PCCP

Accepted Manuscript

This article can be cited before page numbers have been issued, to do this please use: M. Zharnikov, M. Khan and S. Schuster , *Phys. Chem. Chem. Phys.*, 2016, DOI: 10.1039/C5CP07840G.

ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/pccp

View Article Online
DOI: 10.1039/C5CP07840G

Chemical derivatization and biofunctionalization of hydrogel nanomembranes for potential biomedical and biosensor applications

Musammir Khan[#], Swen Schuster and Michael Zharnikov[*]

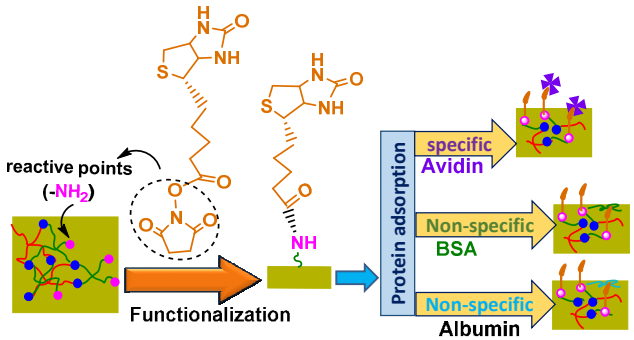
Applied Physical Chemistry, Heidelberg University, Im Neuenheimer Feld 253, 69120 Heidelberg, Germany

[#] Present address: Department of Electronics and Communications Engineering, Tampere University of Technology, Korkeakoulunkatu 3, 33720, Tampere, Finland

* To whom correspondence should be addressed: phone: +49-6221-54 4921, fax: +49-6221-54 6199, E-mail: Michael.Zharnikov@urz.uni-heidelberg.de (M. Zharnikov)

View Article Online
DOI: 10.1039/C5CP07840G

A table of contents entry:



Chemically crosslinked, poly(ethylene glycol) based hydrogel nanomembranes can be functionalized with specific receptors and, subsequently, with specifically bound proteins, providing, at the same time, stable, protein-repelling matrix.

View Article Online
DOI: 10.1039/C5CP07840G

Abstract

Poly(ethylene glycol) based hydrogel nanomembranes (PHMs) are demonstrated to be able to host protein-specific receptors, providing, at the same time, stable, protein-repelling matrix with a characteristic mesh size up to 7-8 nm. The membranes were prepared by crosslinking of amino- and epoxy-terminated STAR-PEG precursors and maintained their hydrogel and protein-repelling properties even at a deviation of the precursor composition from the equilibrium value (1:1). The grafting density of the test avidin protein, specifically attached to the biotin moieties coupled to the free amine groups in the PHMs, varied from 0.45×10^{12} to 1.3×10^{12} proteins/cm^2 within the sampling depth of the experiments (~11.5 nm), depending on the precursor composition, whereas the analogous values for the non-specifically adsorbed proteins were lower by a factor of 4-5. The engineering of PHMs with biomolecule-specific receptors and their loading with biomolecules are of potential interest for sensor fabrication and biomedical applications, including tissue engineering and regenerative therapy.

View Article Online
DOI: 10.1039/C5CP07840G

Introduction

Specifically designed molecular and macromolecular films are of potential interest for biological and biomedical research as well as for a variety of practical applications such as sensor fabrication, medical diagnostics and therapy. In particular, they can be useful for mimicking biological interfaces, cell research, proteomics, fabrication of different assays, and pharmaceutical and medicine screening. Among other systems, biocompatible hydrogel films, based mostly on crosslinked poly(ethylene glycols) (PEGs) have recently attracted significant attention due to flexibility of their design and a variety of potentially useful properties.[1-9] As mentioned in a recent review,[6] these properties include biocompatibility, non-fouling behaviour, non-toxicity, high optical quality and transparency. Possible applications include design of optical sensors,[6] fabrication of model immunoprotective barriers for cell research,[3,8] storage and controlled release of drugs,[5] preparation of biofunctionalized microchannels for dynamic cell adhesion studies,[7] and protein affinity experiments.[1,2,4,9] In the latter case, hydrogel film is typically used as a non-fouling matrix while specific receptors for adhesion of proteins or other biomolecules are imbedded or attached covalently to the molecular network.[1,2,4,9] The mesh size and the extent of crosslinking in the matrix can be additionally varied, affecting the diffusion of the proteins and their final conformation as well as accessibility of the receptors.[3,8]

Recently, we designed a novel type of biocompatible hydrogel films which can also be easily separated from the substrate and serve as ultrathin membranes that are sufficiently robust and extremely elastic.[10,11] These films and membranes were synthesized by thermally-activated crosslinking of amine- and epoxy-functionalized star-branched PEG precursors.[10] Their thickness can be precisely tuned between 4 and 200 nm, by varying the concentration of the precursors in the primary solution.[10,11] The resulting PEG hydrogel films and membranes, abbreviated below as PHFs and PHMs, respectively, can serve as precursors for hybrid nanoparticle-PEG assembles,[10] versatile templates for electron lithography,[12] potential highly sensitive sensing elements in microelectromechanical systems (MEMS) applications,[11] transient membranes for preparation of nanoparticle patterns on arbitrary substrates,[13] and sensing elements of humidity sensors.[14] Among other useful properties, PHFs and PHMs are characterized by high degree of biocompatibility, exhibiting distinct protein-repelling behavior,[10] which, potentially, renders them capable to be a suitable platform for biomedical and biosensor applications, relying on embedding of specific receptors for subsequent binding and sensing of proteins and other biomolecules. The tethering of these receptors should not

View Article Online
DOI: 10.1039/C5CP07840G

involve the PEG network itself, guaranteeing persistent biocompatibility, but rely on a covalent linkage to chemically active groups in the PHFs and PHMs. As such groups, non-reacted, "free" amino and epoxy moieties can be considered. Their density is not high, since most of them react, building the crosslinking network in the course of PHF fabrication,[10] but probably, still sufficient to achieve reasonable grafting density of the receptors.

