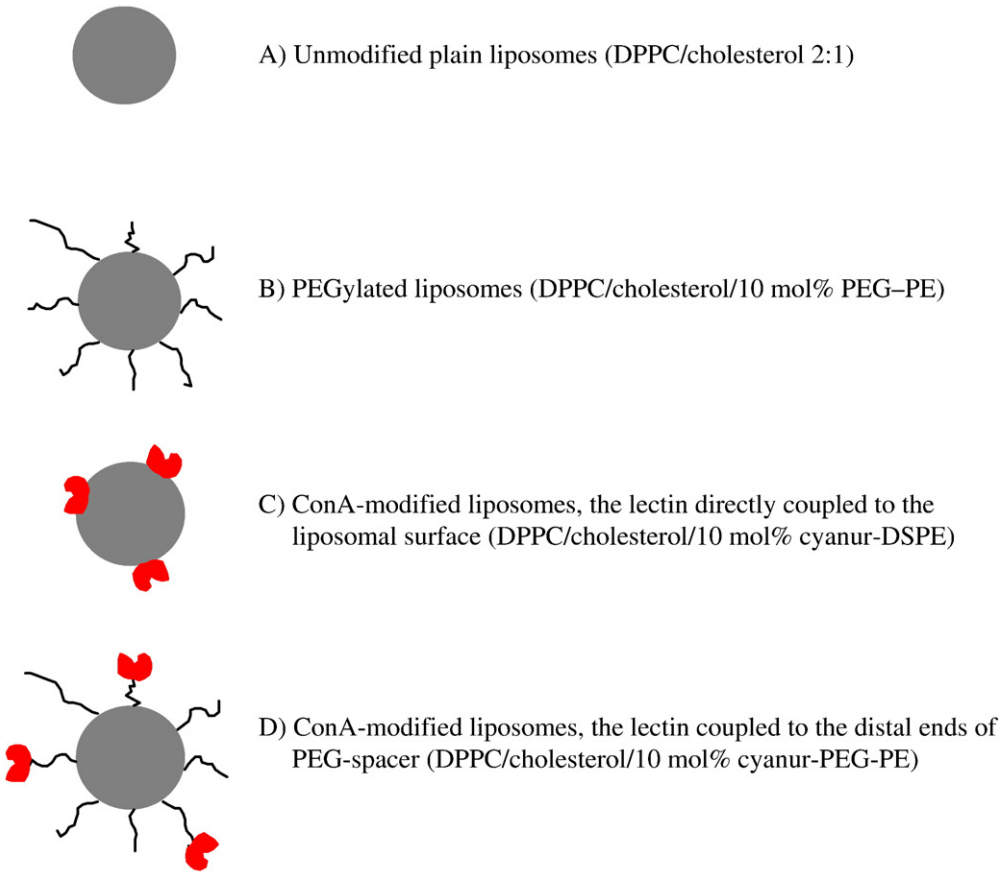


Fig. 1. Schematic of the different liposomal formulation designs.

where $f_0$ [Hz] is the basic frequency (10 MHz), $\Delta m$ [ng] the mass change, A [$cm^2$] the area of the electrode (diameter=6 mm, A=0.283 $cm^2$), mq the shear modulus of the crystal [$2.947\times10^{11}$ $cm^{-1}\times g\times s^{-2}$] and rq the density of the crystal [2.648 $g\times cm^{-3}$]. $\rho_{fl}$ and $\eta_{fl}$ describe the density and the viscosity of the surrounding liquid medium, respectively.

For the measurement of liposome adhesion, the quartz was mounted into a flow cell in such a way that only one side of the slide was exposed to the liquid. The used measuring cell had a small reaction volume (32.4 $mm^3$, width 6 mm, length 12 mm and depth 0.45 mm). All adsorption experiments were performed under constant lamellar flow conditions (200 μl/min) with a shear rate of ~17.5 $s^{-1}$. Liposomes were suspended in PBS to a final phospholipid concentration of 1.33 mM and injected into the system. After an equilibrium of adhesion was reached (i.e., no further frequency shift was detectable), the sensor surface was flushed with PBS under the same shear force conditions (17.5 $s^{-1}$) to test for desorption of liposomes. All measurements were repeated at least six times.

