Stereoselective Interactions of a Specialized Antibody with Cholesterol and Epicholesterol Monolayers

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Abstract: The stereoselective recognition by monoclonal antibodies of two-dimensional monolayers of cholesterol spread at the air–water interface is presented. Using immunofluorescence, we show that one antibody, raised and selected against crystals of cholesterol monohydrate, specifically recognizes monolayers of cholesterol, but not monolayers of epicholesterol—its epimeric form. This demonstrates that stereoselective recognition also applies to protein-surface interactions.

Keywords: antibody recognition · cholesterol · interfaces · monolayers · stereoselectivity

Introduction

The rules that govern stereoselective recognition between proteins at interfaces, or between proteins and exogenous surfaces, are still far from being understood systematically.[1] The interactions among proteins and of proteins with cell membranes and with extracellular matrices, are, however, often determinant in the course of physiological, as well as pathological, conditions. One example of proteins acting interfacially is that of phospholipases. These enzymes bind specifically to biological membranes and hydrolyze phospholipid esters without penetration into the membrane.[2] The interfacial binding is not based on electrostatic interactions, as was previously hypothesized, but rather on hydrophobic interactions.[3] Proteins specifically interact with crystal surfaces. Protein templates can operate as crystal nucleators, as exemplified in several biomineralization processes,[4] or as crystallization inhibitors, in the example of antifreeze proteins.[5–7] Some of these proteins may bind in a remarkably stereoselective manner. The study of their stereoselective recognition may provide more information on the organization of both surfaces involved in the interaction.

Stereoselective recognition has been demonstrated in monoclonal antibodies raised against steroid haptens,[8] enzyme inhibitors,[9, 10] and drugs.[11] Antibodies that catalyze chemical transformations with stereochemical control have been generated.[12, 13] In these examples, the structure of the antibody-antigen complex can be determined at the molecular level, and detailed information about the mode of binding can be thus obtained. The understanding of the binding of antibodies raised against larger haptens such as polysaccharides or polymers is, however, still limited, because the structure of the target entity is mostly unknown.

Here, we report on a specialized monoclonal antibody that stereoselectively recognizes an organized array of cholesterol molecules exposed at the air–water interface in the form of a monolayer. Such antibodies interact within their binding site with more than one molecular component of the monolayer, and are thus expected to bind stereoselectively not only to different molecules, but to different molecular arrangements of the same molecule.

The role of cholesterol in biological membranes has been extensively studied by using various molecular recognition approaches.[14] These studies were targeted, however, exclusively to cholesterol as a molecular component and not as an integral part of organized arrays. The cholesterol molecule exposes mainly the hydroxyl moiety on the membrane surface; this moiety may form structured surface patterns with phospholipids, glycolipids, or other cholesterol molecules. These may be recognized as such by proteins or other extracellular components, thus contributing to the determination of the course of cellular processes.

The present research is part of an ongoing effort in our laboratory toward the understanding of the basic rules that govern highly specific protein–surface interactions. Within this framework, antibodies were raised and selected against crystals of cholesterol monohydrate.[15] The selected antibodies are capable of specifically recognizing defined surfaces of cholesterol monohydrate crystals. They do not recognize, however, the cholesterol molecule itself. The sequence of the variable region of one such antibody was recently determined and the structure of its binding site was predicted by molecular modeling.[16] According to the model, the binding

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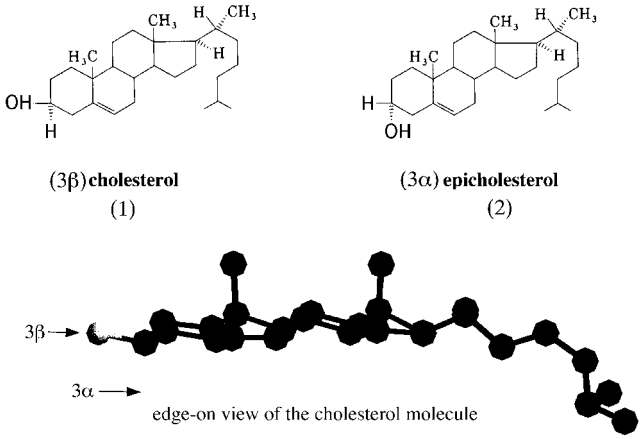
site assumes the characteristic shape of a step with one hydrophobic and one hydrophilic side; these are suggested to interact with hydrophobic and hydrophilic domains exposed at the interface on the specific crystal face recognized by the antibody.

