Morphology of protein polymer hybrid films studied by atomic force microscopy and scanning confocal fluorescence microscopy

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Abstract

Protein–polymer hybrids can act as giant monolayer-forming amphiphiles at the air–water interface. Using biotinylated polystyrene (PS\textsubscript{b}) as the hydrophobic part and streptavidin (SAv) as a hydrophilic end of the giant amphiphile, monolayer formation and subsequent deposition leads to a well defined solid-supported monolayer, in which the remaining free sites of the polymer-bound SAv are accessible to biotin binding. The deposition conditions are evaluated by investigating the supported films by atomic force microscopy (AFM). Imaging of the PS\textsubscript{b} without protein reveals uniform monolayer formation. In situ binding of SAv indicates that the biotin is accessible. Deposition of the SAv-polymer hybrid film results primarily in a very similar morphology, indicative of the dominant role of the polymer in the monolayer formation. High resolution AFM revealed a homogeneously dense packing. This is confirmed by combined AFM and scanning confocal fluorescence microscopy (SCFM) on hybrid monolayers containing rhodamine-red-X labeled SAv. The biotin binding capability of the monolayer was assessed by binding of fluorescein labeled biotin and performing dual color excitation and detection. Biotin binding was uniform and homogeneous. Local spectra revealed that the fluorescein was largely quenched by the rhodamine, indicating an efficient energy transfer.

Keywords: Atomic force microscopy; Giant amphiphiles; Biotin-streptavidin

1. Introduction

Streptavidin (SAv) is a homo-tetrameric protein capable of binding four biotin molecules (vitamin H) in a non-cooperative way \cite{1–3}. The resulting complexes have very high affinity interactions \(K_0 \sim 10^{15}\) \cite{4}, which have even been studied by atomic force microscopy (AFM) \cite{5–8}. These binding properties together with the stability of the protein over a broad range of temperature and pH, makes this ligand-receptor system very suitable for use as a supramolecular tool \cite{9–12}. Biotinylated monolayers of lipids \cite{9,13–17} and thiols \cite{9,18} have been used as substrates to prepare SAv-functionalized surfaces. Electron microscopy (EM) and AFM investigations have shown that SAv can form ordered two-dimensional protein crystals by specific binding to these supports. These crystals can even be imaged with (sub)molecular resolution by EM \cite{13,14,19} and AFM \cite{15}. Biotinylated lipids not only have been used as monolayers, but also as bilayers \cite{20}, vesicles \cite{9,12,21–24} and lipid nanotubes \cite{25–27}. In rare cases, it has been shown that even non-specific binding of SAv can lead to two-dimensional-crystal formation \cite{28,29}. The surface patterning of SAv by microcontact printing \cite{18,30,31}, microfluidic networks \cite{32,33}, polymer masking \cite{34}, e-beam lithography \cite{35} and AFM-manipulation \cite{36} has been reported as a step towards its use in the development of biosensors.

Recently, we introduced a new class of surfactant molecules called ‘giant amphiphiles’ \cite{37}. These molecules consists of a protein or enzyme that acts as a hydrophilic head group and a long polymer chain that
acts as the hydrophobic tail, resulting in amphiphiles several magnitudes larger in size than their low molecular weight equivalents. The ongoing research is aimed at the characterization of the self-assembling properties of these giant amphiphiles and the investigation of their applicability in constructing two-dimensional and three-dimensional ordered structures with well-defined catalytic or sensor activity. These biohybrids differ from other protein–polymer systems described in the literature, in the sense that the protein to polymer ratio is predefined, and the position of the conjugation site is precisely known. Such a biohybrid was constructed by the association of two biotinylated polystyrene chains (PSb, Fig. 1d) and a streptavidin molecule. It has been shown that these hybrids are capable of forming monolayers at the air–water interface [37]. Preliminary AFM and fluorescence microscopy studies have shown that these monolayers can be transferred to a substrate and that SAv is homogeneously bound [37].

