Lessons Learned from Clustering of Fluorinated Glycolipids on Selectin Ligand Function in Cell Rolling[†,‡]

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ABSTRACT: Selectin-induced leukocyte rolling along the endothelium is an essential step in the cellular immune response. Since clustering of binding epitopes is thought to be crucial for selectin−ligand interaction, we focused on requirements of ligand clusters in a flow chamber study. Neoglycolipids bearing the binding epitope sialyl Lewis X (sLeX) were used as artificial ligands in model membranes. sLeX ligands or matrix lipids or both were applied with partially fluorinated alkyl chains to increase the ligand cluster separation tendency. Cluster size, their inner structure, and separation distance were evaluated with high resolution by scanning force microscopy (SFM) and correlated with binding or rolling of E-selectin-expressing cells. Fluorination of only one component, sLeX ligand or matrix lipid, leads to a very high separation tendency and impeded cell rolling, although firm cell adhesion could be observed down to 0.005 mol % ligand concentration. As a sign of total immiscibility, cluster size increased with ligand concentration, and resulting excessive ligand densities within the clusters prevent cell rolling. Fluorination of both sLeX ligands and matrix created small clusters which could serve as rolling patches. Our results confirmed that cluster size and separation distance controlled by a certain miscibility of ligand and matrix as well as a sufficiently diluted ligand concentration within the clusters are necessary for cell rolling. Within this work, selectin ligand clustering and its ability to mediate cell rolling are presented as a balance between multivalency of binding and sufficient flexibility of the single epitopes. This might be helpful for better understanding the function of the natural selectin ligands.

The selectins, a family of three transmembrane glycoproteins (E-, L-, and P-selectin), trigger the emigration of leukocytes from the blood to sites of inflammation by supporting their tethering and rolling along the vessel wall. The underlying principle of rolling is the fast kinetics of selectins interacting with their carbohydrate ligands, which facilitate the rapid association and dissociation of bonds, supported by the impact of shear force. Common minimal binding epitopes of the selectins are the tetrasaccharide sialyl Lewis X (sLeX)[1] and its structural isomers (*1*, *2*). In contrast to natural mucin ligands displaying nanomolar affinity (*3*), the binding affinity of a single sLeX molecule was found to be in the millimolar range (K_D 0.5−1.0 mM) (*4*, *5*). Multivalent arrangement of binding epitopes is thought to explain partly the high affinity of natural ligand binding. The affinity enhancement attained by preparation of rigid multimeric sLeX derivatives complies with this idea (*6*). However, following the structure of physiological ligands, high-affinity binding is rather based on the recognition of a "clustered saccharide patch" (*7*), established by a unique, flexible arrangement (clustering) of sLeX on the ligand peptide backbone.

Cluster formation is a recurring motif in cellular processes. Laterally organized functional microdomains in the plasma membrane, also called rafts, control complex cellular functions such as endocytosis, cell adhesion, and signal transduction. They have a distinct composition and are enriched in sphingolipids, cholesterol, and certain transmembrane or GPI-anchored proteins (*8*−*10*). Several studies demonstrate the significance of clustering for selectin−ligand interaction. Clustering of E-selectin molecules in the vicinity of leukocyte−endothelial cell attachment sites was visualized by confocal microscopy (*11*). E-Selectin is localized in lipid rafts at the cell surface, which is required for signaling during leukocyte−endothelial cell interaction (*12*, *13*). Furthermore, the GPI-anchored molecule CD24, a ligand for P-selectin, is confined to rafts on the cell surface (*14*).

In previous works we established an experimental setup to focus on the molecular binding mechanism of the selectins

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[1] Abbreviations: CHO-E, Chinese hamster ovarial cells containing E-selectin; CLSM, confocal laser scanning microscope; DHPE, 1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; DSPC, 1,2-distearoyl-*sn*-glycero-3-phosphocholine; SFM, scanning force microscopy; sLeX, sialyl Lewis X epitope [α-Neu5Ac-(2→3)-β-D-Gal-(1→4)-[α-L-Fuc-(1→3)]-β-D-GlcNAc-(1→R)].

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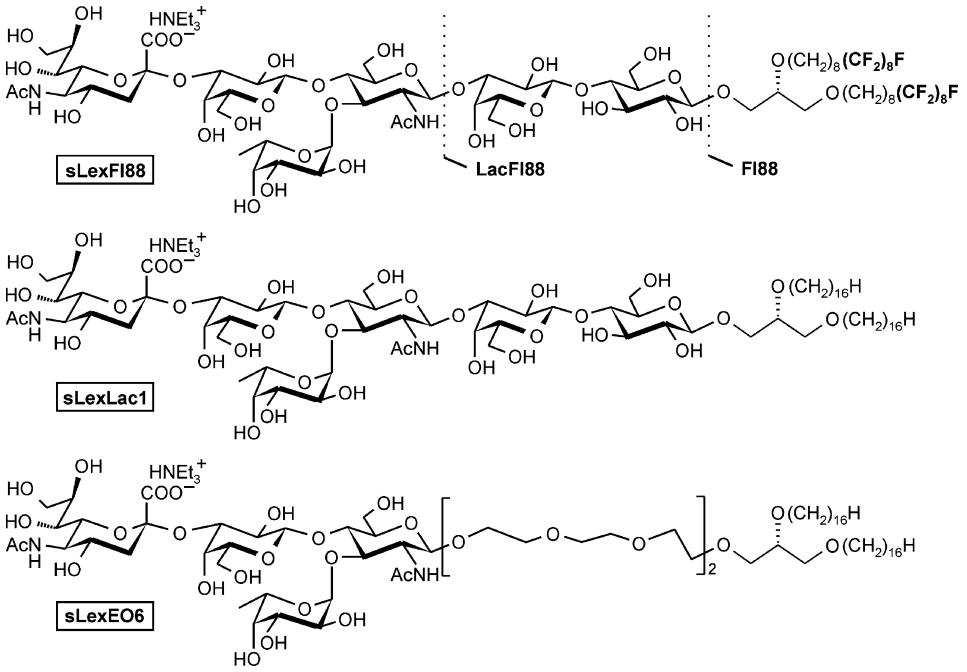


FIGURE 1: Structures of the glycolipids and matrix lipids used in this study. The fluorinated derivative sLexFl88 shows analogy to the nonfluorinated derivative sLexLac1, which has been introduced and compared to sLexEO6 in earlier studies (*17*). The fluorinated matrix lipids LacFl88 and Fl88 differ in the presence of a lactose unit as the headgroup region.

under simulated blood flow conditions. The lateral clustering of the binding epitope sLeX in a model membrane can be correlated with the ability of selectin presenting cells to roll along these membranes. In contrast to other methods creating "immobile" multivalent ligands, our strategy allows for control of the cluster size and distribution by a dynamic accumulation of the sLeX headgroup via self-assembling of each cluster. We could demonstrate that lateral clustering of sLeX glycolipids in a lipid matrix is an essential prerequisite for cell rolling (*15*). Furthermore, we focused on the influence of the mobility and accessibility of sLeX within the clusters (*16*, *17*). Despite the fact that we evaluated the appearance and characteristics of clusters in general, all open questions of selectin ligand functionality could not be answered.

