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SYNTHESIS AND CHARACTERIZATION OF POLYMER BRUSHES FOR CONTROLLED ADSORPTION OF PROTEINS

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SYNTHESIS AND CHARACTERIZATION OF POLYMER BRUSHES FOR CONTROLLED ADSORPTION OF PROTEINS

A Dissertation
Presented to
the Graduate School of
Clemson University

In Partial Fulfillment
of the Requirements for the Degree
Doctor of Philosophy
Material Science and Engineering

by
Olha Hoy
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Accepted by:
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ABSTRACT

Performance of biomedical devices to a large extent depends on the interactions between the device surface and the biological liquids/protein molecules. To achieve controllable interactions between the device and biomolecules and still retain the required mechanical strength on the whole, modification of the surface is often done.

In the present study surface properties were modified through a polymer brush approach. After the modification, surfaces gain tunability toward protein adsorption. Mixed polymer brushes consisting of protein repelling and protein attractive components were used, with a “grafting to” method employed for the synthesis of polymer layers. First, poly(ethylene glycol), the protein repelling component of the mixed polymer brush, was tethered to the surface. Then, polyacrylic acid-b-polystyrene (the protein attractive component) was grafted on top of the previous layer. As one part of this study, the temperature dependence of grafting of the mixed brush components was studied.

Surface morphology and surface properties of the mixed polymer brush were altered by treating the brush with different organic solvents. Changes in surface morphology and properties resulting from the solvent treatment were studied in dry conditions and in aqueous media. Hydrophobic interactions of the mixed polymer brush in different pH environments were also estimated.

Synthesized mixed polymer brushes demonstrated a clear dependency between the external stimuli applied to the brush and the amount of the protein adsorbed onto the brush surface, allowing an effective control of protein adsorption. Attraction forces
between the protein molecules and surface of the mixed polymer brush were measured using AFM and these supported the findings from the protein adsorption studies.

2-D molecular imprinting of the polymer brush approach was used to synthesize a surface with controlled positioning of the protein molecules on the surface. Protein adsorption onto the surface of the synthesized imprints was studied and evaluated using TIRF (Total Internal Reflectance fluorescence) and Fluorescence Spectrophotometry. The studies have shown that the synthesized surfaces may be used for spatial control of protein adsorption.
DEDICATION

This work is dedicated to my husband, Taras Andrukh, who always has been there for me during both the happy and tough times. And my parents Vasyl and Iryna Hoy and sister, Svitlana Matviyiv, who always believed in me.
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I also want to express gratitude to my research team, Dr. Swaminatha Iyer, Dr. Yong Liu, Dr. Karthik Ramaratnam, Dr. Ruslan Burtovyy, Mr. Oleksandr Butrovyy, Mr. Suraj Sharma, Mr. Marius Chyasnavichyus, Ms. Fehime Vatansever, Mr. Kyrylo Chernyshuk, Mr. Michael Seeber and Mr. James Giammarco for their encouragement and support and for all the help and suggestions they provided during my graduate studies.

I am grateful to School of Material Sciences and Engineering, Clemson University for the possibility to work in this incredible team spirit I was exposed to during my memorable research experience.
Finally, I want to thank CAEFF (Center for Advanced Engineering Fibers and Films), NTC (National textile Center) and NSF (National Science Foundation) for the funding of the projects, part of which this work is.
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CHAPTER ONE
INTRODUCTION

A majority of the devices that are used in medicine are made from polymeric materials, a vast fraction of which have a hydrophobic nature. Immersion of such devices into biological liquids is followed by immediate and uncontrollable adsorption of proteins onto the device surface. This protein adsorption may lead to failure or improper functioning of the devices, especially in the case of bioimplants. Thus, the ability to modify the surface layers of implants and other medical devices to control the amount of protein adsorbed is of great importance for today’s biomedical industry demands. Diverse techniques, well documented in the scientific literature, have been applied to date to control protein adsorption on the surface, with a focus on prevention of this adsorption. However, only a few modification techniques have been successfully implemented in the industry, due to the high cost and complexity of the existing techniques. There is a vital demand for a simple, inexpensive approach for the synthesis of a surface with controllable performance toward protein adsorption. Ultrathin polymer layers with tunable properties grafted to a surface can be used to regulate surface properties without altering bulk properties of the material, and, thus, affect the possibility for straightforward generation of surfaces with controllable protein affinity.

The ultimate goal of the current study is to fabricate and characterize thin polymer layers (brushes) prepared by a “grafting to” approach that have tunable properties toward protein adsorption. “Grafting to” is a relatively simple and easy to control surface
modification procedure. It can result in a surface with a durable covering, high grafting
density and known molecular weight of the polymer brush.

Two different strategies were employed for the synthesis of surfaces with controllable protein adsorption. These were construction of surfaces consisting of mixed polymer brushes containing both hydrophilic and hydrophobic fragments for controlling the amount of protein adsorption and construction of molecularly imprinted polymer brushes for control of spatial adsorption of biomolecules.

Chapter 2 provides an overview of polymer brush preparation, mixed polymer brush behavior (switching properties) and the interaction of protein molecules with polymer brushes as they relate to the applications under investigation in this study. Protein imprinting techniques are presented in this chapter to demonstrate the advantages of a surface preparation method that can selectively adsorb protein molecules at assigned sites. Chapter 3 gives descriptions of the experimental techniques used in this work.

In the first part of the current study, the experimental procedure to synthesize a mixed polymer brush by a “grafting to” approach was developed. To achieve uniformity of the coverage and avoid “patchiness” of the surface, grafting was done in two steps. First, poly(ethylene glycol), a protein repelling component, was grafted to the surface. Then, in the second step, polyacrylic acid-b-polystyrene copolymer was grafted through the poly(ethylene glycol) brush. Chapter 4 contains a study of the temperature dependence of the extent of grafting of polymer chains (poly(ethylene glycol) and polyacrylic acid). The influence of the diffusivity of the polymer chains and rates of reactions on the grafting extent was also determined.
**Chapter 5** describes the study of the mixed brush surface properties after subjecting the brushes to organic solvent treatment. Components of the mixed polymer brush having different properties, when subjected to different conditions, segregate and change their surface chemical content as well as distribution of the components inside the brush bulk. Differently sized and differently positioned polystyrene domains are formed inside the brush during solvent treatment. Protein attractive components of the brush (polystyrene and polyacrylic acid) may become exposed to the surface to a variable extent, resulting in different capabilities of protein adsorption. Changes in surface properties change the interaction with the external environment, and AFM and water contact angle measurements were used to study how organic solvents induce surface morphology changes of mixed polymer brushes and their individual components. Based on solubility parameters calculations for brush component and solvents used, together with results of these measurements, the morphology/chemical composition of the surface of mixed brushes were predicted.

All biological liquids containing proteins are water based, thus adsorption onto the surface of bioimplants happens from aqueous solutions of proteins. Water acts in the same way as any other solvent, therefore it also results in changes of mixed brush surface components as well as vertical repositioning of protein attractive hydrophobic components of the brush. In **Chapter 6**, the morphology and behavior of mixed brush surfaces in aqueous environments was studied using AFM in contact mode. Using the AFM images of the mixed polymer brush after organic solvent treatment, the size and concentration of the water insoluble protein attractive component of the mixed polymer
brush – polystyrene was identified. Using a hydrophobically modified AFM tip, hydrophobic interactions between the mixed polymer brush and the tip were measured at different pHs, different ionic strengths of the media and different valencies of metal salt ions. Theoretical predictions of the mixed brush extension are in good agreement with the results of Ellipsometry and AFM scratch measurements.

**Chapter 7** is devoted to the studies of protein adsorption onto mixed polymer brushes and their components. Brushes were first treated with organic solvents and then subjected to a solution of bovine fibrinogen as a test protein. Dependency between the morphology of the brush surface developed after the solvent treatment and the extent of protein adsorption was determined. Results of the adsorption were consistent with the results of adhesion measurements obtained with the protein modified AFM tip and the surface of a mixed brush. The effect of the treatment of a mixed polymer brush with calcium chloride solