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A liposome-based model system for the simulation of lectin-induced cell adhesion

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Abstract

A parallel plate flow chamber with defined wall shear rates was developed in order to study and simulate cellular adhesion to biological membranes as mediated by lectin/carbohydrate interactions. Planar bilayers containing clustered areas of various long-chain alkyl mannosides as carbohydrate ligands and supported on transparent materials were used as model membranes. Their interaction with liposomes bearing Concanavalin A as model cells was observed fluorimetrically by confocal laser scanning microscopy. The use of supported membranes made it possible to study the dependence of adhesion upon different physicochemical parameters of membranes. The liposomes of this model were able to simulate the lectin-mediated adhesion of cells in a shear flow. Once specific receptor-mediated adhesion had taken place, liposomes tended to attach irreversibly to the membrane. This could be avoided by employing lipid compositions which represent a special balance between charged and polyethylene glycol-coupled lipids. This is discussed in term of the interplay between the various attractive and repulsive forces at membrane surfaces. The dependence of liposome adhesion upon the shear rate could be detected. These results were used to evaluate binding forces between lectin-bearing liposomes and ligand-containing planar bilayers.

Keywords: Liposome; Supported planar bilayer; Adhesion model; Concanavalin A

Abbreviations: BCA, bicinchoninic acid; Con A, concanavalin A; DPPE, 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine; DPPG, 1,2-dipalmitoyl-*sn*-glycero-3-phosphoglycerol; DSPC, 1,2-distearoyl-*sn*-glycero-3-phosphocholine; EDC, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide; FITC, fluoresceinisothiocyanate; LUV, large unilamellar vesicles; mannoside 0, 1-*O*-hexadecyl-α-D-mannopyranoside; mannoside III, 1-*O*-(3,6,9-Trioxapentacosyl)-α-D-mannopyranoside; mannoside VIII, 1-O-(3,6,9,12,15,18,21,24-octaoxatetracontyl)-α-D-mannopyranoside; NBD-PC, 1-palmitoyl-2-[12-((7-nitro-2-1,3-benzoxadiazol-4-yl)amino)dodecanoyl]-*sn*-glycero-3-phosphocholine; NBD-PE, 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(7-nitro-2-1,3-benzoxadiazol-4-yl); PEG, polyethylene glycol; SPC, soy phosphatidylcholine

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1. Introduction

Receptor-mediated adhesion of cells to biological membranes in a wall shear field plays an important role in many biological processes. For example, lymphocyte adhesion to the endothelial surface of inflamed tissue during the first response in immunity results from the heterophilic interaction between various receptors and their corresponding ligands on both leucocyte and endothelial surfaces [1]. Of these receptors, the selectins, a family of three carbohydrate binding proteins, mediate the initial adhesion events which results in leucocyte rolling along the vessel wall, followed by leucocyte sticking, which is mediated by the family of integrins [2,3]. Their central role in the adhesion cascade of immune response makes selectins attractive targets for pharmacological and pharmaceutical research. As predicted by analogy with the structure and function of other lectins, selectins bind soluble monovalent carbohydrate ligands with low affinity [4]. Multiple protein-carbohydrate interactions are therefore thought to be the basis of the necessary functional affinity [5]. However, the complex nature of leucocyte adhesion makes it difficult to investigate the function of selectin-carbohydrate interaction and their contribution to adhesion separately.

The aim of this work has been to perform basic experiments on cell adhesion in a flow field using a defined system in which cells are modelled by lectin-bearing liposomes which interact with a ligand-containing membrane fixed to a support. The direct analysis of binding events in this model should illuminate the molecular basis of carbohydrate-induced cell adhesion. The transfer of these results to the process of leucocytes adhesion to inflamed tissue should help us understand and influence these interactions in later experiments, for instance by inhibiting such adhesion or by targeting binding components with drug.

Various groups have performed model experiments to determine the critical parameters of receptor-mediated cell adhesion under flow conditions [6,7], in some cases using lectin-carbohydrate bindings [8]. It could be shown that ligand and receptor density and mobility on the surfaces in combination with the shear forces are the main parameters of adhesion events. Wattenbarger et al. [9] demonstrated the dependence of liposome adhesion on ligand concentration and fluid force in a flow chamber study using glycophorin liposomes and surface immobilized wheat germ agglutinin. In all these experiments, one binding component was attached to solid materials to simulate the conditions on membrane surfaces. Other model experiments were performed to investigate leucocyte adhesion and rolling. Lawrence and Springer [10] thus investigated neutrophil adhesive behaviour on selectin-coated surfaces in viscous shear flow.

We have developed a model system for adhesion consisting of receptor-containing liposomes as model cells and a supported planar bilayer containing carbohydrate ligands as the complementary components. Advantages of our supported bilayer system are their similarity to natural membranes, their long term stability, and their physical dimensions. The Langmuir-Blodgett technique can be employed to prepare supported bilayers in which lipid fluidity, ligand concentration and lateral ligand organization are varied to mimic natural conditions. A lateral ligand concentration is necessary when investigating low-affinity binding events to prove multivalent binding [5].

The advantage of liposomes as model cells lies in their ready and defined preparation, and the possibility of varying the lipid composition to demonstrate the dependence of adhesion on different parameters. The plant lectin concanavalin A (Con A) was choosen as a model receptor, and was coupled (by covalent linkage to a hydrophobic anchor) to the outer surface of preformed large unilamellar vesicles (LUV). Con A is the most extensively studied member of the lectin family. The crystal structure of Con A and Con A-carbohydrate complexes are known, as well as its binding characteristics [11,12]. Furthermore, the binding affinity of Con A to its monosaccharide ligands is similar to the affinity of selectins for their model ligand Sialyl Lewis^x [13]. The use of Con A coupled to liposomes enables the binding characteristics to be determined independently of any contribution from other receptor systems or cytoskeleton. In view of the selective recognition of α-D-mannose, long-chain alkyl mannosides incorporated into the supported bilayers were used as ligands. Our experiments were performed in a parallel plate flow chamber, where under well defined shear forces receptor bearing liposomes were driven along a ligand-containing mem-

brane by a hydrodynamic drag in a temperature-controlled manner. The use of transparent support materials permits the direct fluorimetric determination of binding events by means of an inverted confocal laser scanning microscope. The described characteristics of our model system should enable detection of the interplay between specific receptor-mediated cell adhesion and basic physicochemical parameters (phase state of the matrix lipids, surface charge density, steric repulsion and lateral ligand organisation) of the model membrane.