The aim of this study was to get a deeper insight into the functionality of selectin ligands as well as the cluster-induced cell rolling. Using scanning force microscopy (SFM), we analyzed cluster appearance, size, and distance within a model membrane and provided an insight in the inner cluster structure to evaluate ligand density. In addition to previous studies, in which we induced ligand clustering as a result of hydrophobic mismatch, we employed here partially fluorinated lipids for stronger separation. Fluorination increases van der Waals interaction and thus a separation tendency of fluorinated and nonfluorinated molecules for mimicking the natural situation found in rafts.

In recent years, various studies based on the idea of fluorination induced separation (*18*). Liposomes (*19*) and phospholipid bilayers (*20*) were applied in several biomedical approaches. Although we could show in a previous study that sLeX derivatives with partially fluorinated alkyl chains have a strong separation tendency in various alkyl matrices, the impact on cell binding could not be explained nor the importance of the clusters as a selectin rolling ligand patch (*21*). In this study we focus on a detailed visualization of the cluster characteristics by SFM. These parameters were correlated with the functionality of the clusters, i.e., the ability to serve as a rolling ligand patch and discussed to distinguish requirements for cell rolling compared with mere adhesion.

Our findings confirm that clustering has a strong influence on binding avidity. However, cluster formation of sLeX lipids does not always result in a ligand patch for cell rolling in a parallel plate flow chamber. Size, distance, and inner ligand density of the cluster patches appear to be important factors for cell rolling. This emphasizes that rolling is a highly specific kind of cell binding, sensitively balanced by a certain local presentation and concentration of carbohydrate binding epitopes.

EXPERIMENTAL PROCEDURES

Materials. 1,2-Distearoyl-*sn*-glycero-3-phosphocholine (DSPC) was purchased from Sigma (Deisenhofen, Germany). Texas Red dihexadecanoylphosphoethanolamine (DHPE, headgroup-labeled) was purchased from Molecular Probes (Leiden, The Netherlands). The sLeX-based glycolipids and partially fluorinated matrix lipids (Figure 1) were synthesized as previously described (*16*, *21*, *22*). All substances were used without further purification.

Cell Cultivation. E-Selectin-transfected CHO cells (CHO-E cells) of mice were grown in MEM-α containing 10% fetal calf serum, 2 mM L-glutamine, and 100 nM penicillin/streptomycin. Flasks seeded with 5×10^4 CHO-E cells were incubated at 37 °C in 5% CO_2 for 3−4 days to near confluency. After trypsinization for 3 min with 0.25% trypsin/EDTA, the cell suspension was transferred to slowly rotating plastic tubes. The cells remained in suspension for up to 4 h. Within this time, the rolling experiments were performed in the flow chamber.

Preparation of Supported Planar Bilayers. Supported planar bilayers were prepared using the Langmuir−Blodgett technique. Microscopy slides (glass, diameter of 18 mm,

thickness of 0.2 mm) were used as transparent supports. The slides were treated to achieve a highly homogeneous surface as follows: The slides were incubated in a concentrated 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 min. To increase the density of silanole groups at the surface, a cleaning procedure with $NH_3/H_2O_2/H_2O$ (1:1:5) was performed, followed by a final rinse with ultrapure water and drying of the slides. To form supported bilayers, monochlorodimethyloctadecylsilane (Sigma, Deisenhofen, Germany) was bound covalently to the surface of the slides at 50 °C for 30 min, resulting in a hydrophobic monolayer. A film of DSPC or the indicated matrix lipid containing the desired concentration of a glycolipid ligand was preformed on the Langmuir trough. The monolayer on the slides was completed by transferring the X-type monolayer to the hydrophobic substrate at a lateral pressure of 38 mN/m and a speed of 0.5 mm/min. The transfer ratios were between 0.95 and 1. Freshly prepared supported bilayers were immediately used for experiments in the flow chamber or for the visualization with SFM.

Film Balance Experiments. The investigations of the monolayers were carried out on a purpose-built film balance based on a rectangular Teflon trough (area 140 cm^2, 1 cm depth) equipped with a thermostat. The surface pressure was measured using a commercial Wilhelmy balance system (R&K GmbH, Mainz, Germany). For all film balance experiments, the pure glycolipids as well as their mixtures were dissolved in chloroform/methanol [2:1 (v/v)] at a concentration of about 1 mM. The lipid solution was spread on the air/water interface with a microsyringe. The self-assembled lipid films were equilibrated to zero surface pressure for 10 min before the experiments were started. Compression was performed at 25 °C with a speed of 0.01 $nm^2\ min^{-1}\ molecule^{-1}$.

All experiments were performed at 25 °C. Borate buffer (pH 7.4) containing 150 mM NaCl, 10^{-4} M Ca^{2+}, and 10^{-4} M Mg^{2+} was favored as the subphase, as its compatibility with the flow medium in the cell rolling experiments was confirmed previously.

Laminar Flow Experiments. The parallel plate flow chamber used in these studies was described in detail in our previous investigations (*17*). The flow apparatus was mounted onto the inverted fluorescent microscope Axiovert 135 of a laser scanning microscope (LSM 410 invert, Carl Zeiss).

Adhesion experiments were performed at 25 °C in a temperature-controlled environment to maintain the lateral structure of the model membrane. MEM-α was used as flow medium at a shear rate of about 200 s^{-1}, driven by hydrostatic pressure. For the flow experiments, 10^6 fluorescently marked CHO-E cells (calcein AM; Molecular Probes, Leiden, The Netherlands) in 100 μL of medium were injected into the streaming medium. The flow was stopped to allow interaction of the cells with the supported membrane. After 5 min, shear force was applied, and the adhesion behavior of the cells was monitored by a sequence of images taken every 2 s. To characterize the cell movement, 50−150 cells within an area of 630 × 630 μm were analyzed throughout a 20 s period. Only those cells which adhered to the membrane without contact to other adhered cells were counted and analyzed.