### 2.5. Atomic force microscopy (AFM)

AFM measurements were performed with a Nanoscope IV Bioscope (Veeco Instruments, Santa Barbara, CA). The microscope was vibration damped and installed in an acoustically isolated container. Commercially available pyramidal $Si_3N_4$ tips (NCH-W, Digital Instruments, Santa Barbara, CA) on a cantilever with a length of 125 μm were used. All investigations were performed in tapping mode. The resonance frequency was approximately 220 kHz and the nominal force constant was 36 N/m. The force was always adjusted to a minimum, typically below 1 nN. The scan speed was proportional to the scan size, with a scan frequency between 0.5 and 1.5 Hz. Images were obtained by displaying the amplitude signal of the cantilever in the trace direction and the height signal in retraced direction, both signals being simultaneously recorded. The results were visualized either in height or in amplitude mode. All samples were examined within 1 h after preparation.

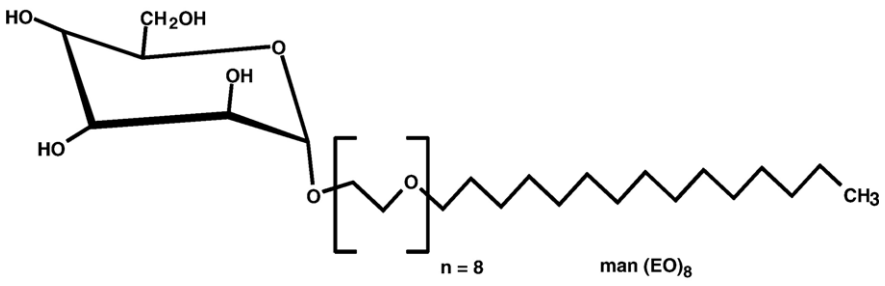


Fig. 2. Structure of the synthetic n-hexadecyl-α-mannoside. Adhesion experiments in this study were performed with lipids containing eight oxyethylene units (man $(OE)_8$).