2. Materials and methods

2.1. Materials

Soy phosphatidylcholine (SPC) was obtained from Lucas Meyer (Hamburg, Germany). 1,2-Distearoyl-*sn*-glycero-3-phosphocholine (DSPC), 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine (DPPE), cholesterol, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), glutaric-anhydride, α-methyl-mannoside, Concanavalin A and FITC Con A were from Sigma (Deisenhofen, Germany). 1,2-Dipalmitoyl-*sn*-glycero-3-phosphoglycerol (DPPG) was a gift from Lipoid KG Ludwigshafen (Germany). 1,2-Dipalmitoyl-*sn*-glycero-3-phosphoethanolamine-N-(7-nitro-2-1,3-benzoxadiazol-4-yl) (NBD-PE), 1-palmitoyl-2-[12-[(7-nitro-2-1-,3 benzoxadiazol-4-yl)amino]dodecanoyl]-*sn*-glycero-3-phosphocholine (NBD-PC) and polyethylene glycol-PE (PEG-PE 2000) were purchased from Avanti Polar Lipids (Alabaster, AL, USA). The substances were used without further purification. The used alkyl mannosides were synthesized in the institute according to Ogawa [14]. All salts and buffers were of analytical grade.

2.2. Vesicle preparation

LUV were prepared by extruding multilamellar vesicles. For this purpose, a lipid film (15 μmol) was suspended in 1 ml 0.15 M NaCl at 60°C. The resulting multilamellar vesicles were extruded six times (Extruder, Lipex Biomembrane, Vancouver, Canada) through a 400 nm polycarbonate membrane (Costar, Bodenheim, Germany). Vesicle size was determined by dynamic light scattering using a Malvern Autosizer II c (Malvern, UK) in mass distribution mode. The lipid composition of vesicles was varied according to the experiments. The basic composition (molar ratio) was: SPC:Chol:N-glutaryl-PE 6:3:1; whereby the addition of 1–10 mol% DPPG diminished the cholesterol content, and the addition of 0.2–4 mol% PEG-PE diminished the fraction of SPC. Additionally, for the purpose of fluorimetric characterization, 1 mol% of the fluorescence marker NBD-PE was incorporated in all preparations.

2.3. Lectin linkage to vesicle surfaces

The lipid N-glutaryl-PE was used as hydrophobic anchor for Con A in the liposome membrane. N-glutaryl-PE was synthesized according to [15] and incorporated in vesicles with 10 mol% for all preparations. To form the protein linkage, 15 μmol vesicles were adjusted at pH 3.5 (0.01 N HCl), and 8 mg EDC were added. After a short incubation period, 92 nmol Con A in 1 ml 0.1M $Na_2B_4O_8$ solution was added. The lectin coupled liposomes were separated from unbound lectin by gel permeation chromatography using Sepharose 4B (Pharmacia, Uppsala, Sweden) eluted with 0.1 M $Na_2B_4O_8$. The coupling yield of approximately 45% was quantified by protein determination using bicinchoninic acid (BCA protein assay reagent, Pierce, USA).

2.4. Preparation of lipid-coated particles

Monodisperse spherical melamin particles (400 nm diameter) were purchased from Aeres GmbH Berlin (Germany). After activation with 200 mM cyanure chloride in dioxane for 6 h at room temperature, particles were washed and then coupled to the headgroups of DPPE to get a monolayer (about 3 mg DPPE/g particle). The bilayer was completed by fusing Con A-liposomes (Section 2.3) to the particle surface in a sonification bath for 30 minutes at room temperature. Non fused liposomes were removed by centrifugation.

2.5. Preparation of supported planar bilayers

Supported planar bilayers were prepared by the Langmuir-technique. Microscope slides (glass, diam-

eter of 18 mm, thickness of 0.3 mm) were used as transparent supports. Slides were first cleaned to achieve a highly homogenous surface. Therefore, slides were treated with conc. H_2SO_4/H_2O_2-mixture (7/3) at 80°C for 30 min under ultrasonic conditions, and were then rinsed with ultrapure water for 30 minutes. To get a homogeneous and highly purified glass surface, a cleaning procedure with $NH_3/H_2O_2/H_2O$ (1/1/5) then followed, before finally rinsing with ultrapure water and drying the slides. The first step in forming a supported bilayer is the covalent binding of monochlordimethyloctadecyl silane (Sigma, Deisenhofen, Germany) at 50°C for 30 min to produce a first monolayer. The bilayer was completed by transfering a second lipid layer using the Langmuir-Blodgett technique, as described below.

For the experiments on the air/water interface, the lipid mixture of DSPC:alkyl mannoside (9:1 molar ratio) were dissolved in freshly destilled chloroform:methanol (2:1/v:v) at 40 to 60 mM. After an equilibrium period of 10 minutes, compression of the monomolecular film at the air/water interface commenced at a velocity of 0.045 cm s^{-1}. The lipid monolayer was transferred at a lateral pressure of 38 mN/m and a speed of 0.5 mm/min by dipping the hydrophobic substrate through the interface from air to water. The transfer ratios were between 0.9 and 0.95. Freshly prepared supported bilayers were immediately used for experiments in the flow chamber. The water used for the film balance subphase was of Milli Q (Millipore, Eschborn, Germany) quality.