Alternatively, images were monitored capturing 25 frames per second with a CCD camera (CSC 795). The video sequences were analyzed by application of a specific software (Imagoquant Multitrack-AVI-2; Mediquant, Halle, Germany), resulting in a detailed analysis of cell number and rolling velocity. For the data presented, experiments were repeated at least four times.

Scanning Force Microscopy. The supported lipid model membranes were prepared as described above. Scanning force microscopy was performed on a digital nanoscope IV bioscope (Veeco Instruments, Santa Barbara, CA) as described in detail elsewhere (*23*). The microscope was vibration-damped. Commercial pyramidal Si_3N_4 tips (NSC 16; Anfatec, Oelsnitz, Germany) on an I-type cantilever with a length of 125 μm, a resonance frequency of about 180 kHz, and a nominal force constant of 30 N/m were used. All measurements were performed in Tapping mode to avoid damage of the sample surface. The scan speed was proportional to the scan size, and the scan frequency was 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 the retrace 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 at a relative humidity of 60%.

RESULTS AND DISCUSSION

Introduction of the Applied Lipids. Selectin-mediated leukocyte rolling is a highly specific process which is based on the fast kinetics of selectin bindings. Further aspects, such as flexibility of the natural ligands, concentration, and local distribution of the binding epitopes, are sensitive prerequisites. We could simulate this under in vitro conditions in plate flow chamber studies. It became obvious that lateral clustering of sLeX lipids such as sLexEO6 or sLexLac1 (Figure 1) in a DSPC matrix mediates E-selectin-dependent cell rolling in a narrow concentration range of 0.05 and 0.0025 mol % (*16*, *17*), whereas higher ligand concentrations lead to firm cell adhesion.

To emphasize the cluster effects and to induce a ligand cluster formation caused by the characteristics of the ligand itself, we employed a sLeX lipid (sLexFl88) displaying fluoro substituents at seven CH_2 groups and the terminal CH_3 group of the alkyl moiety (Figure 1). This approach is based on the idea that a fluorination leads to a stronger and intrinsically triggered separation.

In a recent study we confirmed a strong separation tendency of this sLeX ligand as well as other fluorinated lipids such as LacFl88 or Fl88 (Figure 1) in a DMPC matrix by epifluorescence microscopy in monomolecular films at the air/water interface (*21*). However, preliminary data on selectin binding were difficult to interpret.

Glycolipids with Fluorinated Alkyl Chains in Conventional Matrix Lipids. Our first investigations focused on model membranes composed of fluorinated ligands in alkyl lipids (F/A), namely, sLexFl88 in DSPC, as DSPC had already served as a matrix lipid in cell rolling studies. At a lateral pressure of 38 mN/m, the films were transferred from the air/water interface of a Langmuir−Blodgett (LB) film balance to a hydrophobized glass slide.

The general appearance of phase-separated fractions within the model membranes after LB transfer was visualized by a confocal laser scanning microscope (CLSM). When 0.1 mol

% of the fluorescence dye Texas Red DHPE is added to the lipid mixture, images verify the existence and stability of clusters after transfer at ligand concentrations between 1 and 10 mol %, as illustrated for 5 mol % in Figure 2A. Since Texas Red DHPE preferably accumulates within the fluorinated compartments of the film (*21*), the white spots of irregular shape in a micromolar range can be related to the clustering of the sLeX ligands in a black matrix. Although detailed knowledge about ligand clustering in the membrane and especially the submicron cluster structure cannot be obtained from these images, the cluster patches apparently fulfill the prerequisites to act as cell rolling or adhesion spots.

To get better insight into the phase behavior of the glycolipids as a basis for phase separation and cluster nanostructure in the model membranes, continuous Π/A isotherms of the pure lipid systems used and their mixtures were recorded at 25 °C (Figure 2B,C).

The pure sLexFl88 film shows a continuous curve progression (Figure 2B). No clearly visible first-order phase transition could be observed. The compressibility increased dramatically at pressures above 10 mN/m. This is related to a condensation of the lipids within the film. We conclude that the molecular organization within the film was different below and above this pressure, representing a not well-defined phase transition. The film collapsed at a molecular area of 0.72 nm^2 and a film pressure of 52 mN/m. In the collapse region, more than 80% of the lipids are in the condensed state (epifluorescence microscopic findings; data not shown).

The isotherm of the nonfluorinated analogue sLexLac1 shows a transition between the liquid-expanded and the liquid-condensed phase and a collapse pressure of 53 mN/m (0.68 nm^2) (*17*). Similar to sLexLac1, the nonfluorinated glycolipid sLexEO6 carrying a spacer of six ethoxy units shows also a mean phase transition during the compression (Figure 2C). The phase transition of sLexEO6 occurs at a higher pressure (35 mN/m; molecular area 1.01 nm^2), compared to sLexLac1. This is obviously caused by the more flexible headgroup region leading to lower van der Waals attraction forces. The film collapses at 58 mN/m (0.62 nm^2), where 90% of the whole film area is in the condensed state as demonstrated by fluorescence microscopy (data not shown).

Summarizing these data, despite individual differences in the compression behavior, we could not detect fundamental differences between fluorinated and nonfluorinated sLeX derivatives.

The lipid LacFl88 shows a different compression behavior (Figure 2B). The lift-off area is shifted to a larger value. Additionally, the film shows a first-order phase transition region between the liquid-expanded and liquid-condensed phase behavior at a pressure of about 5 mN/m. At pressures higher as 38 mN/m, the membranes are completely condensed but show no high crystalline order. Isotherms of LacFl88 show a collapse pressure of 56 mN/m and a mean molecular area of 0.65 nm^2. Again, more than 80% of the surface consists of condensed domains at the collapse pressure.