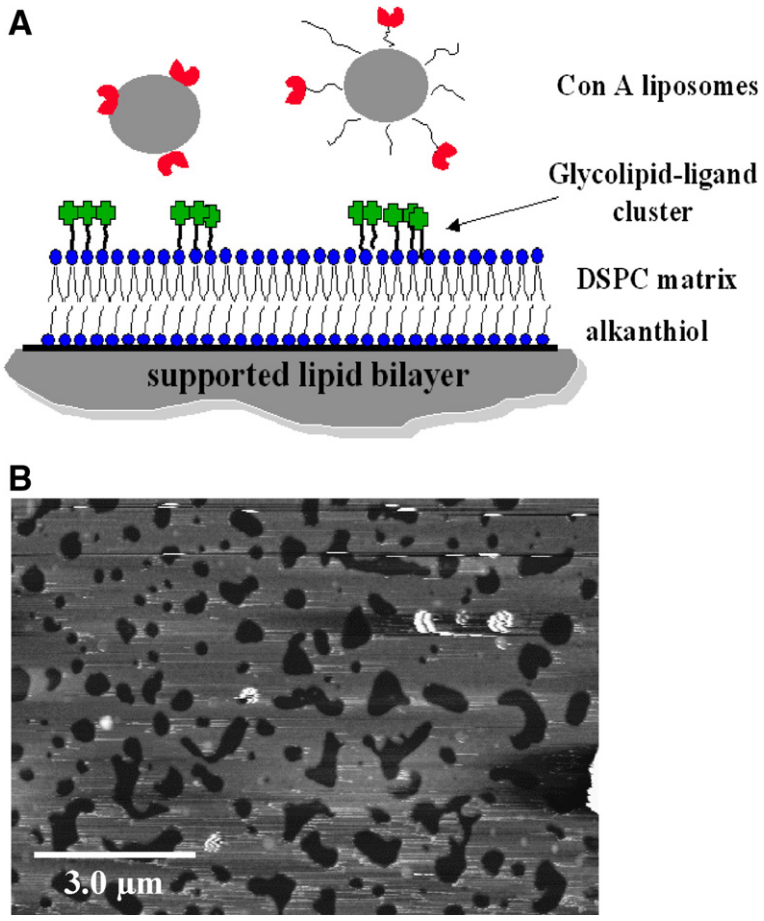


Fig. 3. (A) Schematic of liposome adhesion onto the carbohydrate–lipid–ligand model membrane. (B) AFM image of a transferred model membrane.

### 2.6. Size measurements

Size of the liposomes was determined by photon correlation spectroscopy (PCS) on an ALV 5000 system (Laser Vertriebsgesellschaft mbH, Langen, Germany) at a scattering angle of 90° (sampling time 200 s). Auto correlation was performed using the "contin" method. PCS gives information of the mean diameter of the bulk population (z-average) and the width of distribution via the polydispersity index (PI). For PCS measurements, all samples were diluted 50-fold in distilled water to obtain comparable viscosities. All measurements were carried out at a temperature of 25 °C.

In addition, size and morphology of the liposomes were measured by AFM. Between fifty and one hundred vesicles of each sample were counted manually and measured using the height-mode of the microscope.

## 3. Results and discussion

### 3.1. Characteristics of the model membranes

The model membranes were prepared by mixing synthetic α-D-mannopyraniside and DSPC (10 mol% ligand concentration),

Table 1
Size determination of the different liposomal formulations

| Liposomal formulation | Unmodified liposomes | | After ConA-modification | |
|---|---|---|---|---|
| | PCS diameter [nm] (PI) | AFM diameter [nm] mean±S.D. | PCS diameter [nm] (PI) | AFM diameter [nm] mean±S.D. |
| DPPC/cholesterol 2:1 | 221 (0.157) | 251±18 | N/A | N/A |
| DPPC/cholesterol 2:1+10 mol% $PEG_{2000}$-PE | 246 (0.232) | 254±16 | N/A | N/A |
| DPPC/cholesterol 2:1+cyanur-DSPE 2.5 mol% | 234 (0.187) | 283±27 | 252 (0.344) | 289±31 |
| DPPC/cholesterol 2:1+cyanur-DSPE 5 mol% | 249 (0.256) | 268±26 | 278 (0.421) | 395±37 |
| DPPC/cholesterol 2:1+cyanur-DSPE 10 mol% | 256 (0.208) | 279±29 | 266 (0.349) | 372±44 |
| DPPC/cholesterol 2:1+cyanur-$PEG_{2000}$-PE 2.5 mol% | 207 (0.152) | 251±22 | 231 (0.321) | 271±19 |
| DPPC/cholesterol 2:1+cyanur-$PEG_{2000}$-PE 5 mol% | 235 (0.311) | 244±19 | 227 (0.419) | 273±25 |
| DPPC/cholesterol 2:1+cyanur-$PEG_{2000}$-PE 10 mol% | 232 (0.215) | 217±12 | 257 (0.342) | 256±27 |

Data represent mean±S.D. ($n$=6) in the case of AFM studies and mean (polydispersity index, PI) of $n$=6 experiments in the case of PCS measurements.

followed by pre-organization at the air/water interface. The film was transferred to the thiolated gold-sputtered quartz sensor using the Langmuir–Blodgett technique and the correct deposition of the second glycolipid monolayer could be directly recorded by QCM. Assuming a mean molecular weight of 700 g/mol and a mean molecular area of 0.7 $nm^2$, a theoretical frequency shift of 38 Hz can be calculated for the absorption of such a monolayer. The experimental values obtained by QCM were in the range between 36 Hz and 43 Hz.

