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Formation of protein molecular imprints within Langmuir monolayers: A quartz crystal microbalance study

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Abstract

Protein imprinting leading to enhanced rebinding of ferritin to ternary lipid monolayers is demonstrated using a quartz crystal microbalance. Monolayers consisting of cationic dioctadecyldimethylammonium bromide, non-ionic methyl stearate, and poly(ethylene glycol) bearing phospholipids were imprinted with ferritin at the air/water interface of a Langmuir-Blodgett trough and transferred hydrated to hydrophobic substrates for study. This immobilization was shown by fluorescence correlation spectroscopy to significantly hinder any further diffusion of lipids, while rebinding studies demonstrated up to a six-fold increase in ferritin adsorption to imprinted versus control monolayers. A diminished rebinding of ferritin to its imprint was observed through pH reduction to below the protein isoelectric point, demonstrating the electrostatic nature of the interaction. Rebinding to films where imprint pockets remained occupied by the template protein was also minimal. Studies with a smaller acidic protein revealed the importance of the steric influence of poly(ethylene glycol) in forming the protein binding pockets, as albumin-imprinted monolayers showed low binding of ferritin, while ferritin-imprinted monolayers readily accommodated albumin. The controllable structure-function relationship and limitations of this system are discussed with respect to the application of protein imprinting in sensor development as well as fundamental studies of proteins at dynamic interfaces.

Keywords

Protein adsorption; Molecular imprinting; Langmuir monolayer; Mixed monolayer; Quartz crystal microbalance; QCM; Fluorescence correlation spectroscopy; FCS; Poly(ethylene glycol); PEG; Ferritin

1. Introduction

Molecularly imprinted polymers (MIPs) are typically made by forming a scaffold of functional molecules around a template molecule. This complex is then fixed in place by cross-linking and the template is extracted, leaving imprinted recognition sites, or imprints, behind. Specific rebinding of the template occurs because the imprints are complementary to template surface properties, shape, and structure [1,2]. MIPs have demonstrated potential in a variety of fields where defined recognition is required, including separation [3,4], sensing [5-7], catalysis [8, 9] and drug delivery [10]. Molecular imprinting has previously been applied to small molecules such as amino acids [11,12], toxins [13-15] and drugs [16-18]. Selectivity between enantiomers

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has been demonstrated [19,20], and affinities comparable to those obtained with antibodies have been achieved [14].

While successful for small template molecules, applying traditional bulk imprinting to macromolecules, such as proteins, has proven difficult due to inherent technical problems [21]. These include reduced mass transfer and permanent entrapment of the macromolecule template in the polymer matrix, diminished integrity of the polymer structure, restricted solvent selection, and production of heterogeneous binding sites. To counter this, several groups have proposed using imprinting methods at surfaces [22]. This strategy eliminates problems associated with mass transfer and entrapment in a matrix, while allowing the integrity of the imprinted surface to be maintained following extraction. With these added benefits several surface imprinting protocols for proteins have been reported, most of which are based on adapting bulk polymerization methods to a surface geometry [23-29]. The work presented here describes an alternative strategy in which imprints are formed in self-assembling lipids at the air/water interface, followed by film transfer and immobilization.

The benefits of lipid monolayer imprinting include excellent mass transfer, ease of integration into sensor systems and directed formation of imprint sites. Sagiv was the first to demonstrate *small molecule* imprinting on a monolayer using self-assembled alkanethiols and dyes [30]. The imprint cavities bound molecules of approximate size and shape as demonstrated for several different dyes. Martín and Möbius extended the small molecule imprinting technique by incorporating porphyrins into mixed lipid monolayers at the air-water interface followed by film transfer to preserve the induced lipid charge pattern [31]. Piletsky imprinted cholesterol in a self-assembled layer of hexadecyl mercaptan on a gold surface demonstrating that the template could be selectively rebound to the layer and that the cavities could be used as channels for the reduction of potassium ferrocyanide [32]. A similar system was developed for ibuprofen using a β -cyclodextrin moiety, where competition for binding sites between ferrocene and ibuprofen was observed [33]. Miyahara formed Langmuir films of two polymerizable amphiphiles (one containing a boronic acid group, acting as monomer) at the air/water interface then transferred and polymerized the films on a hydrophobic quartz slide [34]. It was demonstrated that the imprinted layer offered 35% greater affinity in rebinding of the template, 4-nitrophenyl α -D-mannopyranoside, when compared with its non-imprinted counterpart. The use of metal coordination for the recognition of proteins has also been demonstrated [35]. Arnold's group used these interactions to imprint myoglobin within mixed lipid bilayers and Langmuir monolayers containing metal-chelating lipids and Cu^{2+} ions [36]. The chelated copper ions interacted with histidine residues available on the surface of the protein template.