To measure simultaneously the isotherms and domain growth, the trough was integrated within a fluorescence microscope (Olympus, Japan) with a motor driven xy stage, using the dye NBD-PC at 0.5 mol%, that preferentially partitioned into lipids of disordered liquid-expanded (LE) phase.

2.6. Flow chamber

Fig. 1 shows schematically the laminar flow chamber used in this study. The device was constructed from polyacrylate. The flow chamber was cut into the bottom to form a channel of 6 mm width, 12 mm length and 0.45 mm depth to guarantee flow laminarity. The chamber bottom was closed with a microscope slide of 18 mm diameter bearing a supported

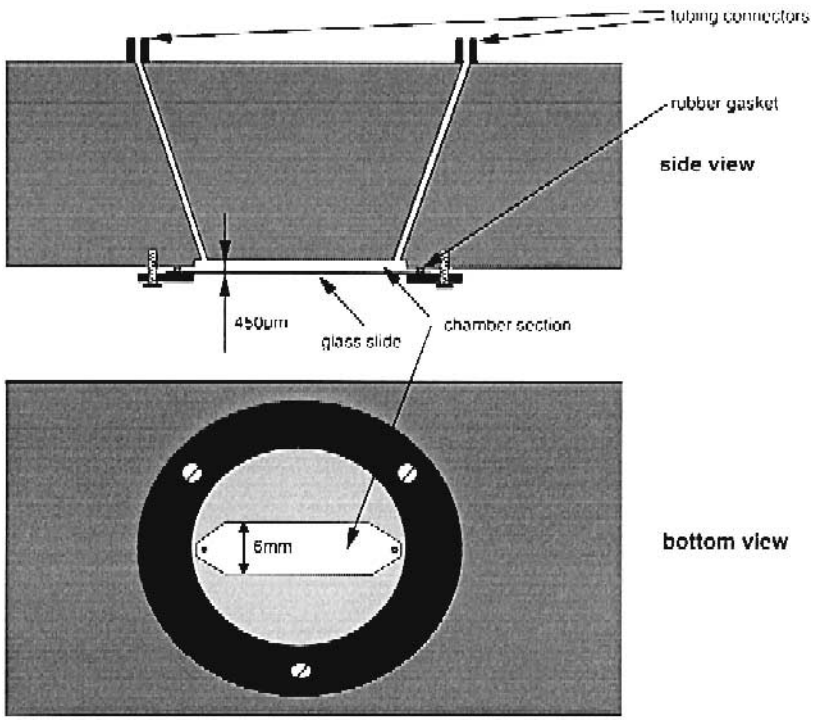

Fig. 1. Schematic presentation of the parallel plate flow chamber.