Cholesterol monolayers at the air–water interface were recently shown to be composed of two-dimensional crystallites with a coherence length of 100 Å.[17] At the boundaries of these domains, three-dimensional structural features similar to those of the crystalline lattice may be reproduced. The antibodies raised against cholesterol monohydrate crystals do indeed interact with monolayers of cholesterol at the air–water interface.[18] In contrast, antibodies raised and selected against crystals of 1,4-dinitrobenzene interact weakly and nonspecifically with the same monolayers.

Stereospecificity was used here to characterize the mechanism of surface–surface interaction and the recognition of these antibodies at the molecular level. In particular, we have tested the recognition of one specific antibody for monolayers of cholesterol (**1**) and its epimer, epicholesterol (**2**) (Scheme 1). Our results, by using immunofluorescence microscopy, show that the monoclonal antibody which recognizes cholesterol monolayers does not interact with epicholesterol monolayers. The stereospecificity in the recognition demonstrates that the interaction is not solely governed by the macroscopic shape or the chemical potential of the binding site.



Scheme 1.

Results

Monolayer morphology: Figure 1 shows the surface pressure/area (π-A) isotherms for monolayers of cholesterol (solid line) and epicholesterol (dotted line). The π-A isotherm of cholesterol gives a limiting molecular area of 42 Å^2 per molecule and a collapse pressure of 43 mN m^{-1}. Epicholesterol gives a limiting molecular area of 35 Å^2 per molecule and a collapse pressure of 30 mN m^{-1}, smaller than that of cholesterol.

A small trough was developed in order to study the morphological behavior of the monomolecular film and to perform antibody assays. The trough was constructed to allow

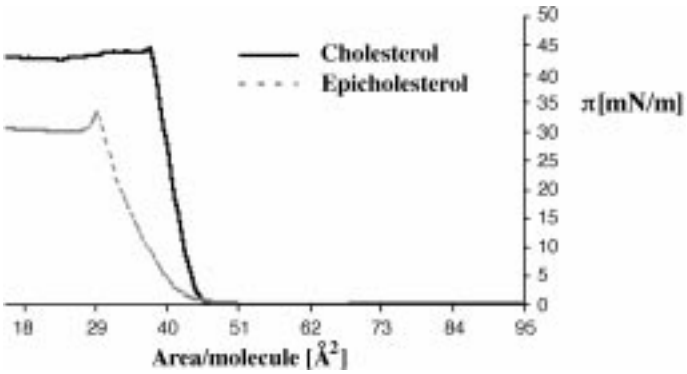


Figure 1. Surface pressure area (π- A) curves for cholesterol (full line) and epicholesterol (dotted line) spread at the air–water interface.

injection of small amounts of antibody solution into the subphase and was equipped with a micro-channel system for exchanging the subphase during immunoassay procedures. The cholesterol solution was deposited on the surface of a solution of PBS (phosphate buffer saline), in amounts calculated from the isotherms to yield complete coverage of the interface. In order to visualize the monolayers under an epifluorescence microscope, the cholesterol solution was doped before deposition with 1 mol% 3β-cholesteryl-rhodamine-B. The labeled monolayers give pressure isotherms identical to those of pure cholesterol. We thus assume that the addition of 1 mol% of fluorescently labeled cholesterol does not substantially modify the monolayer structure. Labeled monolayers of cholesterol appear uniformly fluorescent within the covered regions. Approximately 20% of the surface, however, appears empty (dark), due to incomplete coverage in the absence of applied pressure (Figure 2A). This was confirmed by spreading cholesterol in amounts that were in

