Polyethyloxazoline Monolayers for Polymer Supported Biomembrane Models

Thomas Lehmann and Jürgen Rühe*

Max-Planck-Institute for Polymer Research, Ackermannweg 10, 55128 Mainz, Germany

Abstract

The synthesis of polymer monolayers, which are designed to act as "cushions" for lipid membranes attached to solid surfaces, is described. Monolayers of poly(ethyloxazolines) which could act as a polymeric support for biomimetic membranes have been attached to silicon oxide or gold surfaces following different strategies for the surface-attachment of the polymer chains. The polymers are either bound to the surface through a chemisorption process ("grafting-to") or "grown" on the surface of silicon oxide substrates through a cationic ring-opening polymerization started from the surface of the substrate, creating a surface-attached polymer monolayer *in situ* ("grafting from"). The characterization of the surface attached films by various surface analytical techniques and the swelling behavior of the surface-attached polymer films in moist air are described. The non-specific adsorption of fibrinogen as a typical example for a strongly adsorbing protein onto the surface attached monolayers is studied.

Introduction

Huge efforts have been directed in the last years towards the development of biosensor devices [1]. Such devices are designed to detect very small quantities of biologically or biomedically relevant molecules, which are present in an analyt such as blood for example, even in the presence of chemically or structurally very similar compounds [2-10] and in the presence of compounds, which attach unspecifically to the device surface ("surface fouling"). Accordingly large programs in organic chemistry have been directed towards the synthesis of very complex receptor molecules, which are supposed to react with the desired target molecule in the analyt with high specificity and reliability.

An alternative approach to the problem is to accept mother natures 4 billion years head start on the design and construction of highly specific receptors and use molecules of biological origin instead of synthetic ones. In this case the receptors, mostly proteins, have to be attached

* To whom correspondence should be addressed

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to the surface of a transducer, which transforms the chemical signal of the receptor into an electrical signal. This signal can then be processed further by conventional electronics. A promising way to provide a hospitable environment for such receptor proteins and an approach to orient them in an appropriate way at the surface of the device is to incorporate them into a membrane system, which tries to mimic the plasma membrane of a biological cell. An important task for the design of such a system is to carefully balance the stability and the fluidity of the membrane system. To allow for sufficient mechanical stability the attachment to a solid substrate seems advantageous, however a very rigid immobilization at the surface of a solid substrate will prevent movement in the membrane and prevent the receptor from working properly. To allow for such a compromise between fluidity and stability the concept of a polymer supported biomembrane has been developed.

The polymer layer, which supports the membrane, acts as a molecularly thin buffer between the membrane and the surface of the substrate [1]. It prevents contact between the incorporated proteins and the solid substrate, because such a contact could cause severe denaturing of the receptor molecules. Additionally the polymer "cushion" allows for the adjustment of the mechanical properties of the attached layers. On the one hand the tethering to the solid substrate anchors the membrane to the support and holds the whole assembly in place. It provides mechanical stability to the system and prevents the layer from collapsing of floating off. On the other hand having a soft "cushion" between the immobile substrate and the membrane decouples them and allows for sufficient mobility in the membrane system [2-10]. Important parameters for the properties of the "cushions" are accordingly the swellability of the polymer support in an aqueous environment and the interaction with other polymer molecules or low molecular weight compounds. It has been shown using other polymer monolayer systems, that the polymer conformation can have very profound effects onto the swelling behavior of the films or onto the wetting behavior of such systems [11]. Variations in the graft density can influence the degree of swelling of polymer monolayers, which gives the ratio of the dry vs. the swollen layer, by more than an order of magnitude. Additionally the surface-attachment of polymer molecules can influence the entropic situation in such a case that strongly, that the surface attached monolayers are not wetted even by chemically identical polymer ("autophobic" behavior) [12]. If such a monolayer is coated by an identical layer of non-attached polymer the physisorbed material will form more or less quickly droplets and dewet the underlying polymer coated substrate.

In this paper we focus only on the developement of different strategies for the build-up of the polymeric support layer. We describe the synthesis and characterization of monomolecular layers of poly(2-ethyl-2-oxazoline)s attached to planar, solid substrates. The polymers were chosen as they are hydrophilic, are known to be protein compatible and allow the reaction with lipids and groups, which immobilize the polymer at the surface of substrate. As substrate materials gold and silicon dioxide layers on silicon wafers are employed. We report on the layer thickness and graft density of the surface attached monolayers as well as the swelling behavior of the attached layers in moist air.