In this context, in the present study, we tested specifically the presence of non-reacted, "free" amino and epoxy moieties in the model PHMs, as well as monitored the presence of hydroxy groups, associated with the crosslinking points of the PEG network and being also alternative docking groups for attachment of specific receptors. Along with the characterization of the PHMs prepared at the equilibrium composition of the precursors, PHMs fabricated at different precursor compositions were studied as well, to vary the density of potentially reactive sites for tethering to the specific receptors. The robustness and swelling properties of these PHMs were monitored under controlled conditions, to be sure that they represent suitable and stable hydrogel-like matrix for the derivatization and biofunctionalization experiments. Finally, the receptor functionalized PHMs were tested for both specific and non-specific protein affinity, using several model proteins.

Experimental

Materials. Most of the chemicals were purchased from Sigma-Aldrich (otherwise indicated), viz. trifluoroacetic anhydride (TFAA; ≥99.0%), 4-(trifluoromethyl)benzylamine (TFMBA; 97%), triethylamine (NEt_3; ≥99.5%), trimethylsilyl-imidizole (TMSI; ≥98%), avidin (A9257), bovine serum albumin (BSA; A2153), albumin (Alb; A5503). NHS-Biotin was purchased from APExBIO (A8002).

Nanomembrane preparation. The PHMs were prepared by the previously established technique (Figure 1),[10] from the epoxy/amine-terminated four-arm STAR-PEGs with Mn = 2000 g/mol (Creative PEGWorks), abbreviated as STAR2k-EPX and STAR2k-NH2, respectively. According to the molecular weight, the PEG arms include 10-11 monomers, corresponding to an arm length of 3.5-3.9 nm. The PEG compounds were separately dissolved in chloroform, mixed together with the joint concentration of either 5 mg/mL (thin membranes) or 20 mg/mL (thick membranes), spin-coated onto an Au (111) substrate (Georg-Albert-PVD, Germany), crosslinked by prolonged thermal annealing (6 h, 80°C), and ultrasonicated to remove weakly bound material. In contrast to the previous work where the complementary PEG compounds, serving as precursors for the membrane fabrication, were

View Article Online
DOI: 10.1039/C5CP07840G

exclusively mixed in the equilibrium 1:1 ratio,[10-14] their relative concentration was varied this time, with STAR2k-NH2/STAR2k-EPX (amine/epoxy) ratios of 1:1.25, 1:1, 1:0.8 and 1:0.6. The joint concentration of the STAR2k materials was, however, kept constant, as mentioned above.

Swelling properties. The swelling properties of the PHMs were investigated by a spectroscopic ellipsometer (M-44, J.A. Woollam) at a fixed angle of 75° using the methodology of our previous study[14] adapted, with minor modifications, from ref 15. Briefly, the relative humidity, RH, was varied between 5% to 80% in a controlled fashion by passing nitrogen gas flow through two parallel-adjusted PYREX gas washing bottles filled with pure water and concentrated sulfuric acid, respectively. The branching of the gas flow stemming from these bottles was precisely adjusted by three-way valves (Rotilabo-tubing valves), leading to certain RH values measured by a commercially available hygrometer (BL-20 TRH thermo/hygrometer, RH ± 3.5%).

Chemical derivatization. The tracing of potential docking groups in the PHMs, viz. hydroxy, epoxy, and amine, was carried out by their derivatization, using specific reagents for each of these moieties, as shown in Figure 1. Such a derivatization is a frequently used procedure applied for identification and monitoring of specific functional groups in thin films and on complex organic surfaces.[16-18] In the given case, the tracing of the hydroxyl moieties (crosslinking points) was performed by their derivatization with TMSI using pyridine as solvent (1:4) for 6 h at 60 ^{o}C, with subsequent monitoring of the characteristic Si 2p photoemission signal (steps 2 and 3 in Figure 1). The tracing of epoxy groups was conducted by their derivatization with TFMBA. Accordingly, a pristine membrane was treated with TFMBA in ethanol (300 mM), having 1.5 equivalent of NEt_3, for 24 h at room temperature, with subsequent monitoring of the characteristic F 1s photoemission signal (steps 4 and 5 in Figure 1). At the same time, the above tracing of the epoxy groups served as the first steps in the many-steps procedure for tracing of the amine groups. In the subsequent step (step 6 in Figure 1), all hydroxyl groups, originated from both original crosslinking points and epoxy derivatization, were quenched with TMSI to exclude them from the reaction with TFAA, used for the subsequent tracing of the amine group in the final step of the derivatization procedure (step 7 in Figure 1). Accordingly, the membranes were exposed to TFAA (25%) in anhydrous dioxane at ambient temperature for 24 h, with subsequent monitoring of the characteristic F 1s photoemission signal (step 8 in Figure 1), corrected for the analogous signal of TFMBA (measured in step 5 as shown in Figure 1).

View Article Online
DOI: 10.1039/C5CP07840G

Biofunctionalization. The procedure is illustrated in Figure 2. Among the potentially available docking sites, free amino groups were selected as representative binding sites for the biofunctionalization of PHMs. The procedure was relied on the reaction between these groups in the membranes and the NHS moieties of the NHS-biotin used as the functionalization agent. Such a reaction is frequently used to tether biotin to organic films and solid surfaces.[19-21] In the given case, NHS-biotin (4 mg/mL) was dissolved in DMSO and ultrasonicated for about 5 min to dissolve completely. PHMs were incubated in this biotin solution for 2 h at ambient conditions, as illustrated in Figure 2. After the reaction, the membranes (denoted below as PHM-Bio) were successively rinsed with pure water and ethanol and, finally, dried in Ar.