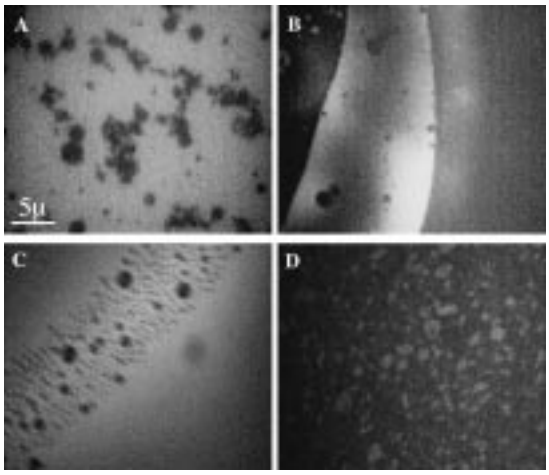


Figure 2. A) Fluorescence image of a monolayer of cholesterol doped with 1 mol% 3β-cholesteryl-rhodamine-B imaged directly at the air–water interface. B) Fluorescence image of cholesterol deposited at the interface in an amount in excess of full coverage. Note the regions of higher fluorescence, that is, multilayers coexisting with regions of lower intensity, monolayer, and with dark regions (empty interface). C) Fluorescence image of a monolayer of epicholesterol doped with 1 mol% 3α-cholesteryl-rhodamine-B. D) Fluorescence image of an epicholesterol monolayer doped with 1 mol% 3α-cholesteryl-Rhodamine-B following washing with Tween 20. The apparent lack of resolution at boundaries of dark regions in all frames is due to monolayer motility.

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large excess relative to the area of the trough. Multilayer formation was demonstrated by the appearance of more intense fluorescence in some regions, while the dark regions did not disappear or increase in relative area (Figure 2B). It is thus unlikely that the dark regions are due to dye exclusion from large crystalline cholesterol domains. Epicholesterol, analogously doped with 1 mol % 3α-cholesteryl-rhodamine-B, also yields a uniformly fluorescent monolayer, with a smaller percent of uncovered regions (approximately 10 %, Figure 2C). Under these conditions, both monolayers are stable, fluid, and retain their uniformity for hours. Both monolayers can be lifted onto glass slides treated with OTS (octadecyltrichlorosilane), by touching the interface with the glass slide, which is lowered from the air parallel to the surface. The monolayer fluorescence can be observed on the slide, and contact angles can be measured. The contact angles measured on the slides after lifting of both cholesterol and epicholesterol monolayers are typically 94°, whilst those of the OTS-treated slides are 115°.

Immunofluorescence assays: The binding of antibody 36A1, raised and selected against cholesterol monohydrate crystals,[15] to monolayers of cholesterol, was first studied by immunofluorescence by using double-labeling of both the monolayer and a secondary antibody tagged with rhodamine-B.[18] The antibody solution was introduced into the subphase after deposition of the fluorescently doped monolayer. The unbound antibody was removed by washing with PBS-Tween® 20 (0.05 %) by using the micro-channel close-flow system. All the incubations and washings were performed inside the trough and do not alter monolayer stability (analogous to Figure 2A). The monolayer was then incubated with a secondary antibody (anti-F(ab)$_2$, labeled with rhodamine-B and washed), and the monolayer–antibody complex was directly imaged under the epifluorescence microscope. New distinct patterns of higher intensity were seen (Figure 3A) under the labeled cholesterol monolayer after incubation with antibody 36A1. The assay was also carried out with unlabeled monolayers of cholesterol in order to exclude the possibility that these patterns are a result of monolayer aggregation. Identical patterns with similar texture were obtained (Figure 3B). As additional control, the monolayer of cholesterol was incubated with the secondary antibody alone. The high intensity patterns were absent under these conditions.

The same procedure, as it turned out, cannot be applied to monolayers of epicholesterol, because the monolayer is degraded after the first washing with PBS-Tween 20. The monolayer fluorescence decreases substantially, leaving only discrete fluorescence patches floating at the interface (Figure 2D). This behavior is independent on whether the monolayer was incubated with antibody before washing. It is observed with all commonly used surfactants, such as Tween® or Triton®, even at very low concentrations (0.0001 %). The effect might conceivably result from detergent-induced selective extraction of the dye from the monolayer. This possibility was, however, ruled out by lifting the putative monolayer onto an OTS-treated glass slide and measuring the contact angle. The measured contact angle was