Results and Discussion

To allow for the attachment of the poly(ethyloxazoline) monolayers to the surfaces of gold substrates, polymer molecules with disulfide anchor groups had to be synthesized as shown in figure 1. Details of the synthesis and the characterization of the monolayers has been published elsewhere [13,14].

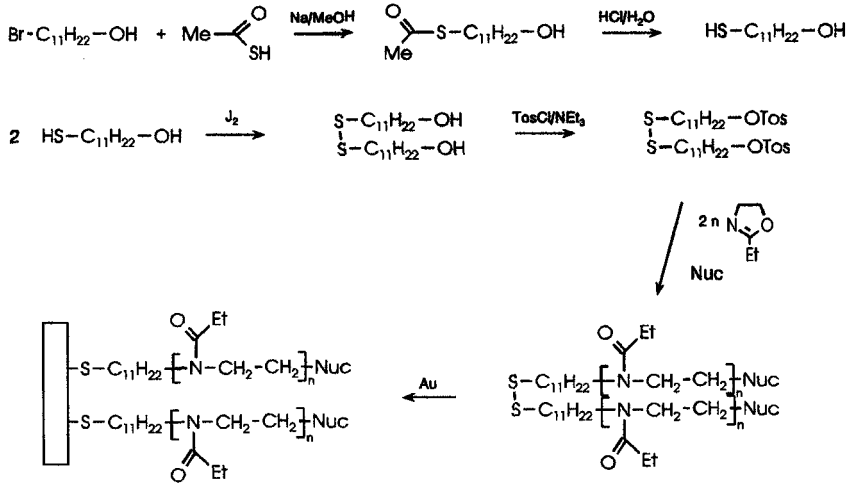

Fig. 1: Synthesis of poly(ethyloxazoline) monolayers attached to gold surfaces

To obtain the desired target polymers firstly tosylates of ω,ω'-dihydroxy alkyl disulfides were synthesized, which were used in a subsequent reaction step as initiators to start a cationic ring opening polymerization of 2-ethyl-2-oxazoline. The initiator was synthesized starting from 11-

bromo undecanol. The bromide was converted to a thiol via nucleophilic substitution with thioacetic acid and hydrolysis of the resulting thioester. The latter was oxidized with iodine to the corresponding disulfide-ω,ω'-diol and tosylated to give bis-11,11'-tosylato-undecyldisulfide, the desired initiator.

This was then used as a bifunctional initiator to start a cationic ring-opening polymerization of 2-alkyl-Δ2-oxazolines in solution. It has been shown, that, if the alkyl spacer connecting the disulfide moiety and the initiating site is long enough to ensure that the structural integrity of the disulfide is conserved during the polymerization process. A series of several poly(ethyl-oxazolines) with varying degrees of polymerization (P_n=5-150) and different endgroups (piperidine, dioctadecylamine and hydroxy) were synthesized (fig. 1). The endgroups were attached after consumption of all monomer by capping the living oxazolinium group with a nucleophile.

The polymers were chemisorbed from ethanol solution onto gold surfaces. The layer thicknesses of the monolayers after Soxhlet extraction were determined by surface plasmon resonance spectroscopy (SPR). With increasing length of the polymer molecules the layer thicknesses increases, but rapidly levels off at about 3.5 nm (Fig. 2).

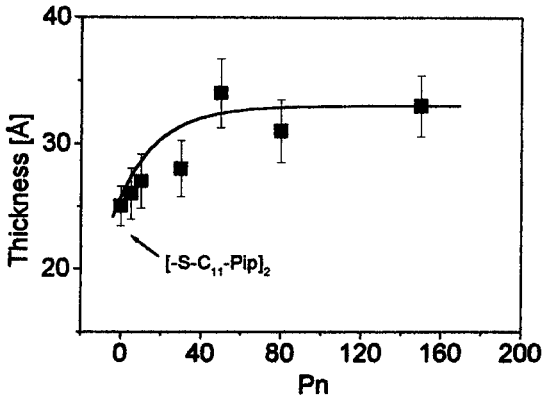

Fig.2: Thickness of chemisorbed poly(ethyloxaline) monolayers at gold surfaces as a function of the degree of polymerization P_n