The phase behavior of the mixed films is dependent on the molecular structure and concentration of the glycolipids. The highly incompressible DSPC matrix lipid becomes more fluid by mixing with either the alkylated or the fluorinated

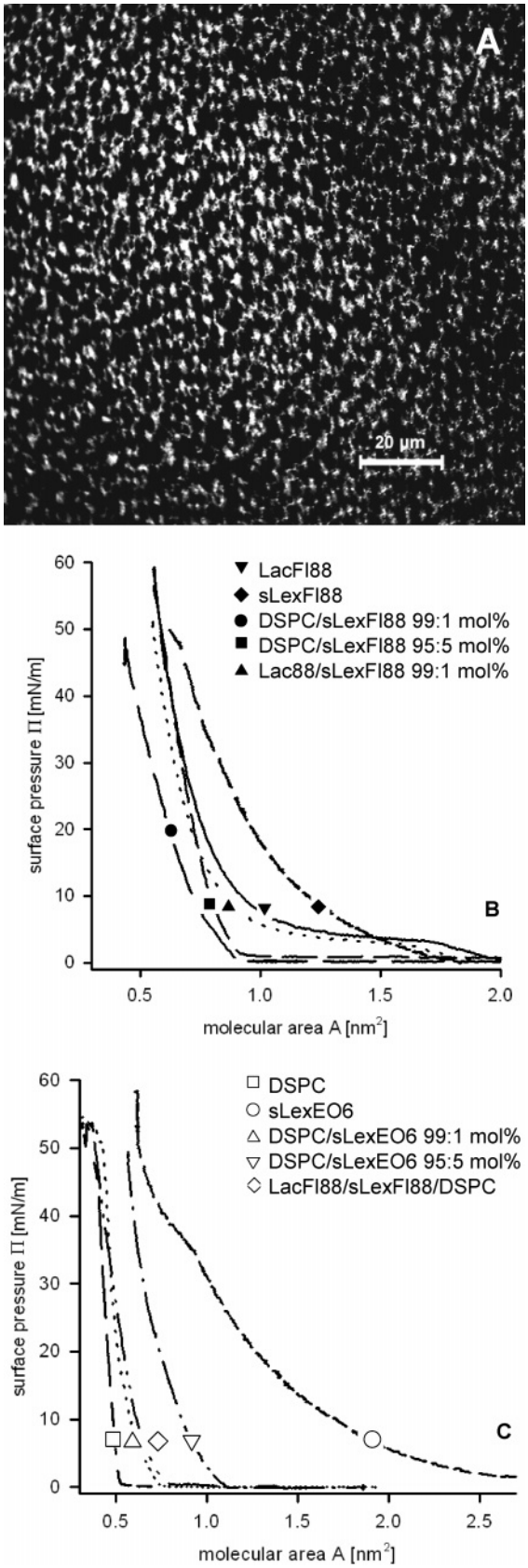


FIGURE 2: (A) Microscopic image of the transferred DSPC film containing 5 mol % sLexFl88. 0.1 mol % Texas Red DHPE was added to accumulate in the fluorinated phase to illustrate the cluster formation. (B, C) Dynamically compressed Π/A isotherms of (B) the pure ligand sLexFl88 as well as mixtures with DSPC and LacFl88 matrix lipids and (C) the nonfluorinated ligand sLexEO6 pure or in mixtures with the DSPC matrix, as well as an isotherm of the F/F/A system sLexFl88 in LacFl88 in a DSPC matrix (0.1/9.9/90).

ligands. The molecular area increases with increasing concentrations of the glycolipids due to the large and flexible headgroup of the ligands. The greatest effect on the area expansion can be observed for lipids with a first-order phase transition (sLexFl88, sLexEO6), which is typical for a lateral phase separation. The collapse pressures are nearly identically for all mixtures and comparable to the pure substances.

The mixed isotherms of the fluorinated lipids show a liquid-condensed film behavior. The compressibility, the collapse pressure, and the resulting molecular area are comparable. No phase transition could be observed. The results are not able to give detailed information about the phase behavior or the molecular organization of these lipid films.

However, we realized that common concentrations of the sLexFl88 (0.01−10 mol %) in DSPC do cause neither adhesion nor rolling of E-CHO cells, which is in contrast to the nonfluorinated system (*16*, *17*). Firm adhesion of CHO-E cells on higher concentrations (up to 100 mol %) of sLexFl88 proves the principle ability of binding. This interaction can be regarded as E-selectin specific because less than 10% of the cells adhered to the pure matrix lipid DSPC. The difference can be related to the limited accessibility of the epitope or a suboptimal cluster formation. Consequently, the reason for the missing rolling movement and the low binding efficiency of the fluorinated ligand clusters has to be searched within the morphology and the inner structure of the clusters.

Therefore, we extended the investigations on using SFM, which allows the analysis of the lateral morphology and inner cluster structure of the pure and mixed lipid films after transfer of the bilayers. Examples of lateral organizations of the pure lipid films are shown in Figure 3. In all cases the film morphology was strictly dependent on the lipid phase behavior and the tendency of the lipids to associate.

The membrane of sLexFl88 appears as a smooth surface in the condensed state without any breaks and domain borders (Figure 3A). High-resolution insert reveals that the condensed phase is not highly ordered. Small amorphous clusters (size range from 20 to 50 nm) of about 200 molecules were homogeneously distributed within the film.

Images of the sLexLac1 films reveal the formation of hexagonally shaped condensed domains with sizes between 1 and 5 μm (Figure 3B). Within these domains, the glycolipids are organized in ordered and tightly packed clusters of 20 nm in size. The amorphous appearance of films consisting of either sLexFl88 or sLexLac1 might be the result of the size differences between the large headgroup and the hydrophobic moieties. This can force the molecule into either a tilted position within a homogeneous area or smaller round clusters, especially in the case of the fluorinated lipids. But the overall appearance of the surfaces shows no significant differences. Since both sLexFl88 and sLexLac1 show comparable images and phase behavior, it can be concluded that the presence of the sLeX headgroup but not the steric organization of the fluorinated hydrophobic tails dominates the systems. This implies that the reason for the missing rolling ability cannot be found in the phase behavior of the pure glycolipid but rather in the interaction with the matrix lipid DSPC.

As described previously (*17*), the pure matrix lipid DSPC exhibited liquid-condensed film behavior during compression (Figure 2C) and displayed large aggregated, irregularly