The films were thermodynamically stable and their morphology independent of the applied shear forces during the adhesion experiments. In Fig. 3, an AFM image of a typical model membrane is shown. The matrix lipid, DSPC, formed a condensed monofilm whereas the glycolipid ligand, which was phase-separated within this film, showed liquid expanded film behavior. Carbohydrate-rich clusters sized between 0.2 μm and 2.5 μm were of fractal shape and homogeneously distributed allover the film. In previously published studies by our group, it was shown that a concentration of glycolipid ligand of 10% within a model membrane was sufficient to achieve saturation of surface adhesion [31,32].

### 3.2. Size measurements

Four different liposomal formulations were tested in this study: plain (i.e., unmodified) DPPC/cholesterol liposomes; sterically-stabilized (i.e., PEGylated) DPPC/cholesterol liposomes; ConA-modified DPPC/cholesterol liposome with directly coupled lectin and PEGylated ConA-modified DPPC/cholesterol liposomes with the lectin covalently attached to the distal end of the PEG-spacer. The size of the liposomes was determined by PCS and AFM measurements. Prior to ConA modification, sizes of 234–256 nm (DPPC/cholesterol/cyanur-DSPE) and 207–235 nm (DPPC/cholesterol/cyanur-$PEG_{2000}$-PE) were measured by PCS. After the coupling reaction, sizes of liposomes increased by approximately 20 nm for all samples (Table 1).

The results of size measurement by AFM showed a similar rank order, but were always ~15% higher compared