Protein adsorption. The procedure is illustrated in Figure 2. Both specific and non-specific protein affinity of the biotin-functionalized membranes was monitored using avidin, bovine serum albumin (BSA), and albumin as test proteins. Note that these particular proteins are well suited and frequently used to check protein adhesion and repelling of thin films and biochemical arrays.[22-24] They have similar molecular weights (66-67 kDA) and compact form and are therefore well suited for the affinity experiments involving comparison of specific and non-specific events. The protein solutions (equimolar) were prepared by dissolving 0.15 mg/mL of the respective proteins in PBS (pH ~7.4, corresponding to physiological conditions). The affinity of PHM-Bio to specific protein adsorption was tested by their immersion into the solution of avidin (Figure 2) which makes specific binding to biotin.[22-24] The non-specific adsorption of proteins on PHM-Bio was tested by their dipping into the solution of BSA or albumin, as illustrated in Figure 2. The immersion was performed for 1 h at room temperature and under ambient conditions. After the immersion, the samples were rinsed with pure water and dried in Ar. They will be denoted as PHM-Bio-Avid, PHM-Bio-BSA, and PHM-Bio-Alb below, referring to the protein being used.

Monitoring of individual reactions. The extent of derivatization, biofunctionalization, and protein adsorption processes was monitored by X-ray photoelectron spectroscopy (XPS), based on the specific signals for the relevant chemical compounds and biomolecules. The measurements were carried out with the MAX200 (Leybold-Heraeus) spectrometer equipped with an MgKα X-ray source and hemispherical analyser. The experiments were performed under ultra-high vacuum conditions, which of course affected the physical state of the pristine and functionalized PHMs but did not changed their chemical composition. The recorded spectra were normalized to the spectrometer transmission function.

View Article Online
DOI: 10.1039/C5CP07840G

The experiments were performed for the thin PHMs only, because of the limited sampling depth of XPS,[10,25] which did not allow to obtain representative data for thick PHMs.

Results and discussion

General comments. Whereas the thin membrane was more suitable for the XPS characterization, due to the limited sampling depth of this technique, its swelling properties could be noticeably different as compared to the thicker one, because of the lower extent of crosslinking.[10] This was the reason why we studied these two particular types of PHMs within the swelling experiments. Note that the PHMs are extremely robust, staying fully intact not only upon repeated immersion into an aqueous solution or chloroform but even upon sonication in the latter solvent, leading to a very limited loss of the physisorbed material only. This has been demonstrated previously for the membranes fabricated at the equilibrium (1:1) composition of the precursors[10-14] but is also the case at a reasonable deviation from this composition, as shown in the present study. The reasons for this robustness are the covalent character of the crosslinking bonds and the 3D network character of the entire system.

Swelling properties. The major goals of the swelling experiments were (i) to prove whether the hydrogel character of the PHMs persists at a deviation from the equilibrium composition of the precursors and (ii) to prove whether such PHMs are robust enough. Ellipsometric thickness of representative thin (25-35 ± 1.5 nm) and thick (170-180 ± 2 nm) PHMs is presented in Figure 3 as function of RH. Accordingly, the hydrogel character of the PHMs persists upon the variation of the precursor composition, which is an important prerequisite in context of practical application. At the same time, both the thickness of the PHMs and their swelling ability depend, to a certain extent, on the precursor composition. The thickness of the PHMs increases with increasing amine/epoxy ratio while their swelling ability decreases, with the swelling ratio varying either between 1.90 and 1.65 (thin film) or 1.63 and 1.52 (thick film). The higher values of the swelling ratio for the thin PHM as compared to the thick ones correlate well with the previous results for the PHMs prepared at the equilibrium composition of the precursors and are explained by the lower extent of crosslinking in the thinner films. The variation of the swelling ratio with the film composition is partly related to the thickness effect (thinner films = higher swelling ratio) but also to the less developed supramolecular network, containing, along with the fully crosslinked precursors, partly crosslinked ones, with non-reacted amine groups. Presumably, these groups are less active in the sense of hydrogel behavior of PHMs, in contrast to the "free" epoxy groups which, according to Figure 3,

View Article Online
DOI: 10.1039/C5CP07840G

contribute constructively to the swelling properties. A possible reason for such a behavior can be hydrolysis of these group resulting in the formation of hydrophilic hydroxyl groups, two for every "free" epoxy group.[4] Further, a "self-addition" reaction of "free" epoxy groups with each other is also possible, resulting in formation of additional hydroxyl groups as well as in an alternative crosslinking path in the PHMs having an excess of the epoxy-terminated precursors.[4] Significantly, the behavior of the PHMs was fully reproducible at the repeating experiments, performed for each sample at random, at high and low RH%. These experiments showed reversible swelling similar to the first reading, underlying the stability and robustness of the PHMs.

As to the comparison between the thin and thick PHMs, the effect of precursor composition on both the membrane thickness and the swelling ability is stronger in the former case as compared to the latter, which can be tentatively attributed to the less developed crosslinking network in the case of the thin PHMs.[10] Note that the extent of crosslinking, dependent in the given case on the precursor composition, affects, generally, swelling behavior of hydrogel films.[26-29]

Apart from the specific composition and thickness dependent effects, discussed above, all PHMs of this study were found to be stable and robust enough as well as maintaining their hydrogel properties even at a deviation of the precursor composition from the equilibrium value (1:1). Accordingly, they were well suitable for the subsequent chemical derivatization and biofunctionalization experiments.