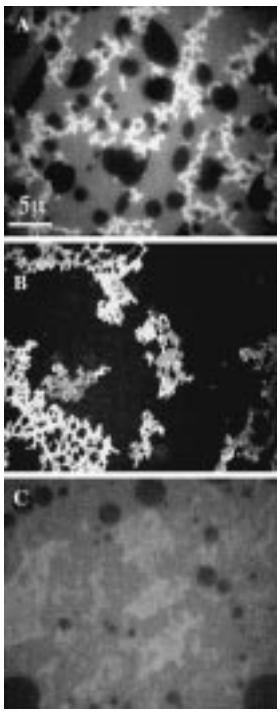

Figure 3. A) Fluorescence image of the (double labeled) antibody-cholesterol monolayer complex formed by antibody 36A1 with secondary labeled antibody. B) Fluorescence image of the antibody(labeled)-cholesterol monolayer(unlabeled) complex formed by antibody 36A1 with secondary labeled antibody. C) Fluorescence image of the antibody-(labeled)-cholesterol monolayer(labeled) complex formed by antibody 36A1 with primary labeled antibody following washing with Tween 20. Note the regions of higher fluorescence which are attributed to the bound primary labeled antibody.

112°, and it was concluded that the monolayer had been destroyed prior to lifting. In contrast to the detergent-induced deterioration, epicholesterol monolayers are remarkably stable to washing with PBS.

Washing with detergent was performed to remove antibody that was bound nonspecifically to the monolayer. A different strategy was therefore developed to study antibody recognition of monolayers, owing to the observed instability of epicholesterol. Antibody 36A1 was labeled directly with rhodamine-B, and was tested with monolayers of cholesterol, to ensure that the dye does not interfere with the binding activity of the antibody. Incubation of labeled monolayers of cholesterol with labeled antibody was followed by washing of unbound antibody with PBS-Tween 20 and direct observation of the monolayer by epifluorescence. Analogous to the case above, distinct regions of higher fluorescence were observed under the monolayer (Figure 3C). These appear more extensive and more uniform than in the previous procedure, presumably because antibody aggregation is limited in the absence of extensive repeated washings. The same experiment could not be performed with epicholesterol monolayers, as these were not stable to washing with detergent.

To avoid washing completely, a competition assay was then developed, in which the labeled antibody was introduced in the subphase concomitantly with excess ($\times$ 10 approximately) of unlabeled anti-HSA antibody. The task of the latter is to remove any nonspecific interactions of the antibody with the

monolayer. The fluorescently labeled antibody (2.5 $\mu g\,mL^{-1}$) was incubated under these conditions with unlabeled monolayers of cholesterol and epicholesterol, and observed directly under the fluorescence microscope, without any washing procedure. A fluorescent pattern was observed, on the background of the bulk solution fluorescence (Figure 4A) for the cholesterol monolayer, but not for the epicholesterol monolayer (Figure 4C). The monolayers were then lifted onto

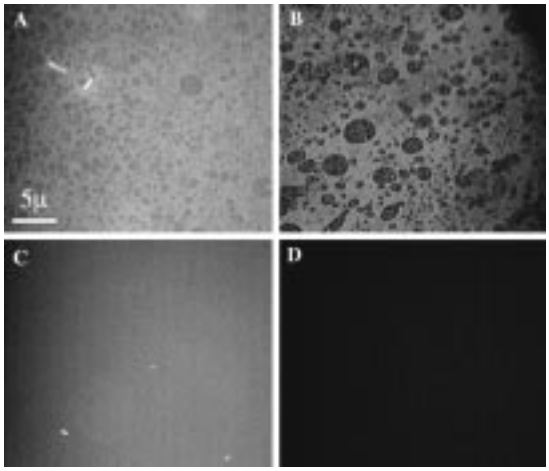

Figure 4. A) Fluorescence image of the antibody(labeled)-cholesterol monolayer(unlabeled) complex formed by antibody 36A1 with primary labeled antibody imaged directly at the air–water interface. The background intensity is due to bulk antibody fluorescence. B) Same as A) after lifting onto an OTS pretreated slide. C) Fluorescence image of the antibody(labeled)-epicholesterol monolayer(unlabeled) complex formed by antibody 36A1 with primary labeled antibody. D) Same as C) after lifting onto an OTS pretreated slide.

OTS-treated glass slides, the slides were observed under the fluorescence microscope, and their wettability was tested by contact angle measurements. All slides had the wettabilities typical of monolayer-covered OTS-glass (95°). Again, distinct fluorescence patterns with morphologies highly reminiscent of the cholesterol monolayer's were observed for cholesterol by using a shutter speed of 0.04 s (Figure 4B). Under these conditions, no fluorescent patterns were observed for epicholesterol (Figure 4D). When the shutter speed was set at 0.32 s (i.e, 8 times slower) weak fluorescence was detected, thus confirming the presence of monolayer on the slide. To avoid artifacts, the experiments were performed each time on the two monolayers in series on the same day and were repeated at least four times independently.