The general concept of protein imprinting on self-assembling ternary lipid films prepared as Langmuir monolayers is illustrated in Fig. 1. Previous work has indicated that protein adsorption to both supported bilayers [37] and multi-component lipid monolayers [38,39] induces local lipid demixing as predicted by mean-field theory [40]. During adsorption, complementary recognition elements are formed as individual lipids reorder to accommodate the template protein in a process analogous to complexation seen in MIPs. This process may be driven through electrostatic interactions between protein residues and lipid head-groups [41]. Previous studies have demonstrated that the acidic protein ferritin could induce lipid restructuring in a mixture of non-ionic methyl stearate (SME) and cationic dioctadecyldimethylammonium bromide (DOMA) lipids at the air-water interface [38,39]. Further, the adsorption process could be controlled by adjusting the SME:DOMA molar ratio. However, a combination of strong protein adsorption and protein aggregation limited the usefulness of the binary SME:DOMA system for imprinting.

For improving protein affinity for the monolayers and reducing protein-protein aggregation, poly(ethylene glycol) (PEG) bearing lipids have been introduced into the SME:DOMA

monolayers [42], as depicted in Fig. 1. Adsorption to and removal of ferritin from these PEG:SME:DOMA films created protein-sized pockets in the monolayer as evidenced by atomic force microscopy (AFM) [42]. Thus, the PEG-lipid addressed the problem of protein aggregation; however, the rebinding of ferritin was not evaluated. This approach to creating protein binding sites in monolayers is in contrast to work by Cai's group, which uses a similar principle of an inert oligoethyleneglycol molecule to shape the pockets in which protein is bound [43]. However, in that case nanolithography is used to form the binding pockets within the fixed monolayer instead of the self-assembly mechanism employed here.

The aim of this work is to fully characterize the formation and transfer of these three-component PEG:SME:DOMA monolayers and assess the rebinding of the template and challenger proteins to the imprint pockets. Langmuir trough techniques are used to characterize the insertion of ferritin into the ternary lipid monolayers as a function of monolayer composition in order to maximize protein-monolayer interactions while restricting protein-protein aggregation. Fluorescence correlation spectroscopy (FCS) is used to examine the mobility of lipids following transfer from the air/water interface to a hydrophobic substrate to ensure preservation of imprint pockets following protein elution. Quartz crystal microgravimetry (QCM) is used to analyze rebinding of ferritin and albumin to imprint and control monolayers to assess imprint site selectivity.

2. Experimental

2.1. Lipids

Methyl stearate (SME) (99% GC, Fluka), dioctadecyldimethylammonium bromide (DOMA) (99% Tech Grade, TCI) and 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[methoxypoly(ethyleneglycol)-350] as an ammonium salt (PEG) (99% in chloroform, Avanti Polar Lipids) were prepared as 1 mM stock solutions in chloroform (99% HPLC grade, Merck). 1-Palmitoyl-2-[12-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]docedyl]-*sn*-glycero-3-phosphocholine (NBDPE) was used as provided (1 mg/ml in chloroform, Avanti Polar Lipids). All lipid solutions were stored at -20°C. These lipids represent non-ionic (SME) and cationic (DOMA) points of interaction for the acidic proteins, ferritin and albumin. For imprinting, lipid restructuring is anticipated to pair cationic DOMA lipid head-groups with acidic residues on the proteins, with SME serving as a spacer molecule to preserve the DOMA charge pattern.

For imprinting studies binary solutions of SME and DOMA were mixed into two molar ratios of 2:1 and 9:1 (alternatively referred to as 2:1 SME:DOMA or SD21 and 9:1 SME:DOMA or SD91, respectively). These ratios were selected to present the protein with surfaces of relatively high (SD21) and low (SD91) charge density. SME:DOMA solutions can be treated as one lipid component for ease of analysis, as previous work shows that they are completely miscible [38,41]. SME:DOMA solutions were then mixed with PEG to give ternary solutions with the molar ratios 6:2:1 PEG:SME:DOMA (2 parts PEG to 1 part SD21) and 20:9:1 PEG:SME:DOMA (2 parts PEG to 1 part SD91). This ratio of PEG to SD lipids was found to limit protein surface aggregation on the monolayers.

2.2. Protein

For titration experiments horse spleen ferritin, type 1 (Fer) (76 mg/ml, Sigma) was diluted to a 10 mg/ml stock. For imprinting studies at the air/water interface a 0.1 mg/ml ferritin solution was prepared in double-distilled, deionized (DDI) water. Bovine serum albumin (BSA) (98%, Sigma) was prepared as a 2.5 mg/ml stock in DDI water and used as required.

2.3. Substrate preparation

For QCM analysis, QCM crystals (5 MHz Au/Ti, Stanford Research Systems) were cleaned initially with ethanol in order to remove any oils and then with a plasma etch (O_2 , 200 mTorr, 50 W, 60 s) (Plasmod, Tegal Corp.) which resulted in a fully water-wettable surface. They were then treated with a solution of 0.1 mM 1-octadecanethiol (ODT) (Fluka) in ethanol (Absolute, AAPER Alcohol and Chemical Co.) for 10 min, followed by rinses in ethanol and water. After drying with a filtered nitrogen gas stream, each crystal exhibited a water contact angle of 100° to 110° (Cam100, KSV Instruments Ltd.). Hydrophobically modified QCM crystals were stored in DDI water until required for use.

