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# Surface plasmon resonance as detection tool for lipids lateral mobility in biomimetic membranes

**Giancarlo Margheri,[1,*] Riccardo D'Agostino,[2] Lucia Becucci,[3] Rolando Guidelli,[3] Bruno Tiribilli,[3] and Mario Del Rosso[2]**

[1]*Institute for Complex Systems of National Council of Researches, Via Madonna del Piano 10, 50019 Sesto Fiorentino, Firenze, Italy*
[2]*Department of Pathology and Experimental Oncology, Viale G.B.Morgagni 50, 50134 Firenze, Italy*
[3]*Department of Chemistry, Via della Lastruccia, 3, 50019 Sesto Fiorentino, Firenze, Italy*
**giancarlo.margheri@isc.cnr.it*

**Abstract**: A procedure based on surface plasmon resonance (SPR) is proposed to monitor the lateral mobility of lipid molecules in solid-supported bilayer lipid membranes (ssBLMs), an essential prerequisite for the formation of important microdomains called lipid rafts (LRs). The procedure relies on the marked tendency of the ganglioside GM1 to be recruited by LRs and to act as a specific receptor of the beta-subunit of the cholera toxin (ChTB). In the presence of both GM1 and ChTB, spontaneous formation of lipid rafts domains in mobile ssBLMs is accompanied by an appreciable increase in the amount of adsorbed ChTB, as monitored by SPR.



**OCIS codes:** (240.6680) Surface plasmons; (170.1420) Biology.

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## 1.Introduction

Solid supported bilayer lipid membranes (ssBLMs) are excellent templates for studying *in vitro* the interactions of the cell membrane with several biological molecules (ionic pumps, signaling agents, etc.) [1]. A satisfactory fluidity allows ssBLMs to reorganize upon interaction with external perturbations, mimicking the functionality of living cell membranes, in which the lateral mobility of lipids is of the order of $10^{-8}$-$10^{-9}$ $cm^2s^{-1}$ [2,3]. In particular, fluidity determines the spontaneous separation of the lipids of the cell membrane (demixing), giving rise to the formation of lipid rafts (LRs) [2]. LRs are "liquid-ordered" ($l_o$), tightly packed microdomains, consisting primarily of cholesterol (Chol) and sphingolipids, such as palmitoylsphingomyelin (Pasm); they are immersed in a liquid-disordered ($l_d$) matrix, consisting primarily of phospholipids with a low melting temperature, such as dioleoylphosphatidylcholine (Dopc).

In these domains, microenvironmental effects on protein functional properties can occur. For this reason, the presence of LRs-like regions in ssBLMs is necessary to better mimic the physiological membrane functions, with particular respect to the important pharmacological implications of such effects [4,5]. Hence, the solid supports on which ssBLMs are grown must ensure the proper mobility for lipids because LRs-like domains may demix efficiently .

Fluidity and lateral mobility of ssBLMs can be characterized quantitatively by AFM and fluorescence microscopy. Each of these techniques exhibits specific advantages as well as some drawbacks. Thus, AFM [6–8] requires very smooth solid supports, such as mica, and expensive equipment for accurate environmental controls. Fluorescence diagnostics [9] need lipids labeled with fluorophores. Besides the typical problem of their short lifetimes (usually of the order of minutes), these markers cannot be in close proximity to metals in order to avoid strong reduction of the light emission [10], that hampers their use in conjunction with electric diagnostics (EIS, cyclic voltammetry).

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Lipid separation has been observed by exploiting quasi-mode propagation in Plasmon Waveguide Resonators (PWRs) [11]. In this case, however, guided modes cannot propagate if the waveguide has a thickness lower than a certain limit (the cut-off thickness) usually of the order of hundreds of nanometers. For this reason, PWRs cannot be exploited to study the assembly of lipids on thin growing platforms (e.g., self-assembled monolayers), that are desirable in several situations [12,13].

In this paper, we propose a different approach to compare the lipid fluidity on different solid supports, based on the use of SPR. Like PWRs, SPR belongs to the family of spectroscopic techniques based on evanescent wave optics, but where light propagates at an interface rather than in a bulky structure. It is commonly used for the determination of refractive index, dielectric constant, and layer thickness with high sensitivity and resolution [14,15]. However, SPR is not capable of directly monitoring the rearrangement phenomena of lipids, because refractive index changes associated with the lipid reorganization are too small. This problem can be circumvented by exploiting the accumulation of the monosialotetrahexosylganglioside (GM1) on mobile ssBLMs with proper composition.