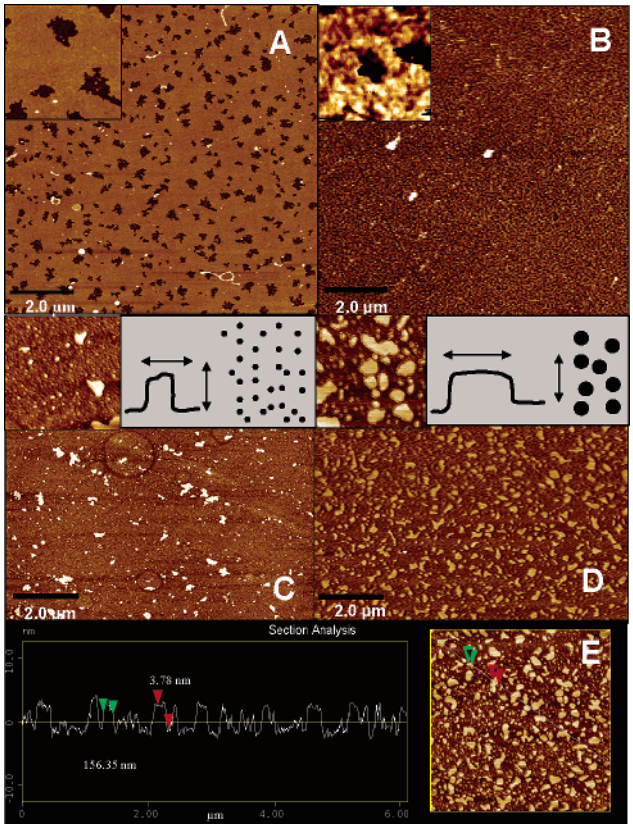


FIGURE 3: SFM images of the pure fluorinated ligand sLexFl88 (A) compared to the nonfluorinated analogue sLexLac (B). The high-resolution inserts (left) show that both lipids form an amorphous clustered arrangement. The mixture of sLexFl88 in DSPC, 1 mol % (C) and 5 mol % (D), results in the formation of atypical clusters that increase with ligand concentration, as schematically illustrated. Clusters protrude around 3 nm above the film surface, visible in the height scan (E).

shaped domains and highly ordered crystallized lipids within the domains (data not shown).

Images of DSPC membranes containing 1 or 5 mol % of sLexFl88 are illustrated in panels C and D of Figure 3, respectively. sLexFl88 is not evenly distributed in the matrix and accumulates in atypical cluster populations, whose number and size increase with rising amounts of glycolipid. Whereas 1 mol % of sLexFl88 formed clusters with an average diameter of 30 nm (Figure 3C), the 5-fold sLexFl88 amount induced much bigger clusters (Figure 3D), as illustrated in the schematic sketch. The clusters protrude around 3 nm above the film surface, visible in the scan of Figure 3E. In comparison to the nonfluorinated analogue sLexLac1, which forms highly condensed surface structures of homogeneous size and distribution in the matrix DSPC (*17*), these results suggest minimal compatibility of fluorinated chains with DSPC.

Summarizing, the system F/A does not generate functional clusters to support adhesion or rolling of E-CHO cells. This could be explained by strong separation of the fluorinated glycolipids from the matrix, resulting in large irregular areas of high ligand density, which, if at all, confer low binding ability.

Functional Glycolipids with Alkyl Chains in Matrix Lipids with Fluorinated Alkyl Chains. To answer the question whether fluorination of membrane components prevent controlled phase separations and functional ligand clusters

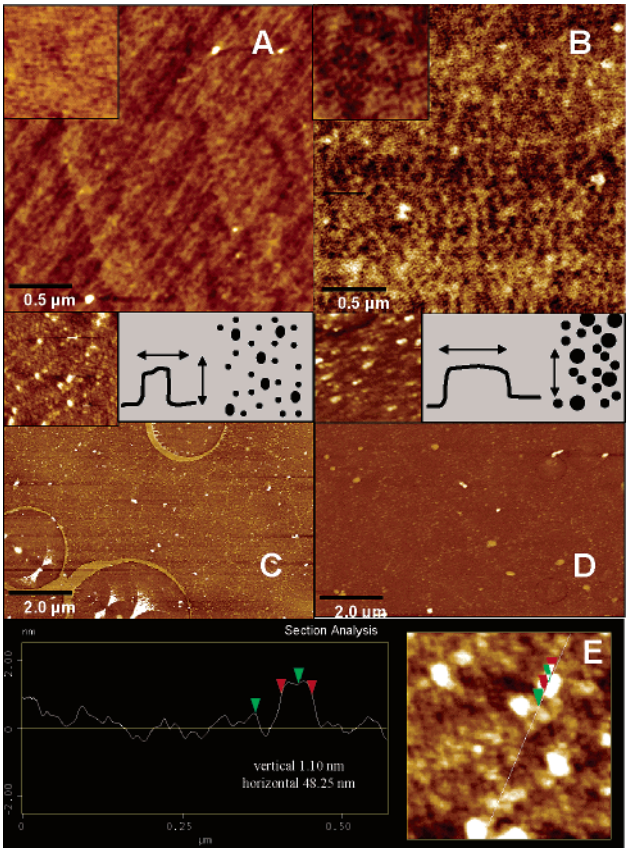


FIGURE 4: SFM images of the fluorinated matrix lipids Fl88 (A) and LacFl88 (B). The nonfluorinated ligand sLexEO6 was inserted into (B) with 1 mol % (C) and 5 mol % (D), respectively, resulting in two populations of clusters, which protrude around 1 nm from the surface (E). In analogy to Figure 3, the number of clusters grows with concentration, while their distance decreases, as illustrated in the sketch.

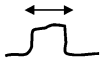
in general, we interchanged the experimental setup from fluorinated ligands in alkyl lipids (F/A) to the alkyl ligand sLexEO6 in a fluorinated matrix (A/F). sLexEO6 is a sufficient ligand with respect to its spacer length and flexibility in DSPC to induce rolling of E-CHO cells. Morphology and phase behavior of pure sLexEO6 films (Figure 2C) have been published previously (*16*). These data show that at the transfer pressure of 38 mN/m a large part of the sLexEO6 film is still in the fluid state.

Fl88 and LacFl88 (Figure 1) were considered as potential matrix lipids. In contrast to Fl88, LacFl88 contains a single lactose unit as headgroup and should therefore offer a larger hydrophilic counterpart to the fluorinated hydrophobic moieties.

The lateral arrangement of Fl88 lipids is characterized by domains of different size, formed by amorphous, but tightly packed aggregates with a diameter of 5.1 ± 0.7 nm (Figure 4A). A single Fl88 molecule occupies a molecular area of 0.5 nm^2. Overall, the film of Fl88 appears homogeneous and smooth. The maximum height difference within the condensed phase amounts to only 0.35 nm. Nonetheless, we observed considerable nonspecific cell adhesion of E-CHO cells on a film of pure Fl88, which might be caused by the imbalance in size between lipid anchor and the small headgroup.

LacFl88 possesses a bigger headgroup moiety. Like Fl88, LacFl88 forms very homogeneous and smooth films with a

Table 1: Lateral Arrangement of the Lipid Membranes Used in This Study[a]

glycolipid / mixture [mol%]			
sLexEO6	18.4 nm ± 1.3 nm	7.8 nm ± 0.7 nm	0.45 nm ± 0.05 nm
DSPC / sLexEO6 (99.9/0.1)	25.9 nm ± 3.8 nm	36.7 nm ± 5.4 nm	0.71 nm ± 0.09 nm
DSPC / sLexEO6 (99/1)	78.9 nm ± 13.4 nm	54.6 nm ± 6.3 nm	0.76 nm ± 0.11 nm
sLexFl88	34.6 nm ± 4.8 nm	14.6 nm ± 1.9 nm	0.35 nm ± 0.04 nm
DSPC / sLexFl88 (99/1)	28.9 nm ± 4.7 nm	45.8 nm ± 8.1 nm	1.1 nm ± 0.15 nm
DSPC / sLexFl88 (95/5)	156.4 nm ± 17.3 nm	200-400 nm	3.72 nm ± 0.7 nm[b]
LacFl88	17.3 nm ± 2.8 nm	22.5 nm ± 2.2 nm	0.35 nm ± 0.08 nm
Fl88	5.1 nm ± 0.7 nm	8.0 nm ± 0.5 nm	0.35 nm ± 0.05 nm
LacFl88 / sLexEO6 (99/1)	24.9 nm ± 6.2 nm 59.7 nm ± 8.3 nm	60.6 nm ± 6.7 nm	1.09 nm ± 0.08 nm
LacFl88 / sLexEO6 (95/5)	36.3 nm ± 4.6 nm 67.3 nm ± 5.2 nm	24.1 nm ± 4.3 nm	1.10 nm ± 0.11 nm
LacFl88 / sLexFl88 (99/1)	13.4 nm ± 1.8 nm	13.9 nm ± 2.9 nm	0.73 nm ± 0.07 nm
DSPC/LacFl88 / sLexFl88 (99/9.9/0.1)	256.5 nm ± 37.1 nm	200-500 nm	4.5 nm ± 0.5 nm[b]

[a] All model membranes were transferred at a surface pressure of 38 mN/m. The size, distance, and height of the domains were calculated from three scanned areas (5 × 5 or 1 × 1 µm in dependence on the domain size), measuring all parameters from the visible surface structures. [b] The measured domain thickness is higher than the calculated monofilm thickness, indicating that the domains represent three-dimensional structures.

maximum height difference of 0.35 nm. Compared to Fl88, larger clusters (molecular area 0.54 nm^2) of a diameter of 17.3 ± 2.8 nm were detected (Figure 4B), which is caused by the increased association tendency of the lipids. Hydrogen bonding between the lactose headgroups provides a sufficient hydrophilic "barrier" on the membrane surface for nonspecific cell adhesion (*17*). Indeed, unspecific adhesion of E-CHO cells to pure LacFl88 could be excluded, and therefore LacFl88 served as the matrix lipid for the following investigations.

sLexEO6 was incorporated in LacFl88 in concentrations expected to be sufficient for cell rolling (5−0.005 mol %), since sLexEO6 in DSPC (A/A) was found to mediate cell rolling between 0.1 and 0.005 mol % (*16*). No ability for rolling could be detected within this concentration range. However, a remarkable strong tendency for cell adhesion down to 0.005 mol % was seen. The detected differences to the A/A system imply that the structure of the clusters is altered by fluorination, as it withdraws their ability to induce rolling, but simultaneously increases their binding affinity.

The SFM investigation of films containing either 1 or 5 mol % sLexEO6 gives insight into the structure of the membranes (Figure 4C,D). Their background is dominated by the smooth surface of LacFl88 (Figure 4B). sLexEO6 is organized in two populations of clusters, which protrude around 1 nm from the surface. At a concentration of 1 mol % they possess a diameter of 24.9 ± 6.2 nm and 59.7 ± 8.3 nm (Figure 4C), which is increased up to 36.3 ± 4.6 nm and 67.3 ± 5.2 nm at 5 mol % (Figure 4D). Simultaneously,

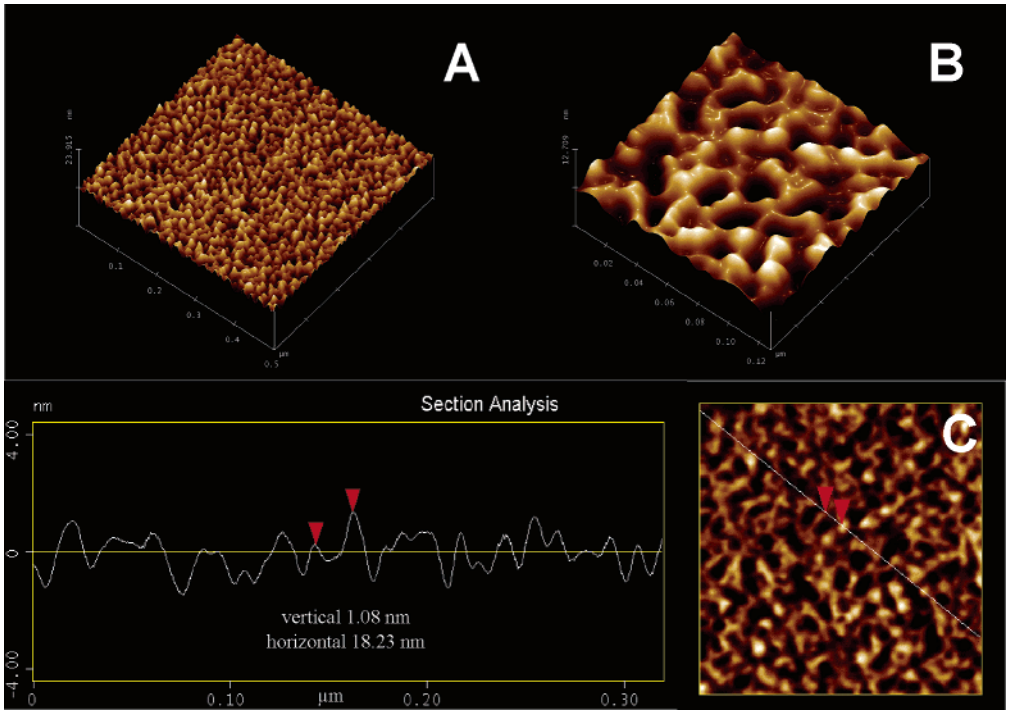


FIGURE 5: SFM image of a membrane composed of 1 mol % sLexFl88 embedded in the fluorinated matrix LacFl88. The higher resolution of the film in (B) reveals a large number of small clusters around 13 nm in diameter. The height of the clusters above the film surface is about 0.73 nm (C).

the number of clusters grows with concentration, while their distance decreases from mean values of 60 nm at 1 mol % (edge to edge) to 25 nm at 5 mol %, schematically sketched in the inserts. The data for the lateral arrangement of the lipid systems are summarized in Table 1.