Silica cover glasses (25 mm diameter No. 2, VWR Scientific or 25 mm diameter No. 1 $\frac{1}{2}$, Erie Scientific) were first washed with ethanol then treated with a 3:1 H_2SO_4 :DDI water piranha solution (96% H_2SO_4 , Mallinckrodt) at room temperature for 1 h, rinsed three times in DDI water, dried with a nitrogen gas stream and then plasma etched as above to ensure a reactive surface chemistry. The coverslips were then treated with 0.5 mM octadecyltrichlorosilane (OTS) ($\geq 85\%$, Fluka) in hexane (96%, EM Science) for 1 h. The slides were rinsed in hexane, then chloroform, and then water for 10 min each and dried with a filtered nitrogen gas stream. The water contact angles of OTS-modified glass coverslips were between 104° and 112° . They were stored in DDI water until use.

2.4. Monolayer formation

A small Langmuir trough with dipping well (KSV Mini-trough, KSV Instruments Ltd.) was used to form all lipid monolayers. The trough was kept free of interfering compounds by subsequent rinses in ethanol and DDI water before and after each experiment. The trough was filled either with DDI water or with 0.1 mg/ml ferritin in DDI water, depending on the experiment. A small, acid-cleaned glass beaker was then submerged in the dipping well for use in sample transfer. The subphase was gently stirred using a Teflon-coated magnetic bar in the dipping well in order to ensure ferritin full dispersion in the solution. A Wilhelmy plate was inserted and the surface was cleaned by compression and aspiration.

Langmuir monolayers were prepared at the air/water interface by drop wise addition of the lipid solutions as described previously [39,42]. Monolayers were allowed 10 min for solvent evaporation and then compressed to 10 mN/m at a rate of 20 mm/min. Compression was stopped at 10 mN/m but it was observed that the surface pressure would spontaneously increase for samples with protein in the subphase due to transient interaction between the protein and the monolayer. This process was not allowed to go to completion in order to reduce protein-protein interactions and ensure that monolayer-associated ferritin was not “pushed out” by the “pancake-brush” phase transition [42]. This surface pressure increase was used to study the insertion of ferritin into the monolayers. Monolayer transfers to QCM crystals were made at a final surface pressure of 15 mN/m for the protein samples.

Lipid monolayers were transferred to the appropriate hydrophobic substrate by horizontal dip (Langmuir-Schaeffer). Briefly, the substrate was slowly translated through the lipid film at the air/water interface and lowered into the beaker in the dipping well. The beaker and substrate were then removed from the trough and added to a large, DDI-filled evaporation dish for further transfer into the FCS or QCM flowcell.

A similar method was used for the production of BSA imprinted layers. A 6:2:1 PEG:SME:DOMA layer was compressed on a stirred water subphase to 10 mN/m. Then 100 μ l of 2.5 mg/ml BSA in water was injected below the surface into the dipping trough. The resulting transient increase in surface pressure was allowed to continue until a surface pressure of 15 mN/m was reached and then the layer was transferred to a substrate as described above.

2.5. FCS on transferred monolayers

$1\frac{1}{2}$ thickness glass coverslips were cleaned and treated with OTS as described above. 2 mol% or 0.02 mol% NBD-PE was added to 6:2:1 PEG:SME:DOMA. NBD-PE-doped 6:2:1 PEG:SME:DOMA lipid monolayers were then prepared at the air/water interface as previously described, followed by horizontal transfers to hydrophobic glass coverslips. The transferred monolayers were finally placed within a coverslip holder such that the lipid-bearing side of the coverslip remained in contact with DDI water during all subsequent handling and observations.

The custom-built instrument used for both confocal fluorescence microscopy and FCS on NBD-PE doped PEG:SME: DOMA monolayers was described previously [44]. For these experiments a 488 nm laser source, 100 $\times$ 1.4NA oil immersion objective, and 50 μ m pinhole were used. For confocal fluorescence microscopy observations, ~7.5 mW excitation laser light was scanned across the 2 mol% NBD-PE-doped sample and the spatial distribution of fluorescence was recorded. For FCS scans the laser was attenuated to 50 μ W, focused onto the monolayer, and a 30 s scan was taken. The total number of NBD-PE lipids in the observation region of the FCS instrument was estimated by finding the total number of lipids in the circular observation region given that its radius is approximately 0.25 μ m and the average area of a single lipid is 50 $\text{\AA}^2$. Hence at 0.02% NBD-PE in 6:2:1 PEG:SME:DOMA there are an estimated 78 fluorescent lipids in the observation region, a number density expected to result in an appropriate signal-to-noise ratio in FCS experiments.¹

2.6. Microgravimetric analysis of protein binding to monolayers

Ferritin imprinted monolayers and corresponding blanks were prepared on QCM crystals and transferred to the flowcell of a 5 MHz quartz crystal microbalance flowcell (QCM200, Stanford Research Systems) underwater to ensure monolayer hydration. The flowcell was then connected to a gravity-driven flow setup such that a larger reservoir could be used for rinsing and protein could be injected upstream with minimal variation of the hydrostatic pressure exerted to the QCM crystal.