We conclude that antibody 36A1, raised and selected against cholesterol monohydrate crystals, interacts selectively with uncompressed cholesterol monolayers at the air–water interface, but not with monolayers of epicholesterol.

Discussion

We have demonstrated that a monoclonal antibody, raised and selected against crystals of cholesterol monohydrate, is capable of stereoselective recognition in two-dimensions of monolayers of cholesterol, but not of epicholesterol. The stereoselectivity is particularly significant as the two monolayers have the same chemical composition, but differ in their molecular structure at the air–water interface.

Antibody complexation was monitored by epifluorescence microscopy, by using a fluorescently labeled antibody introduced into the monolayer subphase. All experiments were performed in series with cholesterol and epicholesterol monolayers several times, using the same antibody solutions, during the same day.

Epicholesterol monolayers are not stable upon washing with solutions containing surfactants, as a result of interaction between the surfactant and epicholesterol. The complex is progressively dissolved in the subphase and is removed during the washing procedure. In the past, fluorescently labeled secondary antibodies have been used in a procedure that requires extensive washing in the presence of detergent.[18] When a fluorescently labeled secondary antibody was used in the assay, the antibody was observed to form large aggregates under the monolayer of cholesterol. The fluorescent aggregates were observed both with fluorescently labeled or unlabeled monolayers. They are thus formed by the antibody and not directly by the monolayer molecules. In contrast, when the assay was performed with labeled primary antibody and unlabeled monolayer, the fluorescence was uniform and followed the characteristic morphology of the monolayer. We assume that the formation of the aggregates is a result of the repeated washings. During the flow of the washing solution, visible turbulence occurs at the interface; this probably induces aggregation of the large antibody complexes. This dynamic process does not alter the monolayer morphology, which remains unchanged after washing is carried out. Negative controls included the use of the labeled secondary antibody alone, with both labeled and unlabeled monolayers. To avoid the formation of these aggregates and to better understand the mode of binding, we opted for direct observation of the fluorescently labeled antibody complexation to the monolayer.

Cholesterol (**1**) and epicholesterol (**2**) (Scheme 1) form stable monolayers at the air–water interface. The monolayers, as observed in our experiments under the epifluorescence microscope in the uncompressed state, are morphologically similar. At the micrometer scale, both monolayers cover continuously extended areas, interrupted occasionally by empty regions. The pressure area isotherm for cholesterol is in agreement with data reported previously.[19–21] In epicholesterol, the effect of epimerization is observed both in the collapse pressure and in the area per molecule, which are smaller than the respective values measured for cholesterol. Although monolayers of epicholesterol have previously been reported in the literature, there are no reports of the corresponding isotherms. For both molecules, the hydrophobic backbones extend out of the water, while the hydroxyl at 3α- (axial) or β- (equatorial) position is in the water. The very different angle between the hydroxyl and the rigid backbone (Scheme 1) must influence the structure and molecular organization of the interface. The structure of compressed cholesterol monolayers at the air–water interface was recently determined by using grazing incidence X-ray diffraction (GID).[17] Cholesterol forms crystalline

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domains of coherence length of 100 Å, with the steroid backbone tilted approximately 15° relative to the vertical. According to the measured area per molecule of epicholesterol, the molecule is presumably even less tilted. This was confirmed by recent measurement of epicholesterol monolayers by GID (L. Leiserowitz, personal communication). The hydroxyl groups must thus be arranged in the water forming a very low angle to the interface.