A possible reason for the A/F system forming clusters, which mediate strong adhesion of E-CHO cells but fail to induce cell rolling, might be the separation tendency of fluorinated compounds from alkyl lipids. In analogy to the behavior of the F/A system, the size of the LexEO6 clusters in the fluorinated matrix increases with concentration.

On the contrary, the cluster size of sLexEO6 in DSPC is smaller and less dependent on the concentration. Furthermore, the area occupied by the clusters did not correspond but is larger than theoretically calculated for the glycolipid concentration in the A/A system. As a part of sLexEO6 is still fluid at 38 mN/m, a partial miscibility of sLexEO6 with DSPC is likely, leading to a dilution of ligand density within the clusters.

Whereas ligand clustering is a prerequisite for selectin binding, binding avidity is controlled by the local ligand density. Considering cell rolling as a steplike process of binding and dissociation along clusters, firm adhesion at high density within the clusters will be conferred to rolling at lower clustered concentrations. This is a new insight in the rolling mechanism; attractive binding spots of a certain size and distance are as essential as a balanced ligand density within these spots. Obviously, the high separation tendency of fluorinated components within this system creates clusters of pure sLexEO6 ligands. They are attractive for firm adhesion up to very low concentrations but do not fulfill the requirements for rolling. Furthermore, size and distances of the clusters contradict cell rolling.

Since the combination of fluorinated and alkyl components leads to strong phase separation and clusters insufficient for rolling, we tested this hypothesis and employed a fluorinated system with more similar characteristics of matrix and ligand. Therefore, sLexFl88 was incorporated in a LacFl88 matrix (F/F).

Membranes with Matrix and Ligand Components Both Fluorinated. Membranes with matrix and ligand components both fluorinated should possess a sufficient separation tendency to form clusters but maintain a weak miscibility leading to a small cluster size.

Figure 5A illustrates the SFM image of a membrane composed of 1 mol % of sLexFl88 embedded in the fluorinated matrix LacFl88. The image reveals a large number of small clusters (diameter around 13 nm). The size of these clusters is considerably smaller than the size of those generated in the system A/F. The clusters are arranged within distances of approximately 13 nm (Figure 5B); the height above the film is 0.73 nm (Figure 5C). Therefore, similar miscibility results in a higher number of smaller clusters in closer vicinity. This size reduction enabled a sufficient ligand density within the clusters for rolling. A slow rolling of CHO-E cells (about 2 μm/s) along the membrane containing 1 mol % of sLexFl88 could be detected. Concentrations lower than 0.5 mol % result in a detachment of all cells, as illustrated in Figure 6A. Consequently, the F/F system displays a comparable behavior to the alkyl system, but shifted to higher ligand concentrations, as schematically summarized in Figure 6B.

This outcome confirms the importance of cluster size and distance for rolling; the postulation on ligand cluster dilution is indirectly supported. Significant evidence for the cluster dilution and a balanced ligand density are area calculations. Clusters comprise around 38% of the total area at concentrations of only 1 mol % of sLexFl88. This suggests the existence of both sLexFl88 and LacFl88 in the clusters due to their partial miscibility. This finding is in agreement with

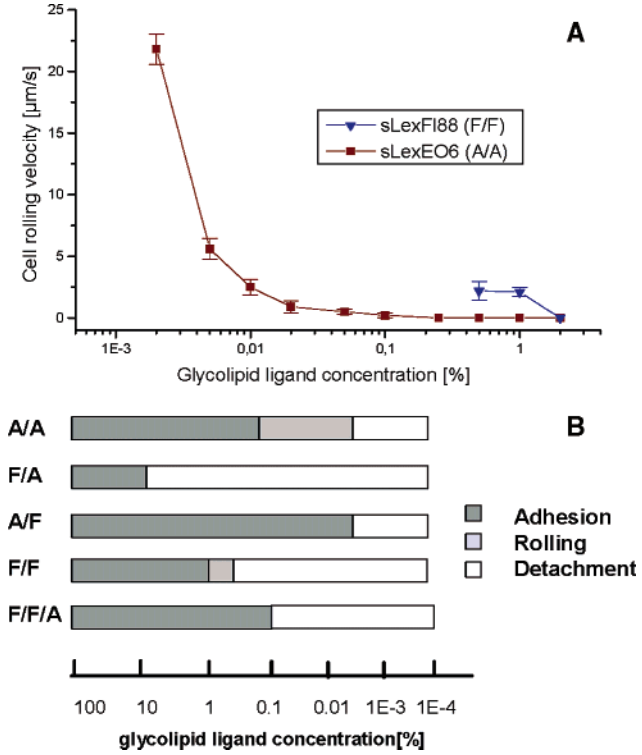


FIGURE 6: (A) Cell rolling velocity of CHO-E cells along model membranes containing the indicated concentration of ligands. The fluorinated ligand sLexFl88 in a LacFl88 matrix (F/F) mediates a slow rolling at higher ligand concentrations compared to sLexEO6 in DSPC (A/A) (*17*). (B) Schematic summary of binding, rolling, and detachment of CHO-E cells on the different model membranes in dependence on the ligand concentration.

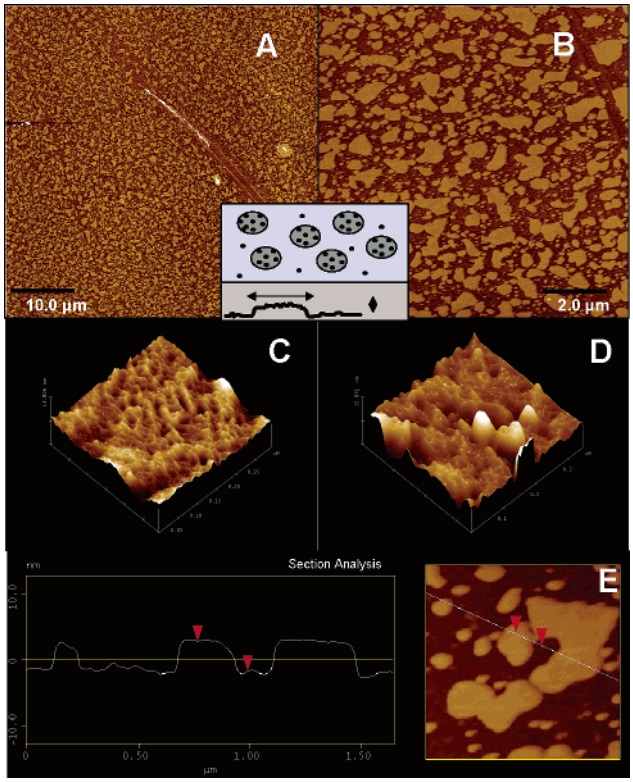


FIGURE 7: SFM images of the mixture of DSPC, LacFl88, and sLexFl88 [90/9.9/0.1 (F/F/A)]. Two different resolutions in (A) and (B) detect very large clusters around 100−500 nm in dimension. The general incompatibility of this mixture is supported by the height scan in (E), indicating a multilayer system with variations of 4.5 nm in height. High-resolution images in (C) and (D) illustrate smaller clusters of approximately 12−15 nm in size within the clustered domains, detecting the clusters composed of sLexFl88 in LacFl88.