Chemical derivatization. The XP spectra of the PHMs are typical of the PEG compounds and are dominated by the O 1s peak of oxygen at ~533 eV and the C 1s emission of carbon at ~286 eV.[10,12] In addition, there is a low intense C 1s shoulder at 284.4 eV stemming presumably from the carbon atoms beyond the PEG chains of the STAR-PEG moieties and a comparably weak N 1s emission of nitrogen at ~400 eV related to the amino groups of the STAR2k-NH2 moieties.[10,12] The spectroscopic signatures of the hydroxyl and epoxy groups are comparably weak and merged with those of the PEG chains. Consequently, they cannot be distinguished, similar to the contributions of the crosslinked and non-reacted amine groups in the joint N 1s peak. Thus, specific information on the chemically active groups, capable of tethering or biofunctionalization cannot be gained from the original XP spectra of the PHMs. However, this information can be obtained in dedicated derivatization experiments, targeting specifically the potentially active hydroxyl groups and non-reacted (and, consequently, still reactive) epoxy and amine groups.

View Article Online
DOI: 10.1039/C5CP07840G

The hydroxyl groups in the PHMs are mostly characteristic of the crosslinking sites as shown in Figure 1 as well as of the possible hydrolysis and self-addition of the free epoxy groups. The tracing of the hydroxyl groups was carried out by their chemical derivatization with TMSI (step 2 in Figure 1;), which is a specific reagent for these moieties.[30,31] The extent of the reaction was then monitored by XPS based on the intensity of the Si 2p emission characteristic of TMSI (step 3 in Figure 1; see Figure S1 in the ESI for a representative spectrum). The respective intensity is shown in Figure 4a as function of the film composition, given by the amino/epoxy ratio. As can be expected, the maximum amount of the crosslinking sites is observed at the equilibrium concentration of the precursors, i.e. an amine/epoxy ratio of 1:1. Upon the deviation from this ratio, both at higher amine and higher epoxy concentration, the amount of the crosslinking sites decreases significantly since some of the reactive groups of the precursor provided in the excess concentration do not find their complementary partners to form a crosslinking bridge and remain "free", i.e. non-reacted.

The tracing of the free (i.e. non-reacted) epoxy groups in the PHMs was conducted by their derivatization with TFMBA (step 4 in Figure 1) which has the same amino group as the original STAR2k-NH2 precursor, reacting specifically with the epoxy moieties without any side products.[17] The extent of reaction was monitored by XPS, using the F 1s emission characteristic of TFMBA (step 5 in Figure 1; see also Figure S1 in the ESI for a representative spectrum). The respective intensity is shown in Figure 4b as function of the film composition. As can be expected, the intensity decreases progressively with the increasing amine/epoxy ratio. At higher concentrations of STAR2k-EPX as compared to STAR2k-NH2, some of the epoxy groups do not find a complementary amine partner for crosslinking, resulting in a higher content of the free epoxy moieties as compared to the equilibrium composition. At a lower concentration of STAR2k-EPX as compared to STAR2k-NH2, the probability to find a complementary amine partner for crosslinking is higher for the epoxy groups as compared to the equilibrium composition, resulting in a lower content of the free epoxy moieties. Significantly, the concentration of the free epoxy groups varies by ±20% only upon the given variation of the precursor composition. Thus, the gain in the amount of the potential binding sites for specific receptors is quite moderate.

The TFMBA derivatized PHMs were subsequently used for tracing of the free (i.e. non-reacted) amine groups by applying several additional steps as described in the Experimental section. First, all hydroxyl groups, originated from both original crosslinking points and epoxy derivatization, were quenched with TMSI (step 6 in Figure 1) to exclude them from the

View Article Online
DOI: 10.1039/C5CP07840G

reaction with TFAA, a derivatization agent used subsequently for the free amine groups. Afterwards, the PHM was derivatized with TFAA (step 7 in Figure 1), with subsequent monitoring of the characteristic F 1s photoemission signal (step 8 in Figure 1; see Figure S1 in the ESI for a representative spectrum), corrected for the signal of TFMBA traced before (step 5 in Figure 1). The respective intensity, associated with the "free" amine groups only, is shown in Figure 4c as a function of the film composition. As can be expected, this intensity increases progressively with the increasing amine/epoxy ratio. At a higher concentration of STAR2k-NH2 as compared to STAR2k-EPX, some of the amine groups do not find a complementary epoxy partner for crosslinking, resulting in a higher content of the free amine moieties as compared to the equilibrium composition. At a lower concentration of STAR2k-NH2, the opposite situation occurs. Significantly and similar to the epoxy case, the concentration of the free amine groups varies by ±20-30% only upon the given variation of the precursor composition. Thus, the gain in the amount of the potential binding sites for specific receptors is quite moderate in the given case as well.

Biofunctionalization. The biofunctionalization was performed for the free amino groups only, representative of the epoxy and hydroxyl binding sites as well. The reaction was conducted with NHS-biotin (see Figure 2), specific for amine that had a sufficiently long (1.35 nm) spacer group allowing for additional flexibility and accessibility of the biotin moiety[19-21] as well as for its possible protrusion out of the membrane.

Protein adsorption. The protein affinity of the biotin functionalized PHMs was investigated for both specific and non-specific case. For the former case, avidin adsorption was tested, relying on well-known biotin-avidin interaction,[22-24] whereas, for non-specific adsorption, BSA and albumin were used. The adsorption of the proteins was monitored by tracing the characteristic N 1s XPS signal, following the established methodology of our previous studies.[23,24,32] The only other species containing nitrogen were the crosslinked and "free" amino groups in the PHMs as well as NHS-biotin moieties. The respective signal could, however, be measured separately, before the exposure of PHMs to the proteins, and corrected for, giving the intensity representative of the adsorbed proteins only. The sampling depth of the XPS measurements is generally determined as 3λ, where λ is the attenuation length of the photoelectrons at the given kinetic energy (~850 eV).[25] Taking the literature value of λ (3.5 nm), determined for the PHMs within a dedicated study,[10] one gets a sampling depth of 11.5 nm. Consequently, only the topmost part of the PHMs (25-35 ± 1.5 nm) was probed in spite

View Article Online
DOI: 10.1039/C5CP07840G

of their thinness. This part, however, constituted a half or a third of the entire PHMs, being, thus, well representative of these moieties.