For each experiment, a QCM crystal with immobilized monolayer was first stabilized in DDI water by passing several sample volumes from the rinse reservoir. A sample volume was defined as the volume required to just replace the volume of solution in the flowcell chamber. Each layer was then treated with three sample volumes of diluted hydrochloric acid at pH 2.1, again allowing for an equilibrium to be reached. Such acid washes have previously been shown to break interactions between SME:DOMA and ferritin without disrupting the structure of the monolayer [39]. In previous control experiments, an HCl solution of pH 2.8 was used, which was not strong enough to remove ferritin from these monolayers [39]. In other controls the acid washes were omitted altogether to ensure the imprint sites remained occupied during rebinding studies.

Following template elution, DDI water exchanges were then initiated and continued until the QCM signal reached equilibrium. Rebinding studies were performed in DDI water (pH 5.5) to allow the results to be compared to previous studies [39,42]. The temperature of the system was maintained at 21 $^{\circ}$ C. Protein was injected into the rinse reservoir at a given final concentration and the flowcell volume was completely displaced with the protein solution. The placement of the flow control valve at the same height as the flowcell, combined with the constant level in the open rinse reservoir ensured constant hydrostatic pressure at the QCM sensor during measurements. The adsorption of protein to the surface was allowed to proceed under same hydrostatic conditions until the QCM signal reached steady state. The rinse

¹The actual number should be higher in the liquid-expanded regions of the monolayer and lower in the liquid-condensed domains due to sequestering [42]. FCS data were taken exclusively from the liquid-expanded regions.

reservoir solution would then be changed to the next increased protein concentration and the run would be repeated, and so on, until the binding titration experiment was concluded. Ferritin titrations were performed in the range of 0.001 to 1 mg/ml. The change in QCM frequency between each solution at steady-state was calculated and used to construct binding isotherms.

In pH specificity studies the solution following the acid wash was changed from water to 50 mM acetate buffer at pH 3.5, which is below the pH 4.5 isoelectric point of ferritin, therefore altering the overall charge of the protein.

3. Results and discussion

3.1. Protein insertion into Langmuir monolayers

The surface pressure isotherms and mixing diagrams for the PEG:SME:DOMA monolayers have been reported previously [42]. Ferritin interactions with these Langmuir monolayers are presented in Fig. 2 as surface pressure transients. In general, ferritin interactions with the monolayer led to an increase in surface pressure over time that was controlled by the lipid composition of the monolayer. The adsorption process was promoted in samples with higher relative DOMA constituency, which is consistent with the hypothesis that the adsorption is controlled in part through electrostatic interactions between ferritin and DOMA. In addition, the inclusion of PEG served to decrease adsorption rates in a controllable manner.

In a previous study, a significant fraction of ferritin was irreversibly bound to SME:DOMA binary monolayers [39], likely through a combination of electrostatic interactions and protein-protein surface aggregation. As seen in Fig. 2, decreasing the relative amount of DOMA and adding a lipid with a protein-blocking PEG head-group discourage this process. This dependence on DOMA is similar to the uptake of myoglobin, which is dependent on the presence of Cu^{2+} in the work of Arnold [36]. In addition, slowing the rate of ferritin interaction with the monolayer leads to an increase in the time available for demixing of the lipids to accommodate the protein in a specific manner, as previously modeled [40]. Finally, the inclusion of PEG leads to a self-limiting adsorption process in which protein interaction with the monolayer increases surface pressure which itself results in a PEG “pancake/brush” transition [45]. Hence, by controlling the monolayer constituency, it was possible to promote the self-limiting formation of ferritin imprints instead of irreversibly-bound ferritin surface aggregates. Thus, having developed a system where protein-monolayer interactions can be precisely tuned, the next questions are: (1) Is the imprinted monolayer structure preserved upon film transfer? (2) Does ferritin interaction with these monolayers lead to formation of protein-specific imprints?

3.2. Microscopy and FCS on transferred monolayers

One of the central assumptions made in lipid-based templating is that the lipids do not spontaneously return to a less-ordered, non-templated state after being transferred to a solid substrate. The substrate is presumed to “lock in” binding pockets formed at the air/water interface. In this work, we have used FCS to characterize the surface diffusion of a fluorescent lipid, NBD-PE, within PEG:SME:DOMA lipid films that have been transferred to hydrophobic substrates.