earlier studies on A/A systems (*17*). Molecular modeling calculations give roughly 500 molecules per cluster for the A/A system and 200 fluorinated molecules for the F/F system. This indicates comparable ligand density considering the different cluster sizes (about 25 vs 13 nm). Obviously, the low rolling velocity of about 2 μm/s vs about 15 μm/s of the A/A system should be related to the smaller cluster separation distance. Consequently, fluorination of only one component leads to total immiscibility, resulting in clusters of pure or very high ligand density. The inability to mediate cell rolling can also be explained with regard to steric organization. Too densely packed ligands within the clusters are less accessible and not flexible enough to induce rolling.

So far, two important parameters have been extracted from the investigations: For optimal rolling, clusters are not to exceed a certain size and distance, and they have to present the ligand epitopes in a loosely packed arrangement.

Fluorinated Glycolipids Diluted by Fluorinated Matrix Lipid Embedded in DSPC. To check the influence of cluster distance and ligand density within the clusters on the rolling velocity, we combined the characteristics of the completely fluorinated system with the immiscibility of fluorinated lipids in DSPC. Therefore, we prepared a mixture of DSPC, LacFl88, and sLexFl88 of 90:9.9:0.1. The idea was to get fluorinated clusters within DSPC, but with a reduced ligand density, diluted by the "second" matrix LacFl88 (F/F/A). In accordance with the conclusions that a smaller cluster size and flexibility within the clusters are necessary to induce rolling, we anticipated rolling with higher velocity. The addition of DSPC would not have an influence on the cluster size directly but would separate the binding patches from each other.

In contrast to reasonable adhesion of the cells, cell rolling could not be observed, and cells detached with reducing the ligand concentration. SFM analysis might explain the contrasting findings, as it detects very large clusters, around 100−500 nm in dimension (Figure 7A,B), with elongated shape. The height scan in Figure 7E indicates a multilayer system with variations of 4.5 nm in height confirming a general instability of this mixture. High-resolution images in Figure 7C,D give an insight into the domains and illustrate smaller clusters of approximately 12−15 nm in size, detecting the clusters composed of sLexFl88 in LacFl88. However, because of the general incompatibility between fluorinated and alkyl structures, this system does not fulfill the requirements for rolling, as stated above.

CONCLUSIONS

Rolling of leukocytes along the endothelium is a unique kind of cell adhesion and a consequence of kinetic and mechanical characteristics of the selectins and their physiological ligands. Natural ligands are mucin-like glycoproteins, which present carbohydrate binding epitopes such as the tetrasaccharide sLeX in a certain arrangement on the protein backbone. It has been proposed that cell rolling is kinetically based on very rapid association and dissociation rates of the selectins with the ligands. Numerous studies in

the past decade provided an excellent insight into the kinetic aspects of the receptor−ligand interactions under in vitro conditions. In most cases, isolated selectins or natural selectin ligands were investigated on a single molecular level using surface plasmon resonance or single molecule force spectroscopy (*24*−*27*). The intrinsic kinetic parameters of the selectin−ligand pairs, the reactive compliance, and tensile strength under shear force were evaluated and explain the cell rolling process. The mechanical properties of the ligands are greatly dominated by the extended flexible appearance of the protein backbone. Despite an enormous increase in understanding the rolling, all aspects, i.e., the high affinity of the physiological ligands in contrast to the binding epitopes (*27*), cannot fully be elucidated. A multivalent arrangement of binding epitopes has been postulated to be the structural prerequisite for high-affinity binding. However, the use of complex natural compounds is not well suited for a correlation of all structural parameters, such as clustering of binding epitopes with cell rolling mechanisms, and would need in vitro simulations.

The aim of this study was to get greater knowledge about the structural prerequisites for selectin−ligand functioning with particular respect to epitope clustering. Special emphasis was laid on the requirements for rolling inducement in contrast to mere adhesion.

The presentation of sLeX on natural ligands was modeled by the experimental setup. For a flexible presentation of sLeX epitopes in clustered arrangements, sLeX binding epitopes were inserted as glycolipids in a lipid membrane. The lipids in this study displayed a partial fluorination of the hydrophobic moiety for a strong separation from nonfluorinated alkyl lipids. A high-resolution imaging of the resulting clusters by SFM in correlation with the binding or rolling of CHO-E cells along these clusters should help to interpret the role of multivalency in selectin function. Therefore, the examination was focused on the analysis of cluster distribution in the membrane, cluster size, and ligand density in correlation to rolling ability.

The fluorination of only one component, sLeX ligand or matrix, led to a strong separation of the lipid phases. It was evident that cluster size increased with concentration of the clustered component in the certain systems, which indicates a total immiscibility of fluorinated and unfluorinated lipids. The inability to induce cell rolling cannot only be explained by the adverse size and separation distance of the clusters, which do not correlate with the postulated step-like mechanisms in cell rolling. The immiscibility resulted in clusters of pure or highly accumulated ligands, which revealed high avidity for a firm cell adhesion. Rolling was observed only in membranes with matrix and ligand components both fluorinated. This system displayed a distinct miscibility, as was derived from calculations of the clustered area and the ligand concentration. Therefore, the small and homogeneously distributed clusters in this system are an indication of partial miscibility. Accordingly, the resulting clusters displayed a lower local ligand concentration, which fulfils the demands for mediating cell rolling with respect to epitope flexibility and accessibility.

Consequently, considering the essential role of epitope clusters for selectin binding, we can derive further specifications; clusters are not to exceed a certain size and they have to present the ligand epitopes loosely packed. In contrast to mere epitope recognition in cell binding, the rolling process is dependent not only on ligand concentration but also on local dynamic epitope arrangement.

Applied to the physiological conditions of the natural mucin ligands, flexibility will be provided by the protein backbone. However, this study underscores the importance of a specific presentation of carbohydrate epitopes. Distances and sizes of clustered epitopes in the nanometer range are decisive for the ability to induce rolling. Considering these findings, it appears that the extended and highly flexible mucin structure is the ideal substrate for cell rolling under shear force conditions.

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