As the layer thickness remains constant upon increasing of the chain length of the attached polymer molecules, the graft density of the chains must consequently be reduced with

increasing molecular weight of the surface-attached chains. The coupling between graft density and molecular weight is intrinsic to all chemisorption processes of polymers to solid surfaces. The reason for such a behavior is as described in the following. Polymer molecules arriving at a later stage of the chemisorption process have to diffuse against the concentration gradient, build up by the chains attached in the early stages of the process, in order to successfully bind to the surface. Once a certain critical segment density within the surface layer is reached, the surface-attachment of the polymer molecules slows down due to this limitation of diffusion and further attachment of chains takes place only on a very slow (i.e. logarithmic) time scale [15] (Fig.3). This kinetic limitation reduces the film thickness which can be obtained under practical experimental conditions to values which are typically between 3 and 5 nm. If now longer polymer chains are used for the chemisorption, the critical segment density at which the kinetics starts to control film growth, is simply reached at a lower graft density. In this case the layer thickness becomes independent of the length of polymer molecules.

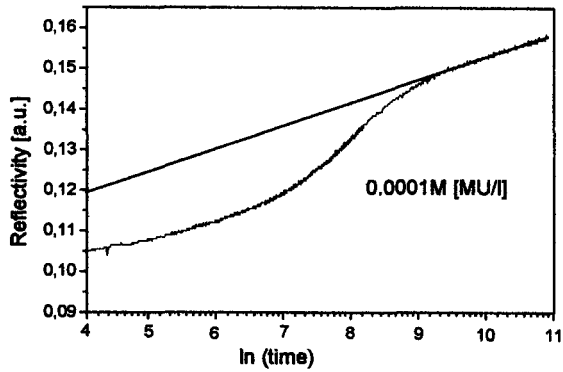

Fig. 3: Kinetics of the surface attachment of a disulfide modified poly(ethyloxazoline) at a gold surface as measured by SPR experiments

The number of molecules attached to the gold surface per surface area can be directly determined if the polymer chains are electrochemically cleaved off from the surface of the substrate and the number of charges flown during this process is determined. In Figure 4 cyclic voltammetry (CV) experiments are shown, in which polymers of different degrees of polymerization were attached to gold electrodes. From the integral of the reduction peak in the CV experiment the number of charges consumed and thus the number of cleaved Au-S bonds

can be directly inferred. It is evident that with increasing chain length of the polymer the area under the peak and thus the number of surface-attached polymer molecules is strongly reduced.

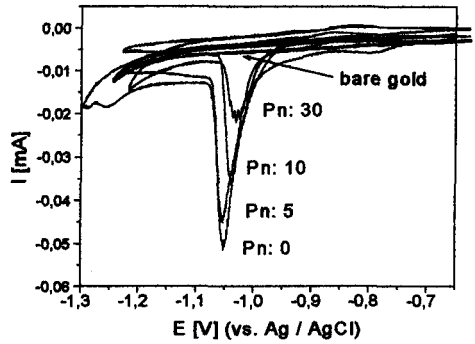

Fig. 4: Cyclovoltammogram of poly(ethyloxazoline) monolayers attached to gold electrode surfaces

One important question for the reproducibility of the synthesis of such systems is how the film thickness is influenced by the concentration of the polymer molecules in solution.

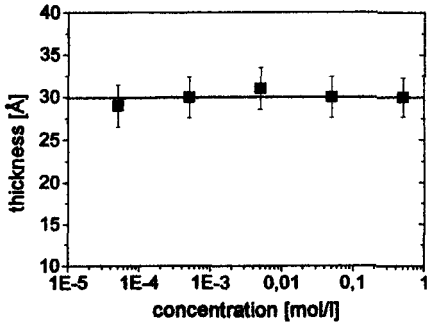

Fig. 5 Thicknesses of monolayers of disulfide modified poly(ethyloxazoline) self assembled from ethanol solution onto a gold substrate at different polymer concentrations

In Figure 5 the thicknesses of monolayers of one polymer are shown as a function of the concentration. It is clearly visible, that the layer thickness is not influenced by the concentration of polymer molecules present during the chemisorption reaction. This contrast strongly to the behavior of the surface-attachment of partially hydrolyzed poly(ethyloxazolines) attached to surfaces modified with appropriate "anchor" molecules in a step-wise chemisorption process resulting in multiple point anchoring of the polymers to the surface (Fig. 6).

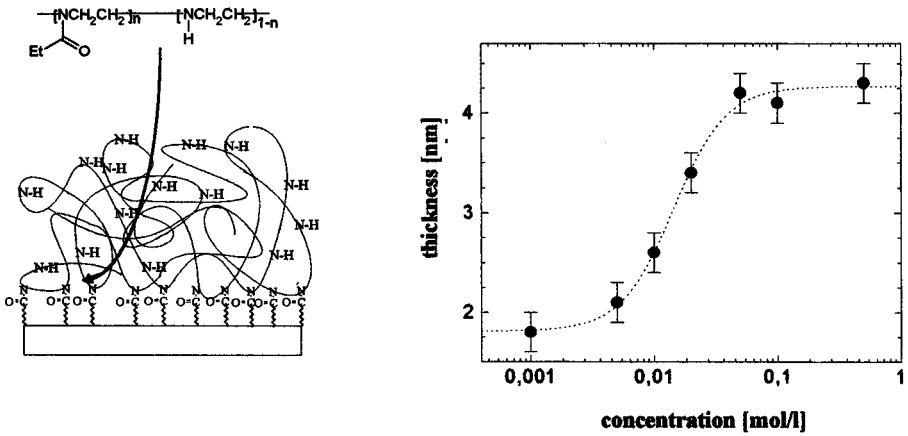

Fig. 6: Thickness of polyoxazoline monolayers obtained by a sequential approach in which poly(ethyloxazoline-stat-ethyleneimine) polymers are attached to N-hydroxy-succinimide self-assembled monolayers

The concentration dependence in the latter case can be explained if at first a situation is envisaged, were one reactive segment of the polymer molecule is attached to a corresponding site at the substrate (Fig. 6a). Other reactive groups, which are part of the same polymer molecule, can now also get into contact with the surface and become attached. As the surface attachment reaction is irreversible, the polymer is "frozen" into a non-equilibrium, pancake like structure. The polymer "rolls out" on the surface and many different segments of the same chain become attached to the surface. As all reactive sites at the surface of the substrate become consumed the attachment of further molecules from solution is prevented.

If in contrast to this the chemisorption process is carried out in highly concentrated solutions segments of other polymer molecules efficiently compete for the reactive sites on the surface. The number of attached molecules per surface area raises and consequently the film thickness increases. This increase, however, saturates at a certain value as at that point the thickness of the layer is no longer limited by the number of available reactive sites at the surface, but diffusion of the polymer molecules through the attached chains becomes the limiting factor as already discussed above. Thus an S-shaped curve is obtained when the layer thickness is plotted as a function of solution concentration (Fig. 6b).

It is important to note, that in both cases described above (chemisorption in one step or stepwise surface-attachment) the polymer is essentially the same, only the conformation of the chains at the surface is different.

"Growth" of poly(ethyloxzaolin) brushes

Independent of the exact nature of the chemisorption process the thicknesses all polymer layers obtained by such a "grafting-to" process are intrinsically limited as already discussed above. Due to kinetic reasons the layer thickness of such monolayers is restricted to a few nanometers for all practical experimental time scales. If higher film thicknesses are required the polymer molecules have to be "grown" at the surface of the substrate *in situ* [13]. To achieve this, a self-assembled monolayer of a tosylate initiator is formed at the surface of a substrate and a cationic ring-opening polymerization is started from this surface-attached monolayer (Fig. 7). In order to obtain a well defined ratio between monomer and initiator concentration "free" initiator is also added, which starts the generation of polymer molecules, which are not attached to the substrate surface.