NBD-PE was previously shown to preferentially sequester to a liquid-expanded phase in PEG:SME:DOMA films prepared at the air/water interface, which were retained upon transfer to glass coverslips [42]. This general phase behavior was verified here on transferred 2 mol% NBD-PE-doped 6:2:1 PEG:SME:DOMA monolayers by scanning confocal fluorescence microscopy (data not shown).

Fig. 3 shows a representative fluorescence intensity transient (upper panel) and correlation curve (lower panel). The lack of noticeable “spikes” in the fluorescent transient, supported by the random distribution of the correlation data around the correlation baseline, leads to the conclusion that the fluorescence observed was not correlated in the characteristic diffusion time range of 140 ns to 30 s, i.e., that transferred NBD-PE lipids were not the subject of translational diffusion. Efforts to use non-linear least-square fitting analysis for the correlation data to a standard FCS two-dimensional diffusion model led to divergence. In a slightly altered experiment in which the fluorescent lipids in the FCS focal region were first photo-bleached, no fluorescence recovery was observed, nor was there any indication of any non-intentional NBD-PE photo-bleaching events. Taken together, this is strong, albeit indirect, evidence that NBD-PE lipids in the transferred monolayer are immobile.

3.3. Rebinding experiments

Prior to imprinting we assessed the affinity of ferritin for binary SME:DOMA monolayers as well as pure PEG-lipid films. Non-imprinted monolayers of these constituent components were formed using the methods described above and immobilized on the QCM electrode. Fig. 4 shows the observed binding of ferritin to these layers. Ferritin showed high affinity towards the pure SD21 layer, mimicking previous work [39], and began to saturate above 10 $\mu\text{g/ml}$ bulk concentration. In contrast, the pure PEG layer bound a negligible amount of protein over this range. The control ODT-modified gold surface also showed no affinity for ferritin in water, matching previous results by Höök [46].

Ferritin rebinding to imprinted and control monolayers is shown in Fig. 5. Significantly less protein adsorbs to these PEG-bearing ternary monolayers as compared with SME:DOMA as was shown in Fig. 4. This decrease in binding capacity of the SME:DOMA:PEG monolayers is a necessary trade-off in order to limit protein aggregation and irreversible binding. When comparing ferritin affinity for imprinted versus control monolayers in Fig. 5, a modest imprinting effect was noted below the imprinting concentration of 100 $\mu\text{g/ml}$. Two ternary mixtures were studied for imprinting: 20:9:1 and 6:2:1 PEG:SME:DOMA. The imprinted 6:2:1 PEG:SME:DOMA layer exhibited almost two times the adsorption of ferritin than the imprinted 20:9:1 PEG:SME:DOMA layer at < 10 $\mu\text{g/ml}$, whereas the non-imprinted layers within the same range were practically identical. This resulted in imprinting factors of 3.8 (20:9:1) and 6 (6:2:1) at 10 $\mu\text{g/ml}$. As the non-imprinted mono-layers were prepared at the same initial conditions it is concluded that the difference in binding resulted from ferritin interactions with the lipid monolayer at the air/water interface, which resulted in the restructuring of the monolayer to form imprints that were subsequently preserved upon film transfer.

Rebinding of ferritin to the films is strongly concentration dependent. This can be attributed to the filling of specific affinity sites, i.e., available imprints, at lower concentrations where the greatest difference in rebinding between imprinted and control films is seen. As indicated by the surface pressure transients (Fig. 2) the imprinting process was stopped prior to reaching equilibrium. Thus, fewer imprint sites were formed than possible for an imprinting concentration of 100 $\mu\text{g/ml}$. Therefore during rebinding, once the imprint mediated binding threshold was reached, non-specific interactions became more prominent and the adsorption contrast between imprinted and control films reduced.

To address whether any residual template protein not removed during acid washing steps might be influencing the rebinding of protein we assessed protein binding to an imprinted film in which the template was intentionally left in place. Fig. 6 shows the rebinding of ferritin to the 6:2:1 PEG: SME:DOMA layers prepared under wash conditions of water only and weaker acid that are known to not remove the original templating ferritin during the washing step [39]. Compared with imprint films rinsed at the lower pH necessary to remove the template, the

overall ferritin binding to these films was dramatically reduced. This suggests that formed binding sites consisting of SME:DOMA surrounded by PEG were not available as they were already occupied with protein, therefore little additional protein could be bound. Thus from the data in Figs. 5 and 6 it is evident that Langmuir monolayers prepared on a protein subphase exhibited a higher affinity for protein versus non-imprinted counterparts, as well as imprinted counterparts in which the imprint sites were blocked by original template protein.

3.4. Protein specificity

To test the imprinted monolayers for specificity towards the original template we conducted rebinding on ferritin-templated samples using BSA as a control, and with the opposite case of BSA imprinted in matching conditions challenged with ferritin, presented in Fig. 7.