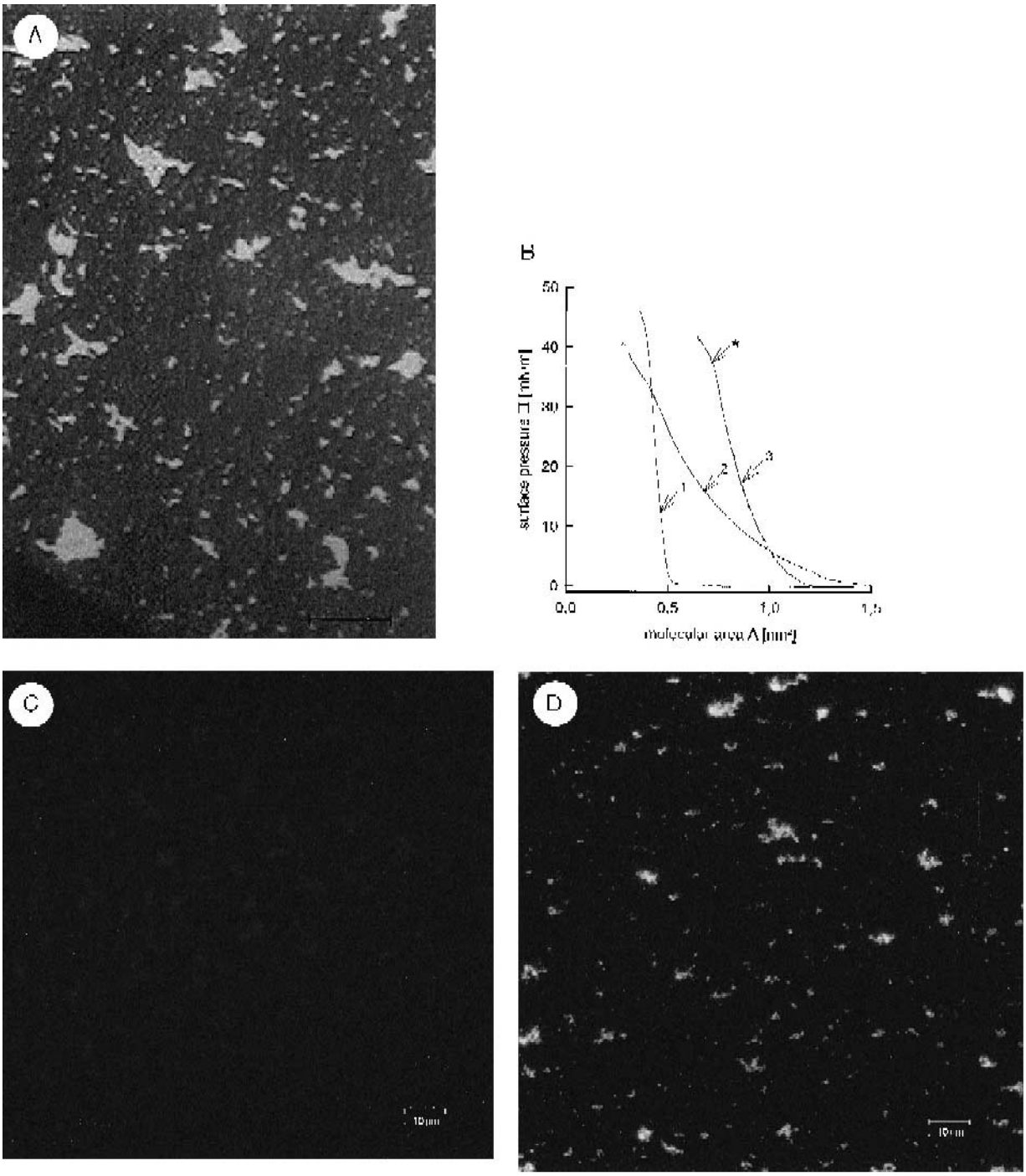

Fig. 2. Comparison of the mannoside containing DSPC-monolayer at air water interface (A) and after transfer to a solid support (C,D). A: Fluorescently marked distribution of 10 mol% mannoside III in a DSPC-monolayer at a Langmuir trough at lateral pressure of 38 mN/m, as indicated in (B). Bar represents 10 μm. B: Comparison of the isotherms of DSPC, mannoside III and their mixture (9:1 molar ratio). Symbol * indicates the lateral pressure where the lipid film was transferred. C: Microscopic view on a slide-transferred DSPC:mannoside III-monolayer (9:1 molar ratio) with fluorescently marked mannoside clusters using NBD-PE as marker for fluid phases. The lipid layer was transferred at 38 mN/m. Bar represents 10 μm. D: Interaction of FITC-labelled Con A with mannoside III clusters in a transferred film shown in (C). Bar represents 10 μm.

bilayer, sealed with a rubber gasket and fixed using a slide holder with three screws. The chamber was rinsed with buffer from a reservoir by hydrostatic pressure with flow velocities of 1-9 ml/min corresponding to wall shear rates G of 83 to 747 s^{-1} according to standard formula

$$G = 6Q/la_2 \quad (1)$$

where l and a are the chamber width and thickness respectively, and Q is the flow rate [16].

2.7. Adhesion experiments

Adhesion experiments were performed at 22°C in a temperature controlled manner. Borate buffered solution (0.15 M, pH 8.4) containing $CaCl_2$ and $MnCl_2$ both 0.001 M, was used as flow medium. Most experiments were performed at a shear rate of about 200 s^{-1} which is slightly below the range of shear rates in capillary venules. The adhesion of liposomes from the flowing medium was detected during a period of at least 10 minutes. The system was then rinsed with pure buffer for 10 minutes, after which inhibition experiments using α-methyl mannoside were performed.

2.8. Microscope investigations

Optical investigations were performed with the aid of a Laser Scanning Microscope Carl Zeiss in combination with an inverted fluorescent microscope Axiovert 135 (LSM 410 invert). The NBD-PE marker was excited with laser light of 488 nm, and emission was detected at 520 nm. The confocal principle permitted measurement of the distance of liposomes from the membrane surface in a range of ± 200 nanometers. Liposomes adhering to the surface appeared as fluorescent points, whereas moving liposomes appeared as fluorescent trails according to scan time and vesicle velocity. Since the flow velocity v is dependent on the distance z from the surface (in the near of the wall) according to the formula

$$v = Gz \quad (2)$$

and the length of streaks is proportional to liposome velocity, it was additionally possible to compare the vesicle velocity near the surface with the free flowing vesicles in the medium.

3. Results and discussion

3.1. Characterization of supported planar bilayers

Because standardization and characteristics of the ligand containing supported bilayers are main factors for the adhesion experiments, we started our work analyzing these model membranes. The experiments should lead to optimal conditions for the preparation of highly reproduceable membranes. As a result of the Langmuir-Blodgett technique, a monolayer with special lateral distribution of the mannoside in the DSPC-matrix should be achieved according to temperature and to lateral pressure, which should thereupon be transferred to the solid support. Local clustering of mannosides in the PL-matrix of the resulting supported membrane should be advantageous for cell binding experiments in which the principle of multivalent, low affine single bindings is to be investigated [5]. To exclude any changes in membrane morphology which might result from different mannoside concentrations, the glycolipid concentration was fixed at 10 mol% in all experiments. On the one hand, the monolayers were most reproduceable at this glycolipid concentration, whilst on the other hand, 10 mol% are sufficient for lectin-induced binding according to our liposome agglutination experiments.

Fig. 2A illustrates the fluorescently-labelled lateral distribution of mannoside III in a DSPC matrix at the Langmuir trough at a pressure of 38 mN/m, indicated in isotherme three, shown in Fig. 2B. At this pressure, fluid mannoside clusters became visible in the DSPC-matrix as bright fluorescent areas whose size is about 5-10 μm. The retention of the clustered structure after transfer to the glass surface is shown in Fig. 2C. To confirm the clustered arrangement of mannosides in this transferred films, the interaction of the resulted membrane with FITC-labelled Con A in solution was investigated under flow in the chamber. Fig. 2D demonstrates this: Con A fluorescently marks areas on the membrane which represent the clustered arrangement of mannosides in the DSPC-matrix in a similar size as shown in Fig. 2A,C.

3.2. Liposome adhesion experiments

The proof of functionality of Con A coupled to vesicle surfaces is a prerequesite for our adhesion

experiments. The interaction of our model cells with mannoside-containing liposomes was therefore analyzed. The agglutination of liposomes resulting from Con A/mannoside bindings could be detected by laser light scattering or by turbidity measurements. Turbidity disappeared upon the addition of soluble α-methyl-mannoside which competes for the receptor, which demonstrates the function of Con A even after coupling procedure (data not shown).