Antibody 36A1 selectively interacts with the {301} faces of cholesterol monohydrate crystals. At the surface of this face the hydrophobic cholesterol backbones are exposed on one side of the molecular steps, while hydroxyl and water groups exclusively are exposed on the other side of the steps. The antibody binding site, as predicted by molecular modeling,[16] assumes the structure of a step with an hydrophilic "pavement", which exposes polar residues, and an hydrophobic "wall". The monolayer probably exposes three-dimensional structural features at domain boundaries akin to the molecular arrangement exposed at molecular steps on the cholesterol monohydrate crystal face. We thus assume that the antibody binds at the boundaries of monolayer domains, with its hydrophobic amino acid side-chains interacting vertically with the cholesterol backbones and its hydrophilic side under the monolayer. Under these conditions, the antibody differentiates between cholesterol and epicholesterol monolayers. Recognition based exclusively on chemical affinity of the hydrophobic and hydrophilic groups may thus be excluded, because both monolayers have identical compositions. The antibody does not interact with cholesterol molecules which are bound to a macromolecular carrier,[15] so that binding akin to that of haptens is also ruled out. The stereoselective nature of binding must thus be attributed to structural, as well as to molecular, organization. One of the consequences of this analysis is that the structure of the binding site of antibody 36A1 must be quite rigid. Conformational freedom would presumably allow the antibody to adapt to the geometrical features of the two monolayer arrays, such as the different dihedral angles of the step boundary. In contrast to the step geometry, lateral rearrangement of the molecules in the monolayer is presumably easy to achieve, because in the uncompressed monolayer the molecules have high lateral motility.

Stereoselectivity in monolayers of cholesterol and epicholesterol has been previously observed with the antibiotic filipin.[22] Filipin interacts specifically with cholesterol by forming a (1:1) filipin-cholesterol complex. Norman et al. showed that filipin interacts stereoselectively with monolayers of cholesterol but not of epicholesterol.[23, 24] Filipin presumably interacts with cholesterol in a manner similar to enzyme–substrate interactions. Other sterols used in this experiment support this model and reveal that the stereoselectivity is preferentially manifested in binding to the hydroxyl group rather than to rings A–D of the steroid backbone. It is expected, therefore, that a change in the molecule, such as epimerization, will prevent binding. We stress however that the filipin stereoselectivity is different from that of antibody 36A1. Whereas filipin discriminates between molecular epicholesterol and cholesterol, antibody 36A1 discriminates between their corresponding structural arrays. Filipin recognizes the molecules as isolated components. In contrast, antibody 36A1 recognizes a pattern of exposed molecules with distinct structural organization.

The idea that an antibody may be structured to stereoselectively recognize an array of molecules, possibly provides a new approach to understanding immunorecognition in submolecular detail. To which degree stereoselectivity can be reached in antibody-surface recognition is yet to be determined. It will be interesting to test whether the antibody is capable of enantioselectively recognizing an array of molecules. In this case, the monolayers to be tested would be structurally identical, but one will be the mirror image of the other (work in progress).

In conclusion, we have shown that an antibody, raised and selected against crystals of cholesterol monohydrate, stereoselectively recognizes monolayers of cholesterol but not of epicholesterol, its epimer. This may provide a recognition tool that could be used in many interesting applications, from recognition of artificial surfaces, such as molecular sensors, to targeting of defined molecular arrays in biological membranes.

Experimental Section

Materials: Epicholesterol was purchased from Steraloid, Rhode Island. Cholesterol (>99%), rhodamine-B-isothiocyanate, cholesteryl tosylate, dry DMSO, and monoclonal anti-human albumin (anti-HSA) were purchased from Sigma. Rhodamine-TRITC-conjugated goat-anti-mouse $F(ab)_2$ was purchased from Jackson ImmunoResearch (West Grove, PA). Square glass slides (20 mm) were purchased from Knittel GLASER (Germany). For flash chromatography, silica gel with a 0.04–0.06 mm particle diameter (Merck) was used.

Antibody purification. Monoclonal antibody 36A1 was raised and selected against crystals of cholesterol monohydrate (for isolation procedure see ref. [15]). The antibody was purified from ascites fluid by affinity chromatography using ImmunoPure® IgM purification kit #44897 (Pierce, Rockford, IL).

3α-cholesteryl-rhodamine-B synthesis. Cholesteryl tosylate (1 g) was dissolved in DMF (10 mL). NaN_3 (180 mg) was added, and the mixture was heated while stirring to 80°C for 3 h. The disappearance of cholesteryl-tosylate was followed by TLC (hexane:chloroform 7:3). Water (50 mL) was added and the solution was extracted with hexane, dried with sodium sulfate, and evaporated to dryness. The crude product was directly subjected to reduction: $LiAlH_4$ (0.6 gr) in dry diethyl ether (60 mL) was added to the white solid and the solution was stirred over night. Diethyl ether was added, and the mixture was extracted with water. After drying and evaporation of the organic layer with sodium sulfate, a green liquid was obtained. Flash chromatography (silica gel, chloroform/methanol 9:1) yielded white crystals of 3α-cholesteryl amine (200 mg). MS-I(+): m/z: 387 I-I; ^{1}H NMR (250 Mhz): $\delta = 5.42$ (d, 1H), 3.43 (s, 1H), 0.65 (s, 1H).