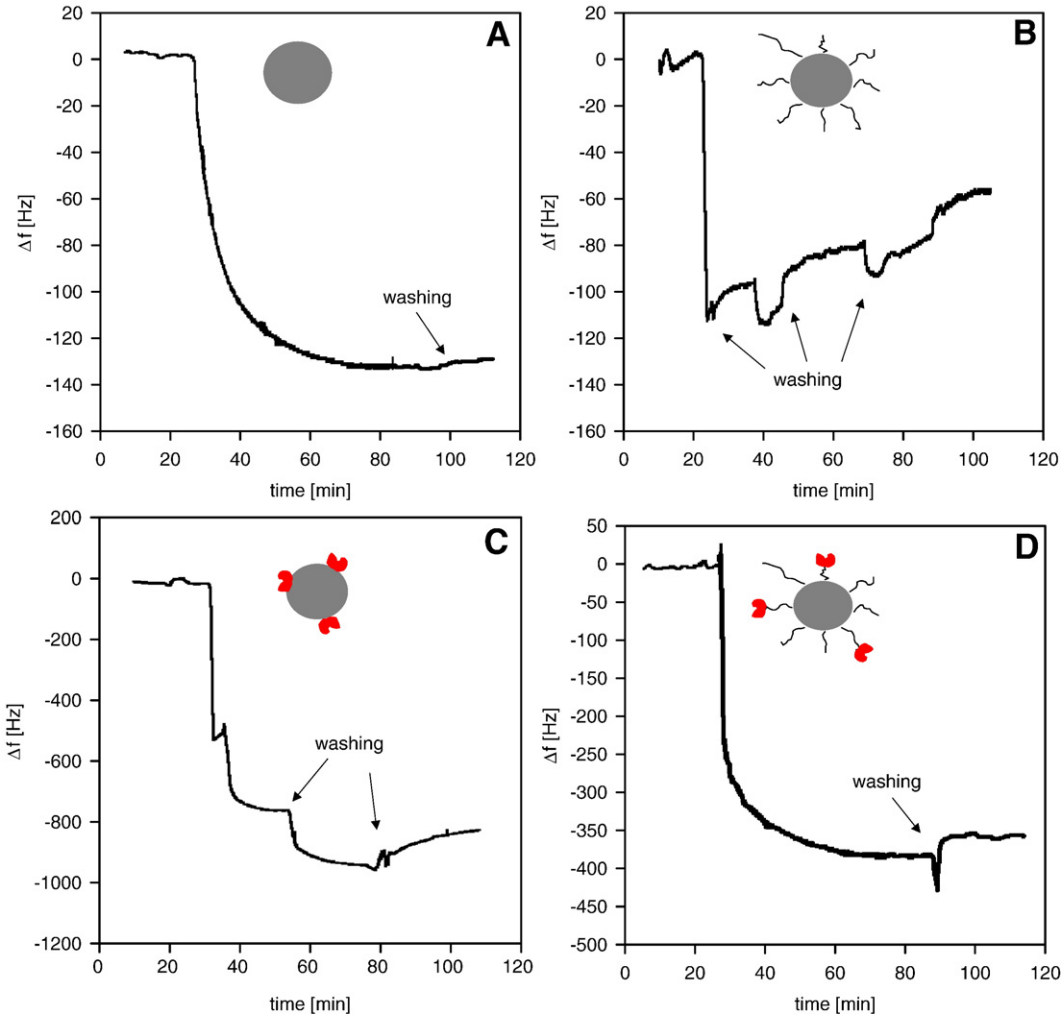


Fig. 4. QCM measurements after adhesion of different liposomal formulations onto the model membrane. (A) unmodified plain liposomes (DPPC/cholesterol 2:1). (B) PEGylated liposomes (DPPC/cholesterol/PEG-PE). (C) ConA-modified liposomes, the lectin directly coupled to the liposomal surface (DPPC/cholesterol/cyanur-DSPE). (D) ConA-modified liposomes, the lectin coupled to the distal ends of PEG-spacers (DPPC/cholesterol/cyanur-PEG-PE).

to the results obtained by PCS (Table 1). While the PCS method works in suspension, AFM samples are always attached to an appropriate support (e.g., silicon wafer). Due to the occurring adsorption, flexible specimens, such as liposomes might flatten and appear slightly larger. These observations are in agreement with previously published data by Jass et al. [37].

### 3.3. Adhesion studies using QCM

The QCM technique was used to study the unspecific/specific adhesion of various liposomes on supported carbohydrate ligand containing model membranes. In Fig. 4, typical frequency shift vs. time curves of liposomal adhesion is shown. After injection of the liposomes, a fast decrease in frequency could be observed. After 15 to 50 min, depending on the composition of the liposomes, a quasi steady state of adhesion was reached.

For plain liposomes (Fig. 4A) a frequency shift of 124± 18 Hz (equivalent to 154 ng adhered mass according to Eq. (1)) were observed. The following washing step with buffer resulted in only a slight increase in frequency (~10 Hz), which was indicative for a stable lipid adhesion on the sensor, likely due to spreading and subsequent fusion of the liposomes. When the shear forces were increased to 70 $s^{-1}$, a further increase in frequency could be detected as a result of the destruction of the model membrane on the sensor surface by the high mechanical stress at the interface (data not shown). Sterical stabilization of the liposomes by incorporation of $PEG_{2000}$-PE led to a decrease in both unspecific adhesion and coalescence. The QCM curve showed rapid decrease in frequency of about 100 Hz (Fig. 4B). The PEGylated liposomes formed a very sensitive layer on the sensor surface. After two washing steps, a final frequency shift of ~40 Hz was measured, suggesting at least partial reversibility of binding. The direct linkage of ConA to the liposomal surface via activated cyanur-DSPE resulted in a high decrease in frequency of 927±125 Hz (equivalent to 1152 ng adhered mass) which was followed by fusion of the liposomes (Fig. 4C). A comparably high frequency shift (1258 Hz) was reported by Liebau and colleagues in their study [27,28] using different liposomal systems. In our experiments, the curve showed a rapid decrease in frequency during the adhesion phase (~800 Hz). After the first washing step, a further decrease in frequency was observed (~200 Hz). Additional washing resulted in an increase of frequency (between 50 Hz and 100 Hz). These changes in frequency can be discussed as a re-organization process of the liposomes on the model membrane surface: Following the injection, liposomes adhere tightly packed onto the sensor surface, keeping their spherical shape. As a result of the first washing step, flattening, spreading and fusion occur, resulting in a densely packed surface consisting of intact liposomes, surrounded by lamellar lipid layers. The higher rigidity of this structure might be responsible for the observed initial decrease in frequency. A comparable effect was observed by Keller and Kasemo [26]. They reported that the formation of a tightly packed liposome layer led to a higher viscosity of the attached film, which caused a decrease in frequency. PEGylated lectin-modified liposomes (i.e., ConA bound to a PEG-linker) showed high specific adhesion and no fusion with a maximal frequency shift of 320±65 Hz (Fig. 4D). The decrease in frequency, however, was significantly lower as observed for liposomes with directly coupled lectin. This effect might be attributed to shielding of the ConA by the PEG chains. Referring to our experiments, the incorporation of PEG lipids could, in addition to changing electrostatic repulsion, suppress unspecific adhesion events.