Liposome adhesion experiments in the flow chamber were started with liposomes containing Con A whose basis composition was SPC:Chol:N-glutaryl-PE 6:3:1, and a DSPC/mannoside III membrane. Many liposomes adhered strictly to the model membrane, made visible as the fluorescent points. Fig. 3 illustrates the situation at a shear rate of 200 s^{-1}, 10 min after start, when an increase of fluorescent areas was already detectable. Flowing liposomes appeared as fluorescence trails with a length corresponding to their velocity (about 20 to 40 μm/s). Ten minutes after start, the liposome-containing solution was replaced by pure buffer. Nevertheless, liposomes continued to adhere to the membrane surface, but further change in appearence from fluorescent points to larger, less fluorescent areas occured up to 30 minutes after start, illustrated in Fig. 4.

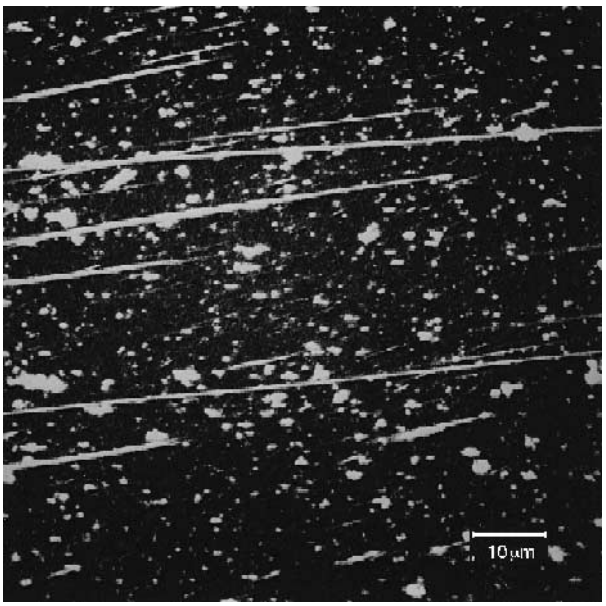

Fig. 3. Interaction of Con A-coupled liposomes (SPC:Chol:N-glutaryl-PE 7:3:1) with a mannoside III containing DSPC supported membrane at a shear rate of 200 s^{-1}, 10 min after start. Adhered vesicles appeared as points, whereas flowing liposomes in the medium are visible as trails. Bar represents 10 μm.

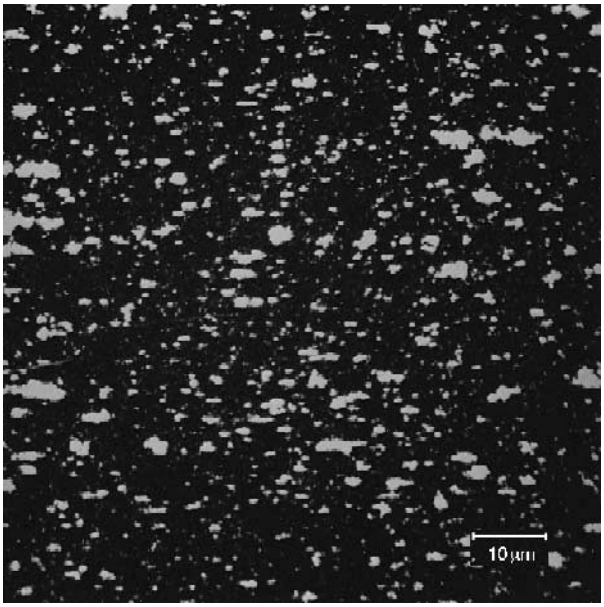

Fig. 4. Adhesion behaviour of liposomes shown in Fig. 3 after 30 min, and rinsing the system with pure buffer at 200 s^{-1}. Liposomes tend to spread on to the surface, indicated by the larger and less fluorescent areas. Bar represents 10 μm.

To demonstrate the specificity of liposome binding via lectin-carbohydrate bonds, their inhibition with α-methyl-mannoside was tested. Despite the high concentration of α-methyl-mannoside (0.1 M), the adhered liposomes could not be detached from the surface, indicating an unspecific kind of adhesion. To determine if these unspecific interactions were initiated by lectin-mannoside bonds, similar experiments with plain SPC/Chol liposomes were performed. These liposomes had no tendency to adhere to the mannoside-containing membrane during the experimental steps.

To further test if adhesion results from unspecific Con A binding, the binding characteristics of Con A-liposomes to a modified model membrane were analyzed. For this purpose, the mannoside III was replaced by mannoside 0, a glycolipid, which was shown in our previous investigations [17] to lack lectin binding capacity owing to the absence of ethoxy units between head group and alkyl chain. Con A-liposomes had only a low affinity for this membrane,

and could easily be removed by washing with buffer (not shown).

In summary, the adhesion of model cells to our membrane in a shear field results from the specific interaction between lectin and carbohydrate. The absence of one binding component prevents adherence. However, within some minutes, specific cell adhesion appears to be covered by unspecific interactions, which cannot be inhibited by antagonists. This behaviour can be interpreted in terms of a spreading or fusion of liposomes onto the planar membrane to create new bilayers, a process which is initiated by the first contact between lectin and mannoside. This postulated mechanism is shown in Fig. 5.