Both BSA and ferritin are acidic proteins having isoelectric points near 4.5 and should thus both bind to DOMA from a solution with a pH greater than 4.5. Hence, in the BSA rebinding experiment, imprint selectivity will be challenged primarily by differences in the size, shape, and distribution of amino acids on the two proteins. When a ferritin-imprinted 6:2:1 PEG:SME:DOMA layer was challenged with BSA, the control protein exhibited a greater affinity for this monolayer than the ferritin. This is not surprising and could be attributed to the smaller protein, BSA, easily fitting into the large imprint sites formed by ferritin. The smaller size of BSA may also allow greater non-specific adsorption as it may be able to better penetrate the PEG layer surrounding the binding pockets than the larger ferritin as previously reported [45]. It is thus apparent that the specificity of an imprint formed against a “large” protein will be intrinsically compromised when challenged with a similarly charged smaller protein. Nonetheless, when challenging an imprint formed against a smaller protein with a larger protein of similar charge a better selectivity is anticipated, which is the case when a 6:2:1 PEG:SME:DOMA layer imprinted with BSA is challenged with ferritin as shown in Fig. 7. These results are comparable to the control experiment when ferritin is presented to a non-imprinted layer. As no appreciable binding is observed we can speculate that the PEG has formed smaller pockets around the BSA template, and these, due to their decreased size, are not able to accommodate ferritin. This demonstrates the steric influence of PEG and its importance in forming a protein-inert matrix around SME:DOMA binding pockets as envisioned in Fig. 1.

To test the imprints with a control protein of the same size as the original template, we challenged an imprinted 6:2:1 PEG:SME:DOMA layer with ferritin in a pH 3.5 solution as shown in Fig. 8. Reducing the solution pH below the isoelectric point of ferritin (pI 4.5) changes the net charge on ferritin from anionic to cationic, thus providing a control for the electrostatic nature of the binding. The rebinding isotherm of ferritin at this lower pH shows that ferritin affinity is dramatically reduced by this pH change when compared to the rebinding of ferritin at pH 5.5.

4. Conclusions

This work highlights the crucial role that monolayer constituency and transfer play in the formation and preservation of protein imprints on multi-component lipid monolayers with both protein attracting (SME:DOMA) and protein repelling (PEG) elements. It was demonstrated that the controllable formation of imprints is aided by a balance between promotion of the protein-monolayer interaction through electrostatics and hindrance through PEG-based steric effects. Upon transfer to a hydrophobic substrate these imprinted lipids are immobilized, thus “locking in” their structure, as supported by fluorescence spectroscopy. When followed by removal of the ferritin template and rebinding studies, this technique resulted in up to a six-fold enhancement in ferritin rebinding to the imprinted versus control monolayers. Dramatically reduced binding to imprinted films in which the binding sites were blocked by

the original template protein further supported the formation of protein binding pockets in the monolayers. The electrostatic nature of the protein-monolayer interaction was assessed by changing the charge on the template protein during rebinding through pH reduction to below the protein isoelectric point. In this case, ferritin adsorption was limited to the level of that seen for non-imprinted monolayers at pH above the isoelectric point.

The lipid monolayer imprinting technique has several limitations, most of which are common to any macromolecule imprinting effort, namely a high degree of non-specific adsorption, and poor discrimination against non-templated protein challenges. In particular, smaller proteins with a similar electrostatic nature to the template are able to effectively compete for imprint sites as was demonstrated for albumin challenges to ferritin imprints; however, the converse was not true, suggesting the steric exclusion of ferritin from albumin imprints by the PEG chains. It is clear that specific binding comes only in simplified environmental conditions conducive to interactions between the layer and protein that are detectable with a very sensitive technique, such as QCM.

Despite these difficulties, lipid monolayer imprinting has inherent potential. Optimization of the lipid types and ratios is a major factor in monolayer imprinting, similar to traditional imprinting where the composition of the polymer mixture is paramount [1]. This is clearly demonstrated in Fig. 5 where two monolayer compositions are directly compared under identical rebinding conditions. For the imprinting conditions employed here, it is observed that the 6:2:1 PEG:SME:DOMA layer (Fig. 5B) offers greater discrimination and less error between replicates than the 20:9:1 PEG:SME:DOMA layer (Fig. 5A).

In addition to optimizing the lipid ratios, employing longer PEG chains to reduce non-specific adsorption may also enhance the lipid-template interaction. While it was demonstrated that the short PEG chains employed here inhibit protein adsorption when prepared as a single component monolayer, this efficacy may be diluted upon mixing with other protein-binding lipids. The observed level of binding to non-imprinted monolayers suggests that the protein is still able to penetrate the PEG layer in the ternary films even though they are immobilized and unable to laterally restructure to best accommodate the protein. A longer PEG chain could restrict this by occupying a greater area. Alternatively, a different blocking chemistry such as a phosphorylcholine head-group or highly charged sugar may be employed in place of PEG. Finally, additional lipid head-group chemistries could be included in an effort to construct a greater number of favorable protein-monolayer interactions beyond the relatively simple ternary monolayer explored here.