In this paper, the characterization of the supported films by AFM and fluorescence microscopy is further elaborated. To allow a thorough study of the different factors affecting the structure of these films, they are investigated using a stepwise increase in film complexity. Firstly, a monolayer of the biotinylated polymer in the absence of SAv was imaged (Fig. 1a). In situ binding of SAv was used to investigate the availability of the biotin moiety for binding. Secondly, a monolayer of the protein–polymer hybrid was investigated by AFM (Fig. 1b). The influence of the deposition conditions on the film morphology was examined. To allow an unambiguous identification of the observed features, fluorescently-labelled SAv was used. This allowed combined AFM–SCFM (scanning confocal fluorescence microscopy) measurements of the same area. Finally, a second fluorescent label was used to visualize the binding of
biotin to a deposited hybrid monolayer (Fig. 1c). In this way the supported films are fully characterized.

2. Experimental section

2.1. Materials

The synthesis of biotinylated polystyrene (PSb, $M_n = 9147$, $M_c/M_n = 1.03$, Fig. 1d) has been described elsewhere [37]. The molecule consists of a polystyrene chain of 90 units whose end is covalently linked to biotin via a hydrophilic ethylene glycol spacer. This compound was used in the described experiments as a 0.61 mg/ml solution in chloroform.

Streptavidin (SAv), streptavidin-rhodamine-red-X (SAvRRX) and fluorescein-biotin (bF) were purchased from Molecular Probes. For the in-situ binding of SAv, 50 μl of a 0.5 mg/ml SAv solution (0.05 M Tris, pH 7) was added to the aqueous environment in the AFM liquid cell. SAvRRX and bF were used as 0.5 mg/ml solutions in PBS.

2.2. Monolayer preparation

The PSb monolayer was prepared by spreading the PSb-solution on a MilliQ subphase in a thermostatted Langmuir trough (6.25 cm). For the PSb–SAv monolayer, 0.5 equivalents of SAv was added to the subphase, 15 min after spreading of the PSb, and incubated for at least 1 h. The monolayer was subsequently compressed (0.7 cm$^2$ min$^{-1}$). The corresponding isotherm characteristics have been discussed previously in Ref. [37].

The PSb–SAvRRX monolayers were prepared on a KSV5000 trough with a reduced surface area (256 cm$^2$) by spreading the PSb-solution (typically 30 μl) on a MilliQ subphase already containing SAvRRX (total concentration of 0.25 μg/ml) until a surface pressure of 10 mN/m was reached.

2.3. Monolayer deposition

Samples were deposited on freshly cleaved highly oriented pyrolytic graphite (HOPG). For the combined AFM–SCFM measurements a transparent hydrophobic substrate was prepared. A glass coverslip was thoroughly cleaned and chemically modified by incubation in a 1% dimethyldichlorosilane solution in chloroform for 10 min. After rinsing with anhydrous acetone and drying under a stream of Ar, the hydrophobic surface was coated with a ZEONEX® film (cyclic olefin polymer) by spincoating from a chloroform solution (1.6 mg/ml). This resulted in a very smooth (roughness only 0.2 nm peak-to-peak), transparent hydrophobic substrate.

The PSb and PSb–SAv monolayers were deposited onto the solid substrates by controlled horizontal deposition (Langmuir–Schaefer technique [38], the substrate was approached parallel to the interface until contact, and immediately retracted without completely immersing the substrate) and allowed to dry.

Labeled biotin binding was accomplished by adding 20 μl of the bF solution to the completely wetted sample before drying. After an incubation of 10 min the samples were rinsed and dried.

2.4. Atomic force microscopy and confocal fluorescence microscopy

Three different AFM microscopes were used: MultiMode SPM (Digital Instruments) (PSb–SAv monolayers), Lumina (Thermomicroscopes) (combined AFM–SCFM) and Picospm (Molecular Imaging) (high resolution AFM on PSb–SAvRRX monolayers).

The MultiMode AFM was controlled with the Nanoscope IIIa controller. For contact mode operation (c-AFM), standard oxide sharpened Si$_3$N$_4$ tips with a nominal force constant of 0.06 N/m were used. Intermittent contact imaging (ac-AFM) was performed using standard etched silicon probes with a nominal force constant of 20–80 N/m, and a resonance frequency of ca. 300 kHz (all supplied by Digital Instruments). Measurements in MilliQ water were performed in a droplet without using the O-ring.

The Picospm was controlled by the SPM1000 control electronics (RHK). Intermittent contact imaging was performed with the acoustic ac-mode (Molecular Imaging) using silicon probes (d-levers, Thermomicroscopes) with a nominal force constant of 1.3–2.2 N/m and a resonance frequency of ca. 100 kHz.