The N 1s intensities for the pristine PHMs as well as for the PHMs functionalized with NHS-biotin and those subsequently exposed to BSA, albumin, or avidin are presented in Figure 5a as functions of the precursor composition; the respective N 1s XP spectra are compiled in the ESI (Figure S2). Both pristine and NHS-biotin functionalized PHMs, containing nitrogen as well, served as references (see above). Correcting the values for the protein loaded samples for the signal of the NHS-biotin functionalized PHM, one gets intensities reflecting solely the protein adsorption onto and into the membranes (Figure 5b). Most importantly, the signal associated with the specific adsorption is significantly higher (by a factor of 4-5) than that related to the non-specific events, underlying the persistence of the protein-repelling properties of the membranes as well as accessibility and performance of the biotin moieties coupled to the PHM framework. Further, the amount of the specifically bound avidin mimics the amount of the available specific binding sites, i.e. the Bio-BSA moieties in the membranes, increasing with the increasing amine/epoxy ratio. But, at the same time, the amount of non-specifically bound proteins increases with increasing amine/epoxy ratio as well, reflecting changes in the PHM composition.

The data presented in Figure 5 allows us to compare the extent of specific and non-specific protein adsorption as well as to establish the effect of the film composition. It would be however useful to get a numerical estimate for the density of the adsorbed proteins. Such an estimate was made using the reference samples of dodecanethiol (DDT) self-assembled monolayer (SAM) on Au(111) prepared according to the literature procedure,[33] exposed to the same solutions of avidin, BSA, and albumin, and carefully washed afterwards. According to our previous experience, this procedure results into a densely packed protein monolayer.[34] Taking the respective N 1s XPS signal as the reference and estimating the characteristic dimensions of the proteins involved in the study from the literature data,[35-38] we got the absolute values for the grafting density of the proteins for the PHMs prepared at the different precursor compositions (Figure 6). Note that the values for avidin lie below monolayer coverage typical, e.g., of such protein-adhesive surfaces as DDT/Au, viz. $\sim 3\times10^{12}$ proteins/cm^2. A reason for the lower grafting density in the case of the PHMs is most likely a comparably low density of the potential specific binding sites, i.e. biotin moieties bound to the free amine groups over the NHS link. The density of the latter groups could be, however, coarsely estimated using the well-defined reference samples of nitrile-substituted

View Article Online
DOI: 10.1039/C5CP07840G

biphenylthiol and terphenylthiol SAMs on Au(111), prepared according to the literature procedure.[39] These samples, having a similar packing density as SAMs on non-substituted alkanethiols on Au(111), viz. 4.63×10^{14} molecules/cm^2,[33,40] provide a suitable reference for the surface density of the nitrogen atoms in the PHMs. Comparing the intensities of the N 1s signals from the above reference samples and the PHMs, with a correction for the self-attenuation of the photoemission signal in the latter case, the density of the nitrogen atoms within the sampling depth of XPS at the given kinetic energy could be calculated. Further, taking into account that the portion of free amine moieties in the PHM prepared at the equilibrium composition of the precursors is ~2.5% of the initial amount of these groups at the given duration of the crosslinking reaction,[10] we could estimate the density of the free amino groups within the sampling depth of XPS at $\sim4\times10^{12}$ groups/cm^2. This value is noticeably higher than the grafting density of avidin for this particular membrane, which, according to Figure 6, is $\sim0.8\times10^{12}$ proteins/cm^2. Accordingly, the amount of the available free amine groups is obviously not the only factor responsible for the limited grafting density of the specifically bound avidin in the PHMs. As other relevant factors, non-100% extent of the amine-NHS-biotin coupling reaction as well as certain sterical constraints for the proteins entering the membrane should be taken into account. Note that the length of the OEG "chains" in the STAR2k-EPX and STAR2k-NH2 precursors is about 3.5-4 nm, which gives an in-plane mesh with an opening of 7-8 nm at the optimal, square-like character of the crosslinking network in a particular plane. In reality, the mesh is most likely not square-like, so that the "efficient" mesh size is somewhat smaller than 7-8 nm, becoming comparable or even smaller with/than the characteristic dimensions of the proteins, viz. ~7.2 nm for BSA and ~6.0 nm for albumin and avidin.[35-38] This can limit the accessibility of the potential binding sites in the inner part of the PHMs, especially if one consider the complex 3D character of the crosslinking network. An additional aspect is preferable attachment of the proteins to the receptors in the topmost part of the PHMs, resulting in a partial closure of the pores. This will hinder the penetration of the proteins to the deeper located receptors, limiting the entire (specific) protein adhesion ability of the PHMs.

Conclusions

We demonstrated the ability of poly(ethylene glycol) based hydrogel membranes (PHMs) to host protein-specific receptors, providing, at the same time, stable, protein-repelling matrix for these receptors as well as for specifically bound proteins or other biomolecules. These

View Article Online
DOI: 10.1039/C5CP07840G

membranes were prepared from the amino- and epoxy-terminated STAR-PEG precursors, relying on their thermally activated crosslinking. The composition of these precursors was varied to some extent around the equilibrium value (1:1), which, luckily, affected only slightly the hydrogel and protein-repelling properties of the resulting PHMs as well as their robustness. On the other hand, this variation allowed to alter the amount of free amine and epoxy groups serving, potentially, as docking sites for protein-specific receptors.

The grafting density of the model avidin protein, specifically attached to the biotin moieties coupled to the free amine groups, varied from 0.45×10^{12} to 1.3×10^{12} proteins/cm^2, depending on the precursor composition, whereas the analogous values for the non-specifically adsorbed proteins were lower by a factor of 4-5. The grafting density was not only limited by the amount of the potentially available specific docking sites but also affected by steric constrains associated with the characteristic mesh size of the crosslinking network of the PHMs. The size corresponding to the most favourable arrangements of the molecular chains in this network was estimated at 8.4 nm, being, most likely, shorter in reality and comparable with the characteristic dimensions of proteins.