The lectin-mediated adhesion could be inhibited in competition experiments, when free carbohydrate (methylmannoside) was added to the liposome suspension prior to incubation with the glycolipid film. In those experiments, only a negligible unspecific adhesion of about 35 Hz could be observed for PEGylated lectin-modified liposomes.

The frequency shift was also found to be dependent on the amount of the cyanur-activated lipid within the liposomes (Fig. 5). Higher levels of the respective cyanur compound in the liposomal formulation led to an increase of adhered mass. This increase, however, was not found to be statistically significant.

### 3.4. Morphological studies using AFM

Morphology changes of the adhered liposomes were characterized by AFM, since this technique allows measurements under physiological conditions, without pre-treatment (e.g., staining or fixation) and with almost molecular resolution (Fig. 6). Unmodified liposomes showed a tendency to spread and coalesce on the model membrane surface (Fig. 6A). The insert shows a single liposome at higher magnification. The effect of liposome spreading was previously investigated by Jass et al. [37]. For drug carrier systems, such spreading is clearly undesired, but represents an elegant method for the preparation

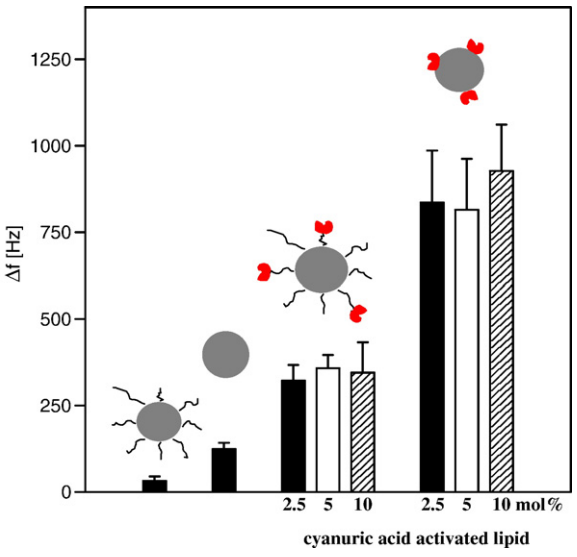


Fig. 5. Effect of ConA concentration on the liposomal adhesion measured by QMC. From left to right (i) PEGylated liposomes, (ii) unmodified plain liposomes, (iii) ConA-modified liposomes (DPPC/cholesterol/cyanur-PEG-PE), (iv) ConA-modified liposomes DPPC/cholesterol/cyanur-DSPE).