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References

- [1]. Wulff G. *Angew. Chem. Int. Ed* 1995;34:1812–1832.
- [2]. Sellergren, B. *Molecularly Imprinted Polymers: Man-Made Mimics of Antibodies and Their Applications in Analytical Chemistry*. Sellergren, B., editor. Elsevier; Amsterdam: 2001. p. 113-183.
- [3]. Bossi A, Castelletti L, Piletsky SA, Turner AP, Righetti PG. *J. Chromatogr. A* 2004;1023:297–303. [PubMed: 14753696]
- [4]. Kempe M, Mosbach K. *J. Chromatogr. A* 1995;691:317–323. [PubMed: 7894656]
- [5]. Haupt K, Norworyta K, Kutner W. *Anal. Commun* 1999;36:391–393.
- [6]. Yamazaki T, Ohta S, Yanai Y, Sode K. *Anal. Lett* 2003;36:75–89.

- [7]. Zhang Z, Liao HP, Li H, Nie L, Yao S. *Anal. Biochem* 2005;336:108–116. [PubMed: 15582565]
- [8]. Wulff G. *Chem. Rev* 2002;102:1–27. [PubMed: 11782127]
- [9]. Heilmann J, Maier WF. *Angew. Chem. Int. Ed* 1994;33:471–473.
- [10]. Alvarez-Lorenzo C, Concheiro A. *J. Chromatogr. B* 2004;804:231–238.
- [11]. Vidyasankar S, Ru M, Arnold FH. *J. Chromatogr. A* 1997;775:51–63.
- [12]. Lin JM, Nakagama T, Uchiyama K, Hobo T. *Chromatographia* 1996;43:585–591.
- [13]. Piletska E, Turner NW, Turner APF, Piletsky S. *J. Controlled Release* 2005;108:132–139.
- [14]. Chianella I, Lotierzo M, Piletsky SA, Tothill IE, Chen B, Karim K, Turner APF. *Anal. Chem* 2002;74:1288–1293. [PubMed: 11924591]
- [15]. Turner NW, Whitcombe M, Malecha M, Karim K, Piletska E, Baggiani C, Piletsky S. *Biosens. Bioelectron* 2004;20:1060–1067. [PubMed: 15556349]
- [16]. Suede R, Srichana T, Saelim J, Thavornpibulbut T. *Analyst* 1999;124:1003–1009. [PubMed: 10736857]
- [17]. Armstrong DW, Ward TJ, Armstrong RD, Beesley TE. *Science* 1986;232:1132–1135. [PubMed: 3704640]
- [18]. Berggren C, Bayoudh S, Sherrington D, Ensing K. *J. Chromatogr. A* 2000;889:105–110. [PubMed: 10985542]
- [19]. Sellergren B. *Macromol. Chem* 1989;190:2703–2711.
- [20]. Yu C, Ramström O, Mosbach K. *Anal. Lett* 1997;30:2123–2140.
- [21]. Turner NW, Jeans CW, Brain KR, Allender CJ, Hlady VH, Britt DW. *Biotechnol. Progr* 2006;22:1474–1489.
- [22]. Nicholls IA, Rosengren JP. *Bioseparation* 2001;10:301–305. [PubMed: 12549873]
- [23]. Bossi A, Piletsky SA, Piletska EV, Righetti PG, Turner APF. *Anal. Chem* 2001;73:5281–5286. [PubMed: 11721930]
- [24]. Chou J, Rick J, Chou TC. *Anal. Chim. Acta* 2005;542:20–25.
- [25]. Shi HQ, Ratner BD. *J. Biomed. Mater. Res* 2000;49:1–11. [PubMed: 10559740]
- [26]. Rick J, Chou TC. *Anal. Chim. Acta* 2005;542:26–31.
- [27]. Burow M, Minoura N. *Biochem. Biophys. Res. Commun* 1996;227:419–422. [PubMed: 8878530]
- [28]. Ramanaviciene A, Ramanavicius A. *Biosens. Bioelectron* 2004;20:1076–1082. [PubMed: 15556351]
- [29]. Rachkov A, Minoura N. *Biochim. Biophys. Acta* 2001;1544:255–266. [PubMed: 11341934]
- [30]. Sagiv J. *J. Am. Chem. Soc* 1980;102:92–98.
- [31]. Martin MT, Mobius D. *Thin Solid Films* 1996;285:663–666.
- [32]. Piletsky S, Piletskaya EV, Sergeyeva TA, Panasyuk TL, El'skaya AV. *Sens. Actuators B* 1999;60:216–220.
- [33]. Chmurski K, Temeriusz A, Bilewicz R. *J. Inclusion Phenom. Mol. Inclusion* 2004;49:187–191.
- [34]. Miyahara T, Kurihara K. *Chem. Lett* 2000:1356–1357.
- [35]. Conrad, PG., II; Shea, KJ. *Molecularly Imprinted Materials: Science and Technology*. Yan, M.; Ramström, O., editors. Dekker; New York: 2005. p. 123–180.
- [36]. Shnek D, Pack D, Sasaki DY, Arnold FH. *Langmuir* 1994;10:2382–2388.
- [37]. Bondurant B, Last JA, Waggoner TA, Slade A, Sasaki DY. *Langmuir* 2003;19:1829–1837.
- [38]. Britt DW, Möbius D, Hlady V. *Phys. Chem. Chem. Phys* 2000;20:4594–4599.
- [39]. Du X, Hlady V, Britt DW. *Biosens. Bioelectron* 2005;20:2053–2060. [PubMed: 15741075]
- [40]. May S, Harries D, Ben-Shaul A. *Biophys. J* 2000;79:1747–1760. [PubMed: 11023883]
- [41]. Goodman T, Bussmann E, Williams C, Taveras M, Britt DW. *Langmuir* 2004;20:3684–3689. [PubMed: 15875400]
- [42]. Dhruv H, Pepalla R, Taveras M, Britt DW. *Biotechnol. Progr* 2006;22:150–155.
- [43]. Gu J, Yam CM, Li S, Cai C. *J. Am. Chem. Soc* 2004;126:8099.
- [44]. Wright, BE. Ph.D. thesis, Bioengineering. University of Utah; Salt Lake City: 2006.