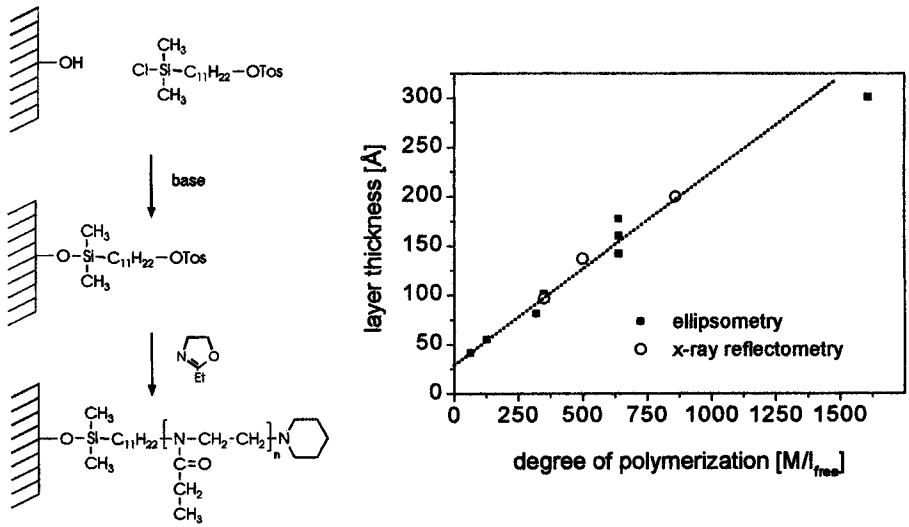

Fig. 7: a) Synthesis of poly(ethyloxazoline) monolayers by a "grafting-from" procedure; b) film thicknesses of the obtained layers

Following this "grafting-from" approach polymer monolayers of 20 - 30 nanometers can be synthesized easily. Thus the layers are about one order of magnitude higher than those, which can be obtained using a simple chemisorption process. The layer thicknesses of the monolayers

can be controlled by adjusting the monomer/initiator ratio (Fig. 7b) as expected for such a polymerization reaction.

Swelling of the Surface-attached Monolayers in Humid Air

One very important property of the synthesized polymer monolayers is how strongly the layers are swollen in an aqueous environment as analysis of biologically active molecules is usually carried out in water. As a first experiment into this direction the swelling of the layers in humid air was studied. The polymers were brought into contact with air, whose relative humidity was controlled by placing small vessels with concentrated salt solutions into the closed measurement cell. The equilibrium water vapor pressure at room temperature was controlled by using different salt solutions. The thickness of the layers was measured by SPR spectroscopy in situ (Fig. 8).

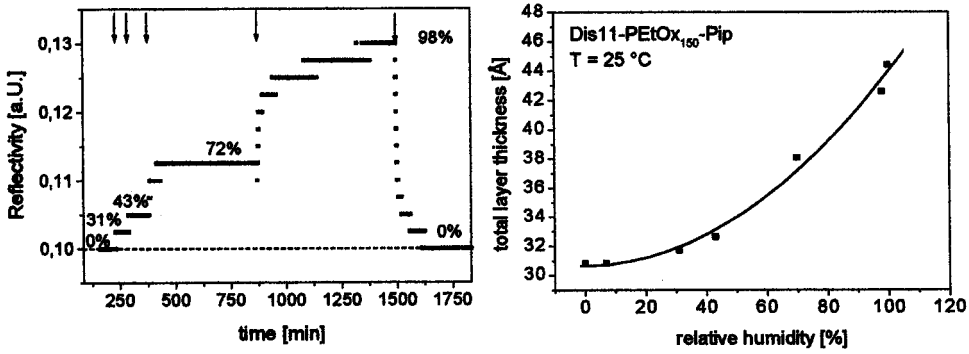

Fig. 8: Swelling of poly(ethyloxazoline) monolayers covalently attached to gold surfaces as a function of the relative humidity at room temperature; layer synthesis according to Fig. 1; a) SPR measurements, arrows indicate change of humidity; rel. humidity values are given in the graph b) thickness values obtained from SPR measurements

It can be seen, that the layer thickness increases up to 50% upon exposure to humid air (Fig. 8b).If poly(ethyloxazoline) brushes are employed even larger absolute increases of the film thickness due to water uptake in moist air can be realized (Fig.8). Experiments on the measurement of the film thickness of the layers, which have been brought into contact with bulk water are currently being carried out.

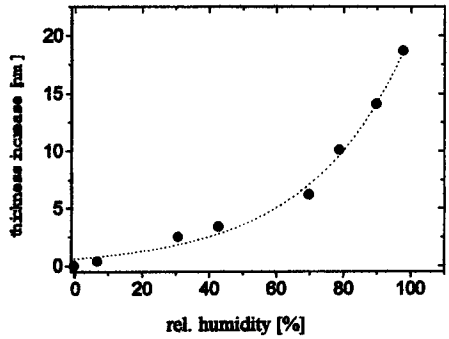

Fig. 9: Swelling of poly(ethyloxazoline) brushed in air having different relative humidities at room temperature; layer thickness at 0 % rel. hum. = 20 nm