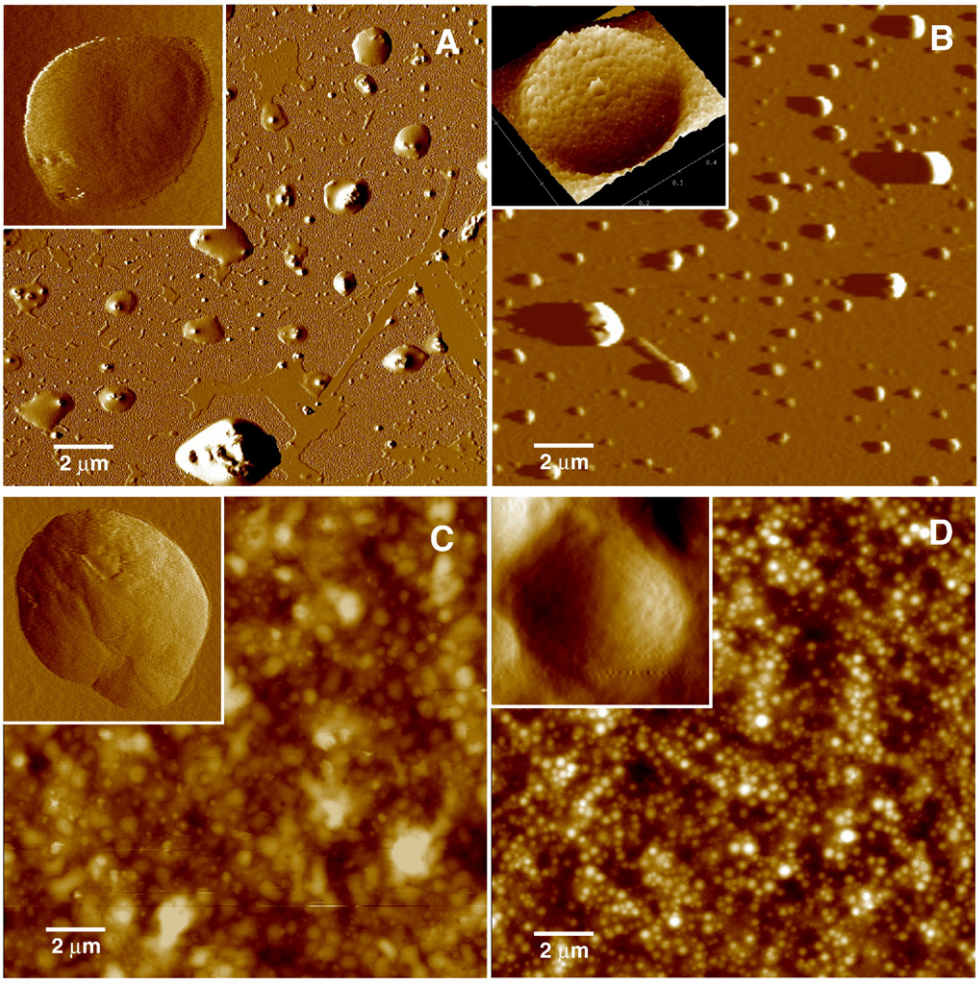


Fig. 6. AFM images of (A) unmodified liposomes (B) PEGylated liposomes, (C) ConA-modified liposomes (DPPC/cholesterol/cyanur-DSPE), (D) ConA-modified liposomes (DPPC/cholesterol/cyanur-PEG-PE). All experiments were performed after equilibration and washing of the quartz surface in tapping mode at 60% relative humidity.

of supported membranes of various compositions. In Fig. 6B, the sterically-stabilized PEG-liposomes are shown. Only few liposomes can be observed on the surface. These liposomes show a rounded morphology and a slightly rougher surface (see insert) compared to unmodified liposomes. The increase in surface roughness is presumably caused by the PEG chains. In the case of un-PEGylated ConA-modified liposomes (Fig. 6C), a densely packed lipid layer can be observed. These liposomes have coalesced and areas of lamellar lipid organization are present on the sensor surface, while PEGylated ConA-modified liposomes still appear round-shaped with no obvious spreading or fusion (Fig. 6D).

## 4. Conclusions

Here, we demonstrated that unmodified DPPC/cholesterol liposomes and ConA-conjugated liposomes adhered to glycolipid membranes at different rates. This adhesion was followed by spreading and fusion of the vesicles. PEGylation of the liposomes abolished unspecific adhesion and, perhaps more importantly, coalescence. The covalent attachment of lectins to the distal end of a PEGylated lipid resulted in stable liposomal carriers with high binding specificity. Both properties are essential pre-requisites for the development of long circulating and site specific drug delivery systems. Moreover, we could establish that quartz crystal microbalance and atomic force microscopy are powerful and complementary methods to characterize adhesion properties of targeted drug delivery systems. QCM allows to follow-up and quantify adhesion in real-time, under physiologic flow conditions and AFM is an excellent method to investigate the morphology of nano-sized drug carriers.

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