GM1 belongs to the group of glycosphingolipids, which contain one residue of the N-acetylneuroaminic acid. GM1 locates preferentially in LRs of the cell membrane, which hosts the two hydrocarbon chains of its ceramide moiety, while its oligosaccharide headgroup sticks out of the membrane. It has important physiological properties impacting neuronal plasticity, repair mechanisms, and the releasing of neurotrophins in the brain [16]. Besides its function in the physiology of the brain, GM1 acts as the binding site for enterotoxin from *Vibrio cholerae*, the cholera toxin (ChT), that in this way can initiate the cholera intoxication process. The binding between GM1 and ChT occurs when the oligosaccharide headgroup of GM1 penetrates into one of the five anchoring pockets of its beta subunit (ChTB) [17], a huge molecule with 11.6 kDa molecular weight, consisting in an annular pentameric ring whose external diameter and height are respectively 6 and 4 nm.

The proposed procedure, outlined in Fig. 1, takes advantage of the following properties: (i) if lipids have lateral mobility, Dopc, Pasm and Chol mixtures (briefly DPC mixtures) demix, giving rise to lipid rafts-like domains; (ii) if GM1 is present in the mixture (DPC:GM1), it is well established that during demixing it accumulates preferentially into the lipid rafts domains [3,18,19]; (iii) thanks to dimensions and mass of ChTB molecule, its accumulation on a GM1-enriched surface can be easily measured with SPR diagnostic [17,20]. Thus, if a lipid bilayer moiety (or even the sole distal lipid monolayer) of a ssBLM consists of DPC:GM1, an appreciable increase in film thickness upon addition of the ChTB, as monitorable by SPR,

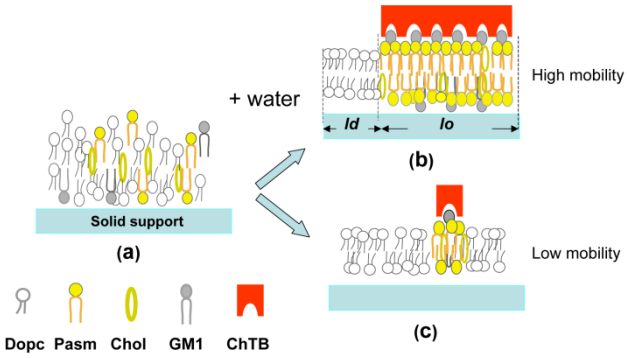


Fig. 1. (a) The DPC:GM1 mixture essiccated onto a solid support, is exposed to water. (b) The formed ssBLM demixes into lipid rafts ($l_o$) and a liquid disordered matrix ($l_d$) if the lipids have high lateral mobility, recruiting a large amount of GM1 in the lipid rafts; (c) a much lower or no demixing, and a correspondingly lower amount of GM1, is observed if lateral mobility is low or absent. The presence of GM1 is recognized by its specific binding to ChTB, easily detected via SPR measurements. Thus, the higher amount of ChTB corresponds to a higher mobility of the ssBLM.

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denotes lipid lateral mobility. To confirm this conclusion, replacement of the DPC:GM1 by a Dopc:GM1 mixture on the same solid support, must give rise to an appreciably smaller thickness, under otherwise identical conditions. An even stronger confirmation is expected from the use of the same raft-forming mixture on a ssBLM that is supposed to lack lipid lateral mobility. In this work the lipids have been deposited onto monolayers of two different hydrophilic "spacer" molecules capable of anchoring to gold and conveying nominal different mobility to the ssBLMs grown upon them.

## 2. Experimental section

### *2.1. Instruments*

SPR instrumentation is a RT 2005 setup (RES-TEC Resonant Sensor Technology) based on the angular interrogation of a Kretschmann stage (a SF4 prism oil-matched to an optical transducer). The deposition processes on the transducers are monitored by acquiring the reflectivity of the stage while spanning over incidence angle (SPR spectra).