The Lumina AFM was built on an inverted optical microscope (Nikon Diaphot 200). The transparent sample is mounted on the microscope platform that acts as a scanning stage. The surface can be investigated by AFM while the microscope objective underneath the sample is used to excite the sample and collect the fluorescence (epifluorescence mode). As excitation light sources, a water cooled Ar-ion (488 nm) and a green HeNe laser (543 nm) were used. Appropriate mirrors and filters were used to collect the 520 (±10) nm emission on one detector (APD, SPCM-AQ-151, EG&G) and the emission approximately 600 (±10) nm on the other (see Fig. 7a for the appropriate spectra). For c-AFM operation the premounted Si$_3$N$_4$ probes (Thermomicroscopes) with a nominal force constant of 0.021 N/m were used. Intermittent contact imaging was performed using premounted silicon probes (Thermomicroscopes) with a nominal force constant of 42 N/m and a resonance frequency of ca. 300 kHz.

AFM scanning was performed with minimal interaction force and a typical scan frequency of 1–2 Hz. The images were analyzed and processed using the standard software supplied with the control electronics. The topography and phase images were leveled by horizontal background subtraction and the color scale was opti-
mized. Fluorescence images are raw data (only color scale optimized in some cases). Roughness values given in the text are typical peak-to-peak values.

3. Results and discussion

3.1. Biotinylated polystyrene monolayers

In order to completely characterize the protein–polymer hybrid films deposited on a solid substrate, the polymer-only monolayers were examined first. For this purpose a biotinylated polystyrene (PSb) monolayer in the absence of SAv was compressed to a surface pressure, sufficiently high to expect a continuous close packed monolayer. It is deposited horizontally from the air–water interface onto HOPG (Fig. 1a) and investigated with contact mode AFM (c-AFM) in aqueous environment. The topography image (Fig. 2a) and a corresponding line profile (Fig. 2c) show that the polymer can form very smooth and extended monolayer patches (roughness approx. 0.3 nm). The size and shape of these patches are rather heterogeneously distributed, with circular patches ranging from 50 nm to several microns in diameter, and ill-defined shapes extending even further. Similar morphologies have been observed for other polymer systems [39]. The thickness of the patches is 7 ± 1 nm. Occasionally, two neighboring patches overlap (brighter areas in Fig. 2a) resulting in a corresponding double step-height of 15 nm. The internal molecular structure of the patches is not clear, but since the thickness is uniform throughout all of our experiments, this layer will be referred to as a monolayer.

The streptavidin (SAv) binding capability of the deposited monolayer was assessed by adding the protein to the contacting aqueous solution and visualizing the binding in situ. Immediately after addition of the protein, the monolayer roughness increased gradually for more than 1 h (from 0.4 to 1 nm, Fig. 2b and d). The protein molecules were too mobile to obtain a stable high-resolution image. The long use of c-AFM in liquid is a very probable cause for this effect, as also has been observed by others [40]. At some sites the onset of densely packed two-dimensional protein layers is visible, though the protein domains only grow laterally up to a few 100 nm within the time frame of the experiment (a few hours). Height measurements of these more dense structures revealed a height of 3–4 nm with respect to the polymer monolayer, as can be expected for a single SAv layer [27,15]. Because of the observed high density binding of SAv to the polymer, it is concluded that the PSb molecules in the precursor Langmuir monolayer have a reasonably high tendency to orient themselves with their biotin moieties to the subphase, despite the small size of this hydrophilic end compared to the long polystyrene chain.

3.2. Biotinylated polystyrene–streptavidin monolayers

After characterization of the morphology of the PSb monolayers, the hybrid PSb–SAv layers were investigated. This was accomplished by spreading PSb on a TRIS–buffered aqueous subphase, subsequently adding SAv to the subphase and allowing it to bind to the polymer. The resulting PSb–SAv complexes were compressed to a predetermined surface pressure to form a monolayer that was again horizontally deposited on HOPG (Fig. 1b) and examined by c-AFM in aqueous environment. The influence of the extent of monolayer compression was investigated by deposition at relatively low (8 mN/m) and high (35 mN/m) surface pressures. The corresponding topography images are shown in Fig. 3a and b, respectively. At low surface pressure the protein–polymer hybrids form distinct monolayer patches with diameters of typically a few hundred nanometers and a thickness of 7–8 nm, which are brought closer together upon compression (Fig. 3b). The thickness of the patches does not change during this process, indicating that the packing of the hybrid molecules is probably not affected, as has also been suggested in similar work [41]. Close inspection of the monolayer surface (Fig. 3c) reveals a certain corrugation (approx. 1 nm) that can be attributed to SAv bound to PSb. Remarkably, the binding of SAv only marginally affects the measured height of the patches. The patches of the hybrid films are only 1-nm thicker than those observed for the pure PSb monolayers, instead of the 3 nm or even more than 4 nm observed by others [27,15]. This can again be explained by the use of the contact mode and the observed mobility of the SAv, which hampers an accurate height determination.

Since contact mode imaging was clearly a limiting factor, the intermittent contact mode (ac-AFM, also known as ‘tapping mode’) was used. The main advantage of this imaging mode is that the reduction of the shear forces can in some cases provide a better resolution. In this work ac-AFM was always used in ambient air since the liquid environment is often troublesome because of lower sensitivity, due to a decreased quality factor and hydrodynamic effects. Constant amplitude scanning results in a topography image. Simultaneous detection of phase variations (between the drive oscillation and detected oscillation) results in a phase image that might provide additional information, often related to material properties [42].

Intermittent contact imaging of the PSb–SAv monolayer revealed very similar characteristics as c-AFM imaging in water, indicating that the air or liquid environment probably does not affect the morphology of the film. Only on a few areas was a somewhat different morphology observed. The large scale image shown in Fig. 4a looks similar to the other areas imaged by c-AFM, with typical monolayer patches extending over micrometers. A slightly higher thickness (8–10 nm
instead of 7–8 nm) is, however, observed, confirming that the height measured in c-AFM is indeed underestimated due to protein mobility. The height is still smaller than the theoretically expected value of 11 nm (7 nm monolayer + 4 nm SAv). Vertical compressibility of SAv might also be a factor worth considering as has also been observed in other ac-AFM measurements on SAv-complexes [43]. Unlike the dominant morphological characteristics described above, the patches do not appear as continuous entities in ac-AFM, but rather seem to be composed of very small ‘globular’ structures. From a line profile (Fig. 4c), it can be clearly seen that occasionally the height falls back to the background level, indicating that these globular structures are not merely proteins on a PSb monolayer patch, but actually small PSb–SAv monolayer aggregates. More detailed examination reveals globular structures of 50–100 nm in diameter that are grouped in larger patches of micrometer dimensions (Fig. 4b). Closer inspection of the background between the large patches reveals a similar but more compact structure suggesting this is not the substrate (HOPG) but a second continuous layer of PSb–SAv (1 nm roughness), corresponding to the first morphology observed.

Phase images of the same area exhibit a clear contrast (Fig. 4d). The smallest globular structures give a dark contrast, while the space in between as well as the background are brighter (corresponding to small and large phase shifts, respectively). This can be interpreted by considering phase shifts to be correlated with energy.

Fig. 2. (a) Topography image of a PSb-monolayer deposited by the LS technique on HOPG at 40 mN/m, obtained by c-AFM in aqueous environment. (b) Approximately the same area after addition of SAv to the measurement solution. (c) and (d) Horizontal line profiles taken from images (a) and (b), respectively, at the positions indicated by the white lines. Scale bars represent 500 nm.
dissipation [44]. Contrary to the reports on other systems these phase images reflect predominantly topographic effects rather than material properties. The monolayer topography causes differences in the size of the interaction area between the tip and sample. If the tip is on top of a small globular structure, it senses only this structure with the very end of the probe, and the phase shift is small (dark). When the tip penetrates the space between two structures, the interaction area increases since it interacts with all the surrounding structures and the background layer, resulting in a higher phase shift (bright). The background layer shows an intermediate and more homogeneous contrast because of the less pronounced topographic characteristics.

An explanation of the second observed morphology is also proposed. The two layers (foreground and background) have the same composition (PSb–SAv). During monolayer compression at the air–water interface one layer slides underneath the other resulting in a double layer after deposition. The top layer is, however, not a continuous layer, but composed of very small patches, that have also been observed by others for diblock copolymer molecules [39]. These small structures have been referred to as ‘dots’ and the extended patches as ‘continents’ [39]. For these diblock copolymers [39], the different morphologies are coexisting, but lower concentrations of the polymer solution favor ‘dots’ while higher concentrations favor ‘continents’. The differences

Fig. 3. c-AFM topography images in aqueous environment of a PSb–SAv film deposited on HOPG at (a) 8 mN/m and (b) 35 mN/m. The line profile (c) is taken from a high magnification image, at the position of the thin white line in image (a). Scale bars represent 500 nm.
Fig. 4. PSb–SAv film deposited on HOPG at 35 mN/m measured with ac-AFM in air. (a,b) Consecutive topography images of the same area. (c) Higher magnification line profile through one of the circular domains visible in image (b). (d) The phase image corresponding to image (b). (e,f) Topography (e) and phase (f) images of a similar area on the same sample. Circles and arrow: see text. Scale bars represent 250 nm (a,b,d) or 100 nm (e,f).
in morphology probably arise immediately after spreading of the PSb when the volatile solvent (chloroform) evaporates and micrometer sized droplets are formed. The local concentration at this point determines whether the molecules will entangle and form an extended monolayer patch or whether dots are formed. Minimization of the surface free energy is probably an important factor in this process. The role of SAv in the subphase is unclear. After ‘continent’ or ‘dot’ formation, the solvent evaporates further, causing the dots to aggregate in larger patches without merging. We are apparently working at a concentration where the extended patches are favored, but ‘dots’ may still appear. The internal molecular structure of these dots is uncertain. The uniform dimensions of these dots suggest a well-defined structure, also supported by studying images Fig. 4e and Fig. 4f in closer detail. These images show a high magnification of an area similar to the one in Fig. 4a–b. From the topography (Fig. 4e) it seems there is more heterogeneity in the diameter of these small patches and the background is flat. The phase image (Fig. 4f), however, is more sensitive to topography and reveals that the larger structures (like the one marked with a large circle) are composed of smaller subunits that have again dimensions similar to the isolated dots (small circle). These dots might be interpreted as two-dimensional equivalents of a micelle, or maybe even single (PSb)–SAv structures. In the background, one can observe very small structures (white arrow). On a larger scale image (not shown), it is clear that this background is again not the graphite but a PSb–SAv layer. Thus, the small structures (up to 1 nm high and 20 nm wide, tip broadened) in the background probably correspond to individually resolved protein molecules of a flat monolayer.

3.3. Binding of fluorescently labeled biotin by biotinylated polystyrene–streptavidin monolayers

The biotin binding capability of the remaining binding sites of the giant amphiphile monolayer has been demonstrated at the air–water interface [37]. Here this property is investigated on a deposited film, an important aspect for their use in the creation of, for instance, biosensor surfaces. Due to the increased complexity of the film, AFM alone is not sufficient to identify all structural properties because it lacks specific contrast. AFM was, therefore, combined with scanning confocal fluorescence microscopy (SCFM), allowing topography and fluorescence imaging of exactly the same area of the sample. For this purpose, SAv with a rhodamine red-X label (SAvRRX) was added to the subphase before spreading PSb. By increasing the amount of spread polymer, a surface pressure of 10 mN/m could be reached without compression. This method and surface pressure were chosen since it was empirically established that these conditions resulted in a good coverage of the substrate without multilayer buildup. After incubation, the biohybrid film was deposited onto hydrophobic glass to allow epifluorescence measurements through the substrate while examining the sample on top by AFM, and dried. The c-AFM topography image in Fig. 5a shows an extended monolayer with a few defects. The measured thickness of the layer (7 nm) corresponds to the monolayer thickness measured in previous c-AFM experiments. Fig. 5b shows the corresponding fluorescence image when exciting at 543 nm and collecting the RRX fluorescence at 600 nm (spectra in Fig. 7a, square symbols). The fluorescence intensity coming from the monolayer is constant, indicative of a homogeneous distribution of the PSb–SAvRRX complexes in the monolayer. The small overlapping areas have a doubled fluorescence intensity, indicating that the defects arise after formation of the stable PSb–SAv complex.

Since the use of contact mode imaging was clearly limiting the resolution, the samples were also examined by ac-AFM. This revealed monolayer patches (image Fig. 5c) with protein molecules at the surface (image Fig. 5d). As expected from previous ac-AFM experiments, the height of SAv relative to the monolayer measured up to 3 nm. The measured width of the small features is still on the order of 20–30 nm (influenced by the tip broadening). These structures most likely correspond to single protein molecules, or clusters of a few molecules at the most. Though the proteins appear to be closely packed, the distance between them is still on the order of a few tens of nanometers. It cannot, however, be excluded that in certain areas the proteins pack more closely, but the relative tip dimensions do not allow molecular resolution.

To visualize the binding of biotin to the free binding sites of the giant amphiphile monolayer (Fig. 1c), fluorescein–biotin (bF) is used since it should allow a spectral discrimination between SAvRRX and bF (see spectra in Fig. 7a). Binding of bF to the monolayer was accomplished by incubating freshly deposited samples in a bF solution. After a few minutes the samples were thoroughly rinsed to remove non-specifically bound bF. Separate detection of bF and (mainly) SAvRRX fluorescence was accomplished using two detectors and the appropriate filter sets (approx. 520 nm and 600 nm, respectively) and switching the excitation light between 543 nm (SAvRRX only) and 488 nm (both SAvRRX and bF). From the bF emission images (488 nm excitation, 520 nm detection, not shown), it was clear that rigorous rinsing was necessary to avoid non-specific binding of bF to the substrate. This extensive rinsing, however, was destructive towards the smaller monolayer patches. As a result, only large patches as shown in Fig. 6a remained more or less intact. The ac-AFM topography image shows a monolayer with defects. The mon-
layer thickness is consistent with previous findings (approx. 10 nm). The background, however, has an increased roughness of several nanometers, which is probably due to random adhesion of polymer and protein molecules after the destruction of the less stable monolayer patches. The SAvRRX emission image (at 600 nm, Fig. 6b) of the same area upon 543 nm excitation reveals that both the monolayer and the background are largely fluorescent, indicating presence of protein molecules everywhere on the surface. The monolayer itself shows typical cracks (no emission) and overlapping parts (double emission). These correlate perfectly with the AFM topography. Excitation at 488 nm using the same detection (600 nm), shows a different picture (Fig. 6c). The background shows only minor fluorescence (RRX absorption at 488 nm is small but non-zero). The monolayer as a whole has an increased intensity relative to the background. This can be explained by assuming that the SAvRRX in the background has denatured and is no longer capable of binding bF. The monolayer has bF homogeneously bound. The bright lines corresponding to overlapping monolayer areas do not exhibit the doubled fluorescence intensity as observed when only RRX is excited. This is easily understood by considering that bF binding occurs after formation of the defects, so only the top monolayer is able to bind bF.

The increased detected monolayer fluorescence in image 6c could come from the bF directly since its emission band tails quite far in the red region of the spectrum (Fig. 7a). However, considering the small
distance between the fluorescein and rhodamine, and the nice overlap between the emission spectrum of the former and the absorption spectrum of the latter, energy transfer from fluorescein to rhodamine is likely to occur. To obtain a conclusive answer to this question, the emission light at 488 nm excitation of a similar patch as in Fig. 6 was collected and recorded with the spectrograph. In this spectrum (Fig. 7b, solid line), one can clearly see the signature of the rhodamine dye and only a small shoulder in the emission region of fluorescein. It can, therefore, be concluded that excitation of bF leads to a substantial energy transfer from bF to SAvRRX and an increased SAvRRX emission. This observation suggests some further interesting experiments using biotinylated enzymes labeled with fluorescein to bind to the rhodamine labeled hybrid monolayers. Changes in enzymatic activity, related to conformational changes of the enzyme, could be monitored by monitoring the energy transfer efficiency between the two labeling dyes.

4. Conclusions

The investigation of the deposited protein–polymer hybrid films by AFM and combined AFM–SCFM allowed a detailed evaluation of the monolayer formation and transfer process. A monolayer of the polymer in the absence of SAv could be prepared in the same
High-resolution AFM imaging of these films revealed a dense packing of the protein molecules, although the packing is not as compact as that observed for monolayers formed from small amphiphiles.

A combination of AFM and SCFM clearly revealed a uniform binding of the labeled protein to the monolayer. Dual color imaging with fluorescently labeled biotin has unambiguously shown that biotin can be specifically bound to a deposited monolayer of the protein–polymer hybrid. This observation opens up the possibility of creating uniform well-controlled functionalized surfaces by application of biotinylated enzymes, antibodies and other functional molecules.

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