Protein Adsorption Studies

As pointed out already above the resistance of the polymer layers against non-specific adsorption of proteins from the analyt is a very important requirement for the properties of a membrane support. If strong adsorption of proteins takes place the device will be covered after a short time with a thick layer of proteins, which limit access to the receptors for the molecules to be detected. To study the non-specific adsorption of proteins at the monolayers fibrinogen was chosen as a model compound, as it is known, that fibrinogen is a particular "sticky" protein, which adsorbs strongly at the surfaces of many different substrates. All experiments were carried out in a 0.1 % solution of the protein in phosphate buffer. The thickness of the protein layer was measured by SPR in situ. At first monolayers of the polymers having different degrees of polymerization were prepared on gold surfaces and brought into contact with the buffer solution (0.02 M phosphate buffer, pH = 7.5). After recording of an SPR spectrum the solution was exchanged against another part of the same solution, however, where fibrinogen has been added. The increase of the film thickness due to adsorption of the protein was followed (Fig. 10). When the film thickness did not increase any longer the sample was rinsed with pure buffer and again an SPR spectrum was recorded. From the angular shift before and after protein exposure the amount of adsorbed protein was calculated.

With increasing length of the polymer molecule the influence of the relatively hydrophobic initiator fragment, which remains in the polymer after completion of the polymerization process, decreases and the amount of adsorbed fibrinogen decreases. Indeed, when a polymer with a degree of polymerization of $P_n = 150$ was employed only 0 - 0.3 nm of fibrinogen could

be adsorbed and the polymer coated surfaces were almost completely resistant against non-specific adsorption of proteins.

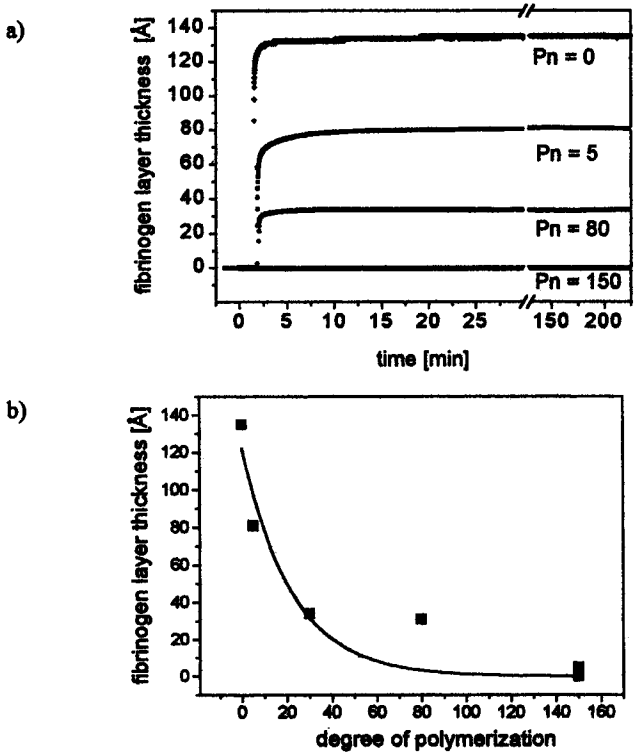

Fig. 10: adsorption of fibrinogen onto poly(ethyloxazoline) monolayers with varying degrees of polymerization; the layers are covalently linked to gold substrats; a) layer thickness as a function of adsorption time as measured by SPR; b) layer thickness as a function of degree of polymerization.

Conclusions

Poly(ethyloxazoline) monolayers have been prepared at the surface of silicon oxide and gold surfaces. While in the latter case the surface-attachment reaction is achieved through disulfide groups contained in the polymer molecules, in the former case the covalent bonds between polymer and substrate are generated through silane chemistry. The structure of the polymer chains, and accordingly the physical properties of the films, depend very strongly on the conditions of monolayer deposition. Under some conditions (multiple anchor points) very flat

conformations of the polymer molecules are obtained. If other monolayer preparation protocols are employed (i.e. "growth" of polymer molecules at the surface) the polymer chains are strongly stretched away from the surface. Accordingly film thickness and graft density of the polymer chains can be fine-tuned in a wide range. This allows to study directly the influence of the conformation of the chain "tethered" to the surface onto the macroscopic behavior of the monolayers.

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