The best fit of the spectra allows to calculate the resonance angles $\theta_{sp}$ corresponding to their reflectivity minima. The laser spot, on which the optical response is space-averaged, has a diameter of ~0.2 mm. A Teflon cell is hermetically sealed on the Kretschmann stage and hosts the solutions to be incubated.

### *2.2. Samples preparation*

As first step, we fabricated the plasmonic transducers by depositing a 50 nm layer of gold (purity 99.95%, Goodfellow, Huntington, UK) onto glass slides (Schott SF4 with laser-finish surface polishing) by electron-beam evaporation under high vacuum conditions (pressure = $5x10^{-6}$ mbar).

Then, the optical transducers were functionalized with two different molecular spacers. One spacer, the hydrophilic tetraoxyethylene glycol-D-L-alpha-lipoic ester (TEGL) [21], is terminated with a hydroxyl group; self-assembling a lipid film on top of it gives rise to a mobile floating lipid bilayer, whose thickness is about 5 nm [12]. The other spacer, the thiolipid 2,3-Di-O-phytanyl-sn-glycerol-1-tetraoxyethylene glycol-D-L-alpha-lipoic ester (DPTL) [22], has a lipid monolayer covalently linked to the hydrophilic spacer; hence, self-assembling a lipid film on top of it gives rise to a lipid monolayer (2- 3 nm thick [2]) that completes the lipid bilayer moiety of the ssBLMs that, for this reason, are expected to be scarcely mobile on the gold-linked hydrophilic spacer. Thus, Au|DPTL bilayer will be the reference growth platform to compare the results obtained with Au|TEGL.

The functionalization of the transducers was performed by incubating them in the SPR cell for 24 hours with an ethanol solution of the DPTL thiolipid (0.2 mg/ml) or of the TEGL spacer ($10^{-3}$ M) to self-assemble a DPTL or TEGL monolayer. They were then washed with ethanol until a steady state SPR spectrum was achieved.

After the functionalization of the transducers, ssBLMs:GM1 were generated atop of them following the procedure outlined in the Introduction.

The DPC mixture was obtained dissolving Dopc, Pasm, Chol (Avanti Polar, Alabaster, Alabama, USA) in hexane in (59:15:26) %mol concentration, typical of mammalian plasma membranes. The stock solution of GM1 (SantaCruz Biotechnology, California,USA), was obtained dissolving the ganglioside in a 4:1 vol. mixture of chloroform-methanol. GM1 was added to DPC or Dopc solutions to a concentration 20% mol to obtain DPC:GM1 and Dopc:GM1 mixtures.

A lipid film was self-assembled on top of the gold-supported DPTL or TEGL monolayer by adding to the SPR cell 30 µl of a hexane solution of 28.6 µg of the desired lipid mixture.

After complete evaporation of hexane, which required about 1 hour, two rinses with water were sufficient for a first reorganization, completed in about 15 hours.

The adoption of a self-assembly procedure starting from dried lipids was chosen in alternative to the widely adopted vesicles fusion to form ssBLMs, because it has been shown that lipid vesicles have a low tendency to fuse on a hydrophobic thiolipid monolayer [12],

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which in our case is the spacer DPTL chosen for the reference experiments. Although less refined than other methods (Langmuir Blodgett and Langmuir Schaefer transfers) it is very easy to apply and has just proven effective to assemble ssBLMs on silver [13].

After the completion of the ssBLM stabilization, the effective recruitation of GM1 in the ssBLM was then checked by injecting in the incubation cell a ChTB water solution, obtained by dissolving ChTB (Sigma Aldrich, St. Louis, Missouri) in MilliQ ultrapure water in a $0.86 \times 10^{-7}$ M concentration. The SPR spectra were taken at different incubation times and the corresponding plasma angles were calculated by their best fit. The association kinetics were then obtained by plotting the resonance angles versus time. At the end of each of ChTB-GM1 association processes, we carried out three rinses with pure water in order to eliminate nonspecific weak binding of ChTB on ssBLMs and evidence any dissociation kinetics of the complexes ChTB-GM1.