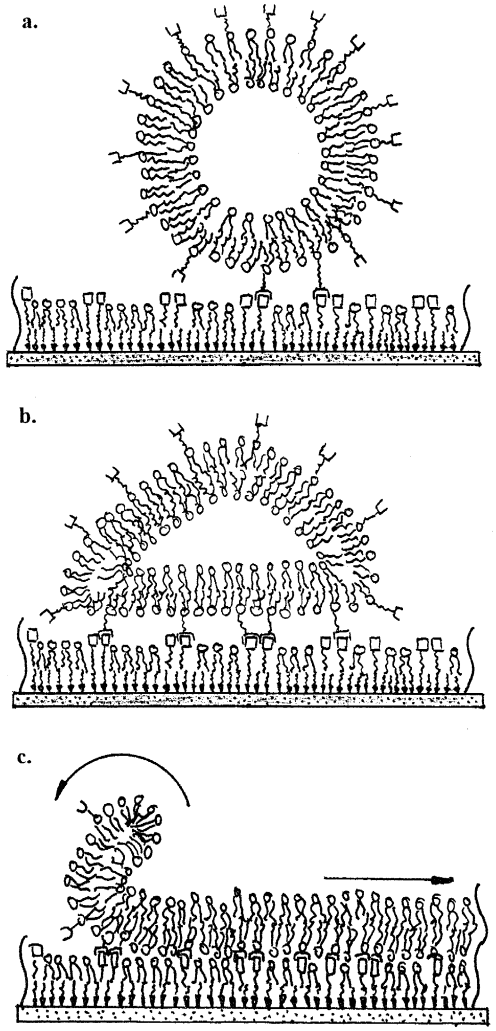

Fig. 5. Postulated mechanism for liposome spreading onto planar hydrophilic surfaces: (a) vesicle adhesion, (b) vesicle spreading, (c) vesicle desintegration.

The fusion or spreading of liposomes onto planar surfaces is a known method of creating supported bilayers [18]. Using this technique, liposome spreading is often initiated by addition of divalent cations. Fluid lipid composition of liposomes are thought to be an essential prerequesite for the fusion process [18]. In an attempt to avoid liposome fusion as an unwanted side effect in these adhesion experiments, we replaced the fluid soy PC by the more rigid hydrogenated soy PC (HSPC). The use of these more rigid Con A liposomes in the adhesion experiments did not prevent the irreversible attachment described for fluid liposomes. Since there was no difference between fluid or rigid liposome, we continued to use fluid SPC for further experiments.

To further investigate if the strong adhesion is caused by multivalent binding of local clustered ligands and receptors and not by fusion process, we replaced liposomes by particles covered with a bilayer analog to the liposome composition. Because the inner monolayer is covalently fixed to the particle, bilayers cannot spread on to our model membrane. Microscopy showed the adhesion of these particles like in the case of liposomes. But, contrary to liposomes, the lipid-like particles could be desorbed by the addition of α-methyl-mannoside. Liposome fusion was therefore confirmed as the most probable side effect of adhesion.

Hydration normally causes a strong repulsive force between electrically neutral bilayers at separations of less than about 20 Å. Regions that bear molecules projecting well out from the bilayer surface (e.g., the coupled Con A) should be the source of smaller separation distances, at which bilayers can adhere and spread upon each other, entering an attractive local free energy minimum because of the effect of interbilayer van der Waals forces [19]. Liposomes of the composition used cannot therefore adequately simulate the receptor-mediated adhesion of cells to membranes. To achieve reversible and defined adhesion events, liposome constituents must be adapted to simulate the composition of biological membranes.

3.3. Effect of charged lipids on cell attachment

Biological membranes have a net negative charge, so that electrostatic repulsion prevents unspecific membrane contacts such as aggregation or fusion.

Electrostatic repulsion is a long-range force that may influence the establishment of short-range interactions. At a bilayer separation greater than 30 Å, repulsion is dominated by electrostatic forces, and the variation of repulsion with bilayer separation and with charge density is well described by electrostatic double-layer theory. We therefore began to incorporate the negatively-charged lipid DPPG into the liposomes and into the model membrane. Whereas the DPPG concentration in the supported bilayer was held constant at 10 mol% (DSPC:DPPG:mannoside III 8:1:1), the DPPG content of liposomes was varied from 10 to 5 to 1 mol%, to detect the dependence of adhesion on charge over a wider range. However, no adhesion events could be detected when these liposomes of various DPPG content were used. Microscope scans demonstrate that these liposomes in the flow medium avoid membrane contact. This means that the incorporation of charged lipids into the binding components prevents unspecific membrane interactions such as fusion, but repulsion in this system is too extensive to permit the formation of low-affinity receptor-ligand interactions.

The use of greater ligands should overcome this problem by shifting the ligand-receptor interactions to a position far from the membrane, so as to achieve a particular balance between charge-induced membrane repulsion and reversible adhesion. We therefore incorporated mannoside VIII instead of mannoside III into the supported membrane. The eight ethoxy units of this compound should position the mannoside headgroup approximately 15 Å above the hydrophilic surface, so that it can interact with the more prominent Con A [12] on the surface at a distance where it is less disturbed by the repulsive barrier properties of opposing membranes. In accordance with our theoretical considerations, ligand elongation indeed results in liposome binding despite the surface charges. We could detect differences in the binding results according to the amount of DPPG in the liposomes. The smaller the DPPG content, the more intensive liposome adhesion occured. Nevertheless, adhering liposomes resist the washing procedure with pure buffer at a shear flow well above 200 s^{-1}.

Fig. 6 illustrates the intensive adhesion of low-charged Con A liposomes (5% DPPG) to the charged membrane which contains mannoside VIII. However, inhibition of these bonds with α-methyl-mannoside