3α-cholesteryl-amine (5 mg, 1 equiv) was dissolved in chloroform (2 mL), rhodamine-B isothiocyanate (7 mg, 1.1 equiv) was added, and the mixture was stirred overnight. The presence of the product was confirmed by TLC (chloroform/ethanol 9:1). The mixture was evaporated to dryness and diluted in chloroform to the appropriate required concentration.

Antibody-rhodamine conjugation: Sodium carbonate buffer (1N, pH 9, 30 μL) was added to the purified antibody (0.657 mg mL^{-1}, 270 μL). Rhodamine-B-isothiocyanate dissolved in dry DMSO (1 mg mL^{-1}, 40 μL) was added to this solution dropwise over 2 h, whilst being stirred. The mixture was stored at 4°C for 6 h and NH_4Cl (10 μL, 50 mM) was added. The solution was stored for additional 2 h and then subjected to dialysis (4 changes, PBS). The average degree of labeling was eight rhodamine molecules per antibody molecule, as estimated by measurement of

absorption of protein ($\lambda = 280$ nm, $\varepsilon = 675\,000$) and rhodamine ($\lambda = 572$ nm, $\varepsilon = 92\,000$).

The trough: Two syringes, which were connected to the Teflon trough ($20 \times 20 \times 4$ mm^3) by Teflon tubes, were positioned one opposite to the other so that they formed a closed flow system. Synchronous and homogenous solution transport under the subphase was performed when one of the syringes served as an input reservoir, while the second simultaneously collected the output solution.

Monolayer deposition: Monolayers are deposited by spreading a solution of cholesterol (2.5 μL) in chloroform (2×10^{-4} M) on phosphate buffer (PBS, pH 7.4, 3 mL).

Surface pressure measurements: Surface pressure was measured by the Wilhelmy plate method by using a thermostatic trough (KSV, Finland).

Immunolabeling with a secondary fluorescent antibody: In each immunofluorescence assay, the labeled or unlabeled monolayer was incubated for 1 h in the trough with the specific antibody, diluted in PBS to a concentration of 2.5 μg mL^{-1}. Unbound antibody was removed by washing with 0.05 % Tween 20 in PBS (20 mL). The monolayer was then incubated for 1 h with 3 mL rhodamine-TRITC-conjugated goat-anti-mouse F(ab)$_2$ diluted 1/100 from its concentrated solution in PBS. The subphase was exchanged again by flushing with 0.05 % Tween 20 in PBS (20 mL). The monolayer was imaged under a fluorescence microscope with a rhodamine filter setting (Zeiss, Germany) by using a video camera equipped with an integration attachment (Applitec, Inc. MSV-700L). Shutter speed was set at 0.32 s. Magnification $\times 80$ or $\times 160$.

Immunolabeling with a primary fluorescent antibody: In each immunofluorescence assay, the monolayer was incubated for 1 h with the labeled antibody diluted in PBS to a concentration of 1.25 μg mL^{-1} jointly with anti-HSA antibody diluted 1/10 in PBS. The monolayer was imaged under a fluorescence microscope as specified above (the shutter speed was set at 0.04 s). The monolayer was lifted onto an OTS slide and its fluorescence image was documented (shutter speed was set at 0.04 s). At least five images were randomly taken from different areas of the monolayer.

OTS coating: OTS coated slides were prepared according to the procedure of Sagiv et al.(ref. [25]).

Lifting procedure: The monolayers are lifted onto OTS coated slides by touching the solution interface from the air side with the OTS slide previously cleaned with chloroform.

Contact-angle instrument: Static contact angles (advancing) were measured under ambient conditions with an NRL contact-angle goniometer (model 100, Rame-Hart) by using the sessile drop method. The reproducibility of these measurements is ca. $\pm 2^\circ$ in the range above 90°.

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