## 3.Results

Figure 2a shows the evolution of the SPR angular spectra recorded in a typical test performed assembling a DPC:GM1 on Au|TEGL and subsequently injecting a ChTB solution for the GM1 recognition. TEGL chemisorption on gold caused an angular shift of the resonance

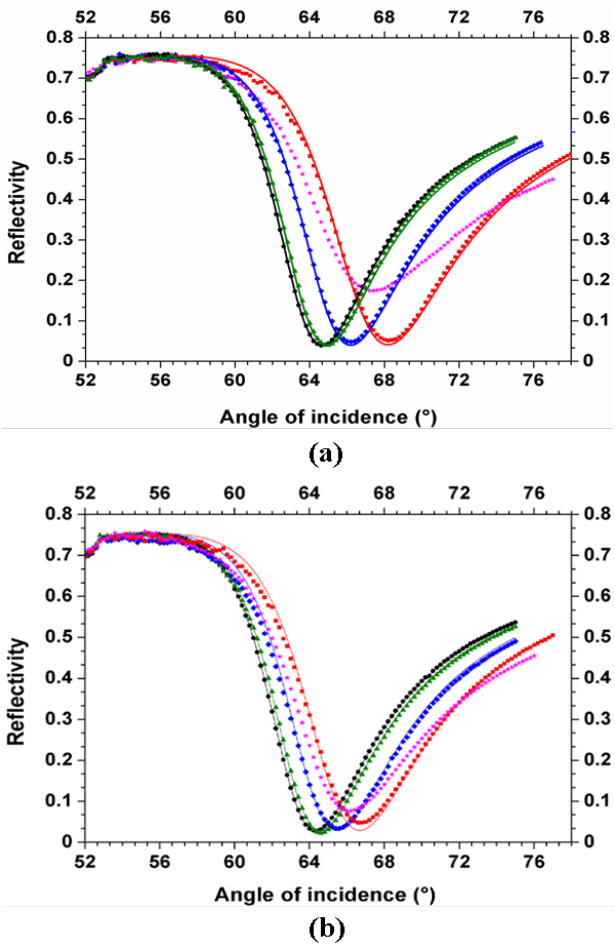


Fig. 2. (a) Experimental SPR spectra (dotted curves) and their best fits (solid curves) at a 50 nm thick Au layer without (black ●) and with (green▲) TEGL, with Dopc:GM1 on TEGL before reorganization (pink ★), after 2 rinses with water and 15 h incubation (blue ♦), and after 20 h of incubation with ChTB in water (red ■). (b) the same steps with Dopc:GM1.

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angle by 0.25° (green curve ▲) with respect to that (black curve ●) of the sole gold film.Upon ascribing to TEGL a refractive index of 1.50 [23], this shift yields a thickness of 1.5 nm for the TEGL monolayer, which is consistent with the calculated length of this molecule [23], while the almost parallel shift of the spectra suggest a nice uniformity of TEGL bilayer, close to that of the sole gold.

Subsequently, a DPC:GM1 solution is allowed to dry on the Au|TEGL bilayer, and the multilayer Au|TEGL|DPC:GM1 is exposed to water. At the onset of the lipid organization in presence of water, the SPR spectrum (labeled with pink ★ in Fig. 2a) revealed the formation of a thick, nonuniform layer that, after rinsing twice with water, became thinner and more uniform. The subsequent phases of the membrane stabilization are very slow. In fact, the SPR spectra attained stabilization (blue curve ♦) after an incubation time of 15 hours. The resonance angle was right shifted by 1.3° with respect to that for the Au|TEGL|water multilayer, while the minimum of the SPR reflectivity curve (~0.02) and full width at half-maximum (FWHM) (3.1°) changed only slightly. Upon ascribing to the refractive index of the lipid the value of 1.47 [24], the shift in $\theta_{sp}$ yields an average value of (6.0 ± 0.3) nm for the lipid film thickness, in good agreement with that, 5.0 nm, reported for a lipid bilayer on top of a thiopeptide hydrophilic spacer [22]. Moreover, the FWHM value, being close to that of the sole TEGL, indicates a uniformity of the lipid bilayer comparable with that of the TEGL monolayer.

After ascertaining the stabilization of the SPR spectrum with two further water rinses, an aqueous solution of ChTB was injected into the cell. The progress of ChTB binding to GM1 was monitored by following the position of the resonance angle $\theta_{sp}$ as a function of time $t$. The shift of the resonance angle with respect to its value just before the binding (that will be referred to as angular shift), is proportional to the mass of the bound ChTB [14]. The plot of the resonance angle shift versus time shown by curve (blue ■) in Fig. 3, attains a regime value of 2.1°, corresponding to the minimum of the spectrum (red ■) of Fig. 2a.

In further tests, the DPC:GM1 mixture was replaced by a Dopc:GM1 (80:20) %mol mixture.

By performing exactly the same sequence of steps, the assembling of Dopc:GM1 membranes was monitored by SPR in the various phases. The spectra, reported in Fig. 2b, show that after two rinses and 15 hours incubation time Dopc:GM1 membranes are smooth and stable.

The plot of angular shift versus time resulting from addition of the ChTB water solution reached a regime value of 1.1°, as shown by curve (green ●) in Fig. 3. This indicates that, during the formation of the lipid bilayer, about one half of the GM1 present in the initial mixture did not incorporate into the bilayer, due to the lack of LRs-like domains, but accumulated at the edge of the gold slide.

To obtain further evidence in favor of LRs-like domains formation on the Au|TEGL support, all measurements carried out on Au|TEGL were also repeated on a Au|DPTL support. As already mentioned, lipid reorganization on top of the Au|DPTL support is expected to give rise to a single distal monolayer. This was actually confirmed by a maximum limiting shift of the resonance angle by 0.46° with respect to that for the Au|DPTL|water multilayer (data not shown), yielding an average thickness of (2.1 ± 0.1) nm for the lipid film on top of the DPTL monolayer. The shift of the resonance angle as a function of time, following the addition of an aqueous solution of ChTB on top of a Au|DPTL|DPC:GM1 multilayer, is shown in Fig. 3, curve (red □); it reaches a steady value of 0.48°. The analogous plot following ChTB addition on top of a Au|DPTL|Dopc:GM1 multilayer is shown in Fig. 3, curve (black ○), and attains a steady value of 0.38°.

The satisfactory repeatability of the measurements is shown by the error bars in Fig. 3. The small difference (0.1°) between the maximum limiting values of the angular shift at a DPC:GM1 mixture and at a Dopc:GM1 monolayer, both stabilized on the same Au|DPTL support, shows unequivocally that the distal lipid monolayer on such a support allows only a small lipid lateral mobility. At the same time, the difference between the angular shifts at a

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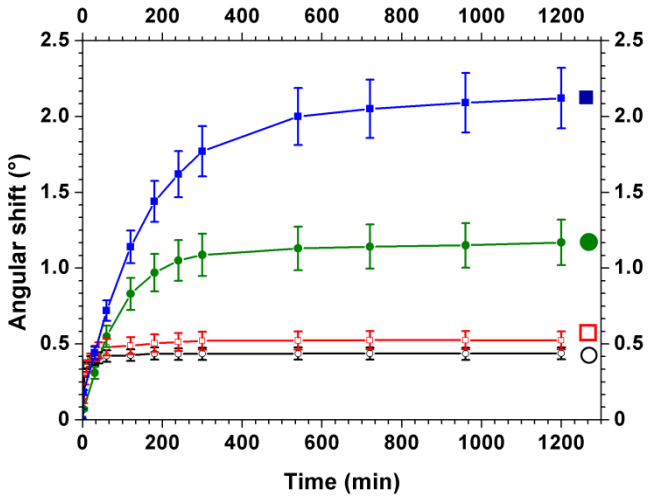


Fig. 3. Shifts of the resonance angle against time during the incubation of a $0.86 \times 10^{-7}$M water solution of ChTB, for DPC:GM1 on Au|TEGL (blue ■) and on Au|DPTL (red □), for Dopc:GM1 on Au|TEGL (green ●) and on Au|DPTL (black ○).

DPC:GM1 mixture and at a Dopc:GM1 monolayer on the Au|TEGL support being about 1°, confirms that the lipid bilayer on top of this support has an evident lateral mobility.

Water rinse cycles carried out at the end of the ChTB binding kinetics had no detectable effect. This indicates that nonspecific ChTB binding is negligible and represents a minor level of concern for the present measurements. This conclusion is in agreement with the lack of adsorption of bovine serum albumin (BSA) on a palmitoyloleoylphosphatidylcholine (Popc):GM1 monolayer, as opposed to a clear specific adsorption of ChTB [20]. The dissociation kinetics of the ChTB-GM1 bond, albeit expected after a series of water rinses, could not be detected with our measurements. Evidently, the well-known strength of the ChTB-GM1 [17,20] binding would have required too much time to allow the detection of resolvable dissociation phenomena.

In the kinetics of Fig. 3 it is worth noticing the different rise times of the association processes of ChTB with GM1 on Au|TEGL and Au|DPTL (curves blue ■ and red □ in Fig. 3).

The time dependences were fitted on the basis of exponential monophasic kinetics describing the pseudo-first order model of equilibrium binding reactions in SPR sensors [25] to calculate the apparent association constants $k_{ass}$ of the ChTB-GM1 complex. In this model, the association constant describing the exponential time evolution of the complexes formation is proportional to the receptors surface density, while the time evolution of the dissociation is described by the dissociation constant $k_{diss}$ . The ratio $K_D = k_{diss}/k_{ass}$, usually referred as the apparent equilibrium dissociation constant, increases when the binding strength is lower. The value of $k_{ass}$ on the Au|DPTL support equals $4 \times 10^4$ $M^{-1}s^{-1}$ and is much higher than that, $1 \times 10^3$ $M^{-1}s^{-1}$, on the Au|TEGL support. This is an unexpected result, as the model predicts a lower association speed for a lower GM1 concentration.

The difference between the observed $k_{ass}$ values on the Au|TEGL and Au|DPTL supports can be justified in light of reports describing a weakening of the ChTB-GM1 bond with an increase of ganglioside concentration in phospholipid membranes [26–29]. For example, it has been shown that binding of ChTB to liposomes containing 2% mol GM1 is tighter than for those containing 4% mol GM1 [27], while in [28] it was demonstrated a similar effect for CT binding to an intestinal microvillus membrane loaded with different GM1 amounts. The effects of GM1 loading on the binding kinetic with ChTB have been systematically investigated by Shi et al. using Popc:GM1 solid-supported membranes [29]. This study clearly demonstrates that the binding process is weakened by the clustering of the GM1 molecules. More specifically, the clustering evidenced by the AFM analysis suggests that the close

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proximity of ganglioside molecules in the phospholipid matrix favors H-bond formation between their oligomer headgroups. The steric hindrance of the coalescent headgroups hampers the penetration of the complexed moieties into the binding pockets of ChTB, lowering its binding probability to GM1. As a result, the association process slows down ($k_{ass}$ decreases) and the dissociation fastens ($k_{diss}$ increases), producing an overall increase of $K_D$ . Actually, the tests reported demonstrates an increase of ~9 times of $K_D$ when the GM1 concentration passes from 0.02% mol to 10% mol. A comparison with these data is not possible in our case, because of the lack of dissociation kinetics. However, upon making the rough assumption that the dissociation constant of the ChTB-GM1 complex on the Au|DPTL support is equal to that on the Au|TEGL support, a 40-fold increase of $K_D$ is estimated in passing from the Au|TEGL to the Au|DPTL support. This increase is of the same order of magnitude as that reported in [29], pointing to the GM1 clustering as a significant slowing factor in the association process.

## 4.Conclusion

In conclusion, our results show that lipid lateral mobility in ssBLMs on a solid support sustaining propagating surface plasmons can be verified via the GM1 enrichment of ssBLMs that occurs during the $l_o$-$l_d$ demixing. The amount of inglobated GM1 is proportional to the bound beta-subunit of the cholera toxin, whose abundance is easily detected by SPR *a posteriori*, after the ssBLM:GM1 reorganization has occurred. The present procedure relies on space-averaged measurements, and does not allow the real time monitoring of LRs-like microdomains nor quantitative mobility measurements, achievable by more sophisticated imaging systems, like fluorescence imaging or AFM.

Nonetheless, it is a label-free technique, has no constraints imposed by the thickness or smoothness of the solid support of the lipid film, it can be exploited to study ssBLMs in conjunction with electrical techniques (EIS, cyclic voltammetry), and it does not require expensive equipment or particular expertise in the hardware handling. This procedure is thus an easy route to select the proper substrates on which LRs-enriched biomimetic membranes have to be grown.

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