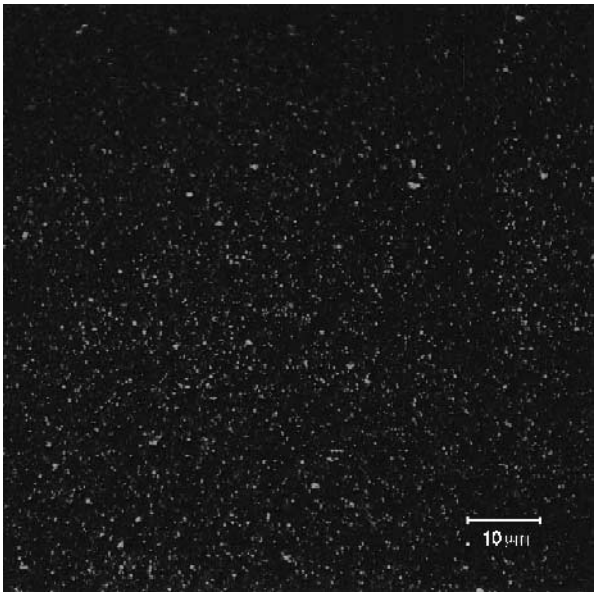

Fig. 6. Adhesion of charged Con A liposomes (5 mol% DPPG) at a model membrane containing mannoside VIII and DPPG at a shear grade of 200 s^{-1}, after washing procedure with pure buffer. Bar represents 10 μm.

was again unsuccessful, which could be attributed to the contribution of unspecific binding. These unspecific interactions should nevertheless contribute to a lesser extent than in the uncharged preparation, because no obvious spreading of liposomes on to the planar surface could be detected in the fluorescence scan in Fig. 6.

Taking together, the incorporation of charged lipids into the binding areas drastically decrease both the specific binding capability of cells and unspecific side effects such as fusion. Nevertheless, the cell binding is possible if larger ligands are used whose binding domains project more prominently from the hydrophilic surface. Receptor-induced adhesion leads to unspecific binding mechanisms which cannot be detected optically, and which are therefore apparently weak. The incorporation of charge is one step towards simulating the behaviour of natural cells.

3.4. Use of lipids derived from polyethylene glycol (PEG lipids)

PEG lipids are widely used as liposome membrane components, because these polymer-lipids cause a

drastic increase in liposome blood circulation half-life in vivo [20]. This extended lifetime appears to result from steric repulsive barrier properties of PEG lipids [21] and from changes in the fixed aquous layer around vesicles [22]. The polymer chain extension from the bilayer and their surface organization could be detected by X-ray studies [23] or Zeta-potential determinations [24,25] with PEG containing liposomes. The organization and extension of PEG lipids from the bilayers depend on the molecular weight and the quantity of the polymer. It could thus be shown that the repulsion between charged liposomes with PEG lipids of higher molecular weight (2000 or 5000) is dominated by steric repulsion at higher pressure and small fluid separations. For example, a PEG lipid with a polymer moiety of 2000 g/mol extends about 50 Å from the liposome surface [24], and a strong repulsive barrier at small fluid separations results at this distance. At large fluid separations in the range of 200 to 600 Å and at low applied pressure, repulsion can be explained solely by electrostastic repulsion [26].

Referring to our experiments, the incorporation of PEG lipids could, in addition to changing electrostatic repulsion, depress the unspecific adhesion events even at small bilayer separations, thus simulating the function of a glycocalix at the surface of natural cells. The hydrophilic vesicle coatings resulting from a PEG 2000 lipid were slightly smaller than the natural glycocalix, thought to lie at approximately 100 Å around the cells. The size of our 'glycocalix' of about 50 Å, should be favourable considering the dimension of a Con A monomer [12] or Con A tetramer as used in our experimentals. Because the extension of the PEG moiety is of the same order as a Con A monomer, the binding ability of Con A at vesicle surfaces to ligands should not generally be suppressed, but close contact between membrane surfaces as initial fusion event should be prevented. PEG lipids were not incorporated into the supported bilayer in view of the relatively small extended mannosides. We incorporated increasing amounts of PEG-lipids into liposomes, (i) to illustrate the steric repulsion of differently-coated surfaces, and (ii) to examine the dependence of protein linkage and activity at the surfaces in the presence of PEG.

It became evident that coupling of Con A at the liposome surface was only slightly suppressed in the presence of 0.5 to 4 mol% PEG-PE (2000). According to the protein determination, PEG-PE decreased the coupling yield by 10-15%, regardless of the four different concentrations. These results deviate from previous investigations by other groups [27,28] of protein interaction at vesicle surfaces which contain different fractions of PEG.

Using the charged (10 mol% DPPG) and PEG-containing liposome populations, no adhesion of liposomes to the charged supported bilayer could be observed. We concluded that the addition of the long range repulsive effects of 10 mol% DPPG on both sides together with the PEG effect of liposomes is to strong for adhesion to occur. To achieve a balance between repulsive and attractive forces, we diminished the fraction of DPPG in the liposomes. As illustrated in Fig. 7, liposomes containing 5 mol% DPPG and 4 mol% PEG PE adhered to the charged supported bilayer. The extent of liposome adhesion is decreased compared to the adhesion of PEG-free preparation in Fig. 6. The decrease of PEG content to 2, 1 or 0.5 mol% did not change the extent of adhesion shown in Fig. 7 for the preparation containing 4 mol% PEG. In further modifications, liposomes

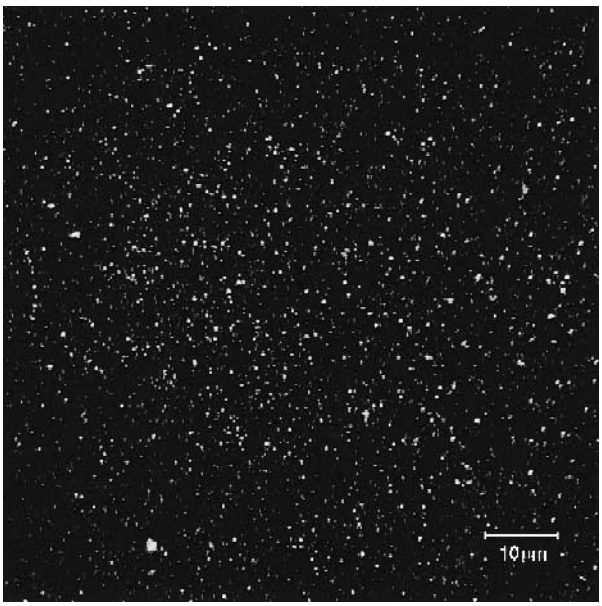

Fig. 7. Adhesion of liposomes which contain a composition balanced between charged (5 mol% DPPG) and PEG lipids (4 mol% PEG 2000) at a charged and mannoside VIII-containing supported membrane at a shear rate of 200 s^{-1}. Bar represents 10 μm.