The engineering of PHMs with biomolecule-specific receptors can be of potential interest for sensor fabrication, e.g. for biomedical diagnostics, while successive functionalization of these membranes with biomolecules can be of importance for medicine, including tissue engineering and regenerative therapy. A special advantage of PHMs as compared to other potentially useful biocompatible hydrogel films is the possibility of their transfer to any substrate or particular place, which facilitates their combination with other functional units of a device or their application at a specific site, useful, e.g. for wound curing. In addition, the size of hydrogel mesh can be varied to some extent, relying on the commercially available STAR-PEG precursors with higher molecular weight. Finally, the PHMs are sufficiently robust in aqueous solutions, due to the covalent character of the crosslinking bonds and the 3D network character of the entire system, which is particular relevant for the application involving protein sensing and/or adhesion.

Acknowledgements

This work has been supported by the German Research Foundation (DFG; grant ZH 63/17-1) and Innovation Foundation FRONTIER of the Heidelberg University (project number D.801000/13.060).

View Article Online
DOI: 10.1039/C5CP07840G

References

1 A. Larsson, T. Ekblad, O. Andersson and Bo Liedberg, *Biomacromolecules* 2007, **8**, 287-295.

2 P. T. Charles, V. R. Stubbs, C. M. Soto, B. D. Martin, B. J. White and C. R. Taitt, *Sensors* 2009, **9**, 645-655.

3 L. M. Weber, C. G. Lopez and K. S. Anseth, *J. Biomed. Res. A* 2009, **90**, 720-729.

4 F. Pippig and A. Holländer, *Macromol. Biosci.* 2010, **10**, 1093-1105.

5 T. Hussain, N. M. Ranjha and Y. Shahzad, *Design. Monom. Polym.* 2011, **14**, 233-249.

6 A. Mateescu, Yi Wang, J. Dostalek and U. Jonas, *Membranes* 2012, **2**, 40-69.

7 S. Kruss, L. Erpenbeck, M. P. Schön and J. P. Spatz, Circular, *Lab Chip* 2012, **12**, 3285-3289.

8 V. Hagel, T. Haraszti and H. Boehm, *Biointerphases* 2013, **8**:36.

9 E. Cambria, K. Renggli, C. C. Ahrens, C. D. Cook, C. Kroll, A. T. Krueger, B. Imperiali and L. G. Griffith, *Biomacromolecules* 2015, **16**, 2316-2326.

10 N. Meyerbröker, T. Kriesche and M. Zharnikov, *ACS Appl. Mater. Interfaces* 2013, **5**, 2641-2649.

11 N. Meyerbröker and M. Zharnikov, *Adv. Mater*. 2014, **26**, 3328-3332.

12 N. Meyerbröker and M. Zharnikov, *ACS Appl. Mater. Interfaces* 2013, **5**, 5129-5138.

13 N. Meyerbröker and M. Zharnikov, *ACS Appl. Mater. Interfaces* 2014, **6**, 14729-14735.

14 M. Khan, S. Schuster and M. Zharnikov, *J. Phys. Chem. C* 2015, **119**, 14427-14433.

15 J. Feng, L. Peng, C. Wu, X. Sun, S. Hu, C. Lin, J. Dai, J. Yang and Y. Xie, *Adv. Mater.* 2012, **24**, 1969−1974.

16 F. Pippig, S. Sarghini, A. Holländer, S. Paulussen and H. Terryn, *Surf. Interface Anal.* 2009, **41**, 421-429.

17 C. Nietzold, P. M. Dietrich, S. I. Pankov, A. Lippitz, T. Gross, W. Weigel and W. E. S. Unger, *Surf. Interface Anal.* 2014, **46**, 668-672.

18 N. Meyerbroeker, P. Waske and M. Zharnikov, *J. Chem. Phys.* 2015, **142**, 101919.

19 H. K. Hunt, C. Soteropulos and A. M. Armani *Sensors* 2010, **10**, 9317-9336.

20 C. M. Yam, C. M. Pradier, M. Salmain, P. Marcus and J. Jaouen, *J. Colloid Interface Sci.* 2011, **235**, 183-189.

21 E. H. Williams, A. V. Davydov, A. Motayed, S. G. Sundaresan, P. Bocchini, L. J. Richter, G. Stan, K. Steffens, R. Zangmeister, J. A. Schreifels and M. V. Rao, *App. Surf. Sci.* 2012, **258**, 6056-6063.

View Article Online
DOI: 10.1039/C5CP07840G

22 L. S. Jung, K. E. Nelson, P. S. Stayton and C. T. Campbell, *Langmuir* 2000, **16**, 9421-9432.

23 N. Ballav, A. Terfort and M. Zharnikov, *Langmuir* 2009, **25**, 9189-9196.

24 Y. L. Jeyachandran, A. Terfort and M. Zharnikov, *J. Phys. Chem. C* 2012, **116**, 9019-9028.

25 B. D. Ratner and D. G. Castner, in *Surface Analysis—The Principle Techniques*, ed. J. C. Vickerman, chapter 3, pp 43-98, Wiley & Sons, Chichester, 1997.

26 J. N. Hiremath and B. Vishalakshi, *Der Pharma Chem.* 2012, **4**, 946-955.

27 E. P. Chan, A. P. Young, J.-H. Lee, J.-Y. Chung and C. M. Stafford, *J. Polym. Sci. Part B: Polym. Phys.* 2013, **51**, 385-391.

28 S. Khan and N. M. Ranjha, *Polym. Bull.* 2014, **71**, 2133-2158.

29 S. Lee, X. Tong and F. Yang, *Acta Biomaterialia* 2014, **10**, 4167-4174.

30 M. G. Horning, A. M. Moss and E. C. Horning, *Biochim. Biophys. Acta* 1967, **148**, 597-600.

31 S. Y. Park, B. X. Kang, Q. Li, H. S. Kim, J. Lee and J. Hong, *Bull. Korean Chem. Soc.* 2009, **30**, 1497-1504.

32 Y. L. Jeyachandran, T. Weber, A. Tefort and M. Zharnikov, *J. Phys. Chem. C* 2013, **117**, 5824-5830.

33 F. Chesneau, J. Zhao, C. Shen, M. Buck and M. Zharnikov, *J. Phys. Chem. C* 2010, **114**, 7112–7119.

34 T. Winkler, N. Ballav, H. Thomas, M. Zharnikov and A. Terfort *Angew. Chem. Int. Ed.* 2008, **47**, 7238–7241.

35 M. W. Laney, G. L. Christina and S. A. Kristi, *J. Biomed. Mater. Res.* A 2009, **90**, 720-729.

36 B. H. Jered, K. D. Neal, A. H. Scott, L. Hakho and W. Ralph, *Nat. Nanotechnol.* 2010, **5**, 660-665.

37 C. Kotsmar, T. Sells, N. Taylor, D. E. Liu, J. M. Prausnitz and C. J. Radke, *Macromol.* 2012, **45**, 9177-9187.

38 V. Liljeström, J. Mikkilä and M. A. Kostiainen, *Nat. Commun.* 2014, **5**, 4445.

39 N. Ballav, B. Schüpbach, O. Dethloff, P. Feulner, A. Terfort, and M. Zharnikov, *J. Am. Chem. Soc.* 2007, **129**, 15416-15417.

40 F. Schreiber, *Prog. Surf. Sci.* 2000, **65**, 151-256.

View Article Online
DOI: 10.1039/C5CP07840G

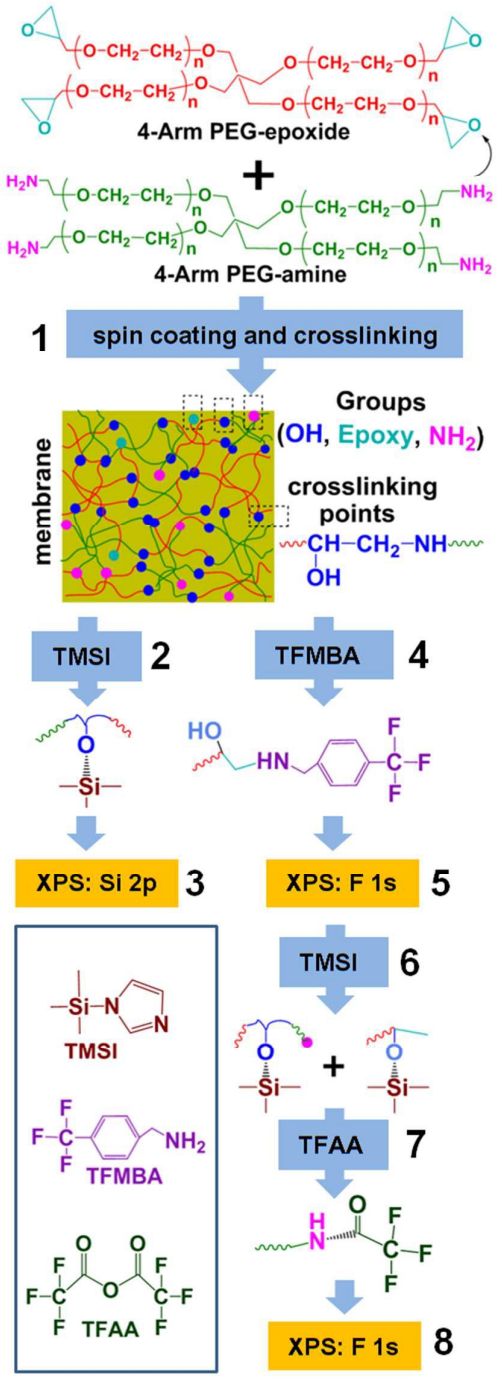


Figure 1. Fabrication (1), chemical derivatization (2, 4, 6, 7), and characterization (3, 5, 8) of the derivatized PHMs. OH groups were traced with the steps 2 and 3, "free" (i.e. non-reacted and, consequently, still reactive) epoxy groups with the steps 4 and 5, which were additionally followed by the steps 6-8 to trace the "free" (i.e. non-reacted and, consequently, still reactive) amine groups; see text for details. The results of the derivatization are schematically shown after the respective derivatization steps. The chemical reagents used for the derivatization are depicted in the bottom left rectangle.

View Article Online
DOI: 10.1039/C5CP07840G

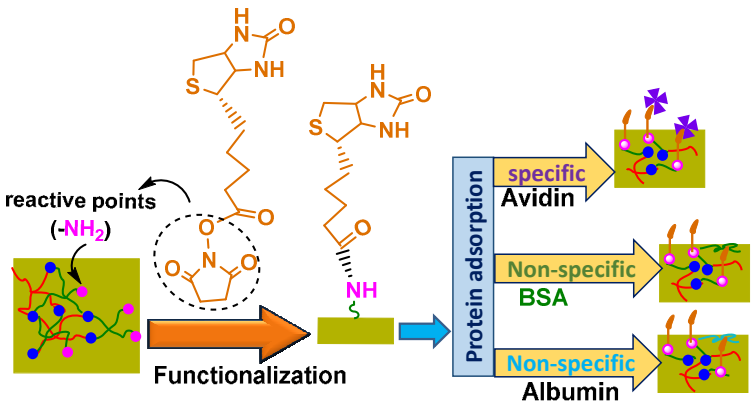


Figure 2. Biofunctionalization of the reactive sites (free amine groups in the given case) of the PHMs with NHS-biotin, and the protein adsorption assay for the specific (avidin) and non-specific proteins, viz. BSA and albumin.