- [45]. Jogikalmath, G. Ph.D. thesis, Materials Science and Engineering. University of Utah; Salt Lake City: 2003.
- [46]. Hook F, Rodahl M, Brzezinski P, Kasemo B. J. Colloid Interface Sci 1998;208:63–67. [PubMed: 9820749]

Fig. 1. Schematic of protein imprinting in Langmuir monolayers. The native PEG:SME:DOMA monolayer at the air/water interface (A) interacts with the protein to form imprints (B) that are complementary in shape and charge to the protein. The monolayer is locked into place when transferred to a hydrophobic support (C), followed by removal of the template protein and rebinding studies (D).

Fig. 2.

Surface pressure transients following the insertion of ferritin into Langmuir monolayers at the air/water interface from a 0.1 mg/ml ferritin subphase. Four lipid monolayers are shown: 2:1 SME:DOMA (A), 9:1 SME:DOMA (B), 6:2:1 PEG:SME:DOMA (C), and 20:9:1 PEG:SME:DOMA (D). All layers were compressed to 10 mN/m before recording the transient surface pressure increase to 15 mN/m.

Fig. 3. FCS results for 0.02% NBD-PE-doped 6:2:1 PEG:SME:DOMA transferred monolayer. A representative fluorescence intensity transient, $I(t)$, and correlation curve, $G(\tau)$, are shown. Correlation curves on these samples were consistently and randomly distributed about the correlation baseline, indicating no observed diffusion events in the range of 140 ns to 30 s.

Fig. 4. QCM measured ferritin binding in water (pH 5.5) to non-imprinted SME:DOMA (2:1) layer (squares); ODT modified gold only (diamonds); and non-imprinted PEG layer (triangles).

Fig. 5. QCM measured ferritin rebinding in water (pH 5.5) to (A) imprinted (closed circles) and control (non-imprinted) (open circles) ternary mixture monolayers with the ratio 20:9:1 PEG:SME:DOMA, at 15 mN/m; (B) imprinted (closed squares) and control (non-imprinted) (open squares) ternary mixture monolayers with the ratio 6:2:1 PEG:SME:DOMA, at 15 mN/m. The error bars show $\pm$ one standard deviation. For 20:9:1 layers: $n = 2$. For 6:2:1 layers: $n = 3$.

Fig. 6.

QCM measured ferritin rebinding in water (pH 5.5) to 6:2:1 PEG:SME:DOMA layers at 15 mN/m. Non-imprinted washed with acid (pH 2.1) (open squares); imprinted layer washed with acid (pH 2.1) (closed squares); imprinted layer washed with acid (pH 2.8) (open triangles); and imprinted layer washed with water only (pH 5.5) (closed triangles).

Fig. 7. Protein rebinding to imprinted ternary mixture monolayers with the ratio 6:2:1 PEG:SME:DOMA, at 15 mN/m, measured by QCM. BSA in water (pH 5.5) to a ferritin imprinted layer (closed diamonds) and ferritin in water (pH 5.5) to a BSA imprinted layer (open diamonds).

Fig. 8. Protein rebinding to imprinted ternary mixture monolayers with the ratio 6:2:1 PEG:SME:DOMA, at 15 mN/m, measured by QCM. Ferritin in water (pH 5.5) to a ferritin imprinted layer (closed squares) and ferritin in 50 mM acetate buffer (pH 3.5) to a ferritin imprinted layer (open squares).