with 1 mol% DPPG and varied PEG content were investigated, and exhibited similar adhesion behaviour to preparations which contain 5 mol% DPPG. Competition experiments using α-methyl-mannoside should demonstrate a possible effect of PEG lipids in preventing irreversible liposome adhesion. These experiments demonstrate that adhesion of liposomes which contain 4 or 2 mol% PEG-PE is clearly diminished by the soluble antagonist (Fig. 8), whereas the major fraction of liposomes of lower PEG content resist inhibition (not shown). These observations indicate that liposomes which contain a sufficient proportion of PEG lipids (2 or 4 mol%) in the presence of small amounts of charged lipids (≤ 5 mol%) represent a sensitive balance in achieving specific adhesion without unspecific adsorption effects when the shear conditions are near-physiological. Such liposomes can thus mimic the adsorbance of cells on to membrane surfaces. To characterize the adhesion of such liposomes under modified conditions, consecutive microscope scans of preparation with 2 or 4 mol% PEG at different times and higher shear rates were compared. A permanent exchange of adhered vesicles was detected, thus demonstrating the reversibility of binding and their low affinity. At shear rates exceeding 700 s^{-1}, no liposome adhesion could be detected.

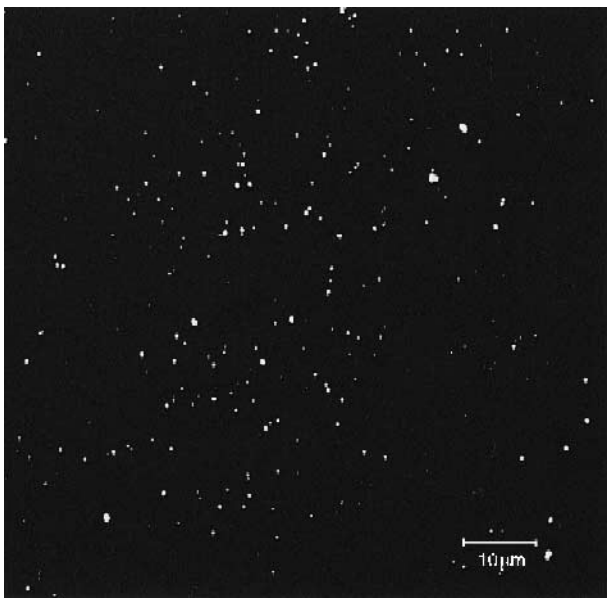

Fig. 8. Inhibition of liposome binding shown in Fig. 7 by added α-methyl-mannoside solution (0.1 M) at a shear rate of 200 s^{-1} during 1 min. The decreased extent of adhered liposomes proves receptor-mediated adhesion of liposomes. Bar represents 10 μm.

A shear force-induced rolling of cells mediated by carbohydrate-lectin bindings, as described for the selectin-controlled leucocyte rolling, results from an equilibrium between shear and binding forces, which was not possible to simulate in our experiments. Therefore, the rapid bond formation and rupture of low affine lectin bonds could not explain the rolling process alone.

Considering the dissociation constant for Con A to soluble ligands in the range of 10^{-4} M [14] and a binding length from 5 to 10 nm, an equilibrium binding force of 10 to 50 pN should be estimated for single receptor-ligand binding events. Contrary, liposomes with a diameter of about 300 nm are applied to a shear force of only about 1 pN under the described conditions in our model system, estimated according to the theory of receptor-mediated cell adhesion to surfaces by Hammer and Lauffenburger [29]. Since the binding force for one Con A-ligand interaction is 10 to 50 times stronger than the force that the passing fluid applies to liposomes, and considering that about 10 to 100 receptors participate simultaneously in liposome binding, a very strong liposome adhesion should result resist all shear conditions.

But at the end of our experimental effort, we could demonstrate a weak and reversible liposome binding. This indicates that a state narrow to the force equilibrium has been achieved, which is required for rolling. Therefore, the combination of charge-induced and sterical repulsion suppressed not only nonspecific adhesion, specific ligand-receptor interactions were weakened too.

Although binding forces as preconditions for rolling are fulfilled, liposomes do not roll along the surface. Therefore, other important factors should mediate a rolling movement, e.g. the presence of highly extended and flexible binding structures as proposed for selectins and their ligands. In context with the increase in ligand size, the elastic properties of cell membranes have to be taken into consideration as described in the theoretical basis of cell rolling [30].

The influence of more extended and flexible ligands and modification of liposome elasticity on liposome rolling should be tested in future studies with this model.

4. Conclusions

This study introduces a model system for the investigation of carbohydrate-induced cell adhesion at biological membranes. A supported planar bilayer containing carbohydrate ligands functions as a model membrane which offers many advantages when modifying conditions to detect the dependence of adhesion upon various physicochemical membrane properties. Liposomes containing the covalently-linked lectin Con A act as model cells in a shear flow which interacts with ligands on the model membrane. A local clustering of ligands in this membrane should support the principle of multivalency of binding.

Reversible binding events of liposomes under certain shear conditions could be achieved following a sensitive balance between receptor-mediated adhesion and steric and electrostatic repulsion. The model could therefore be used to evaluate binding forces and to discuss the influence of repulsive forces on binding strength.

Since liposomes thus modified could adequately mimic carbohydrate-induced cell adhesion, the prerequisites for a receptor-mediated cell rolling at surfaces could be specified.

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