View Article Online
DOI: 10.1039/C5CP07840G

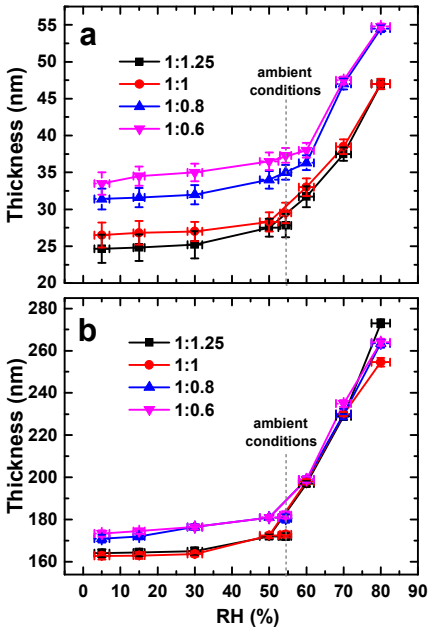


Figure 3. Ellipsometric thickness of the thin (a) and thick (b) PHMs as function of relative humidity. The PHMs were fabricated at different compositions of the precursors as marked and color-coded in the figure. RH corresponding to the ambient conditions is marked by the vertical dotted lines. The thickness of the PHMs is 25-35 ± 1.5 nm (a) and 170-180 ± 2 nm (b).

View Article Online
DOI: 10.1039/C5CP07840G

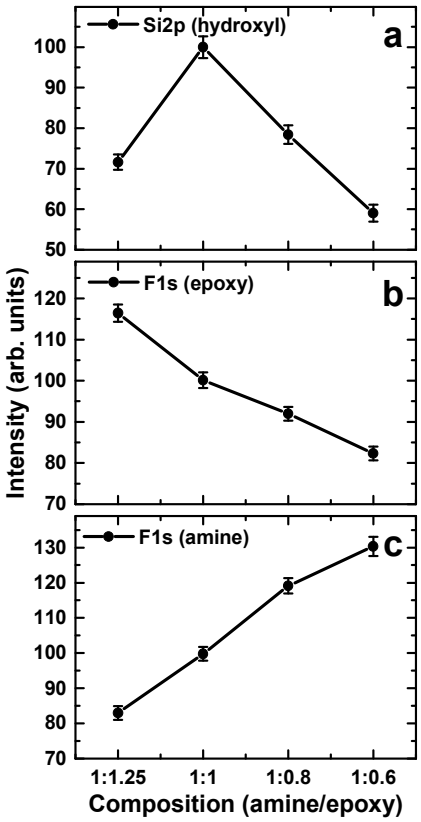


Figure 4. Intensities of the characteristic photoemission signals for the hydroxyl groups (a) as well as for non-reacted and, consequently, still reactive epoxy (b) and amine (c) groups for the PHMs fabricated using different compositions of the precursors, given by the amino/epoxy ratio. The intensities are normalized to the values for the PHM fabricated at the equilibrium composition of the precursors (1:1).

View Article Online
DOI: 10.1039/C5CP07840G

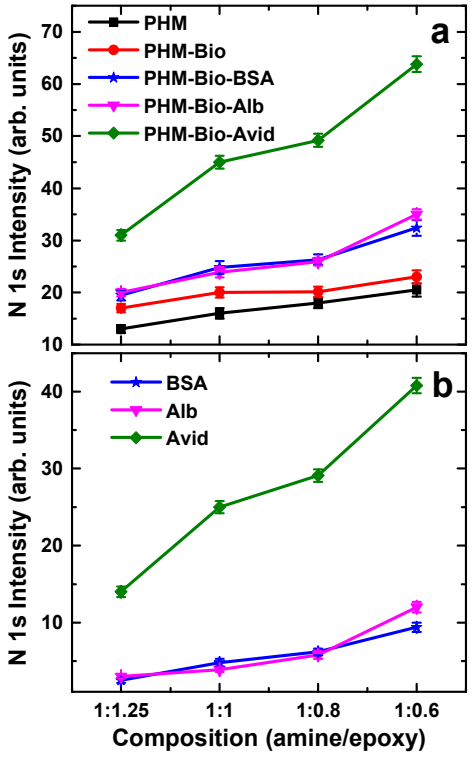


Figure 5. Intensities of the characteristic N 1s photoemission signal for the pristine PHMs (black squares) as well as for the PHMs functionalized with NHS-biotin (red circles) and subsequently exposed to BSA (blue stars), albumin (magenta down triangles), or avidin (green diamond). The composition of the precursors, given by the amino/epoxy ratio, was varied. (a) As measured intensities; (b) Intensities for the protein-loaded PHMs corrected for the PHM-Bio signal.

View Article Online
DOI: 10.1039/C5CP07840G

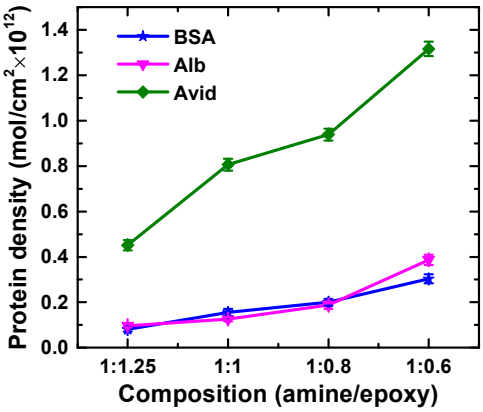


Figure 6. Grafting density of specific (avidin; green diamond) and non-specific (BSA; blue stars and albumin; magenta down triangles) for a series of the PHMs functionalized with NHS-biotin. The composition of the precursors, given by the amino/epoxy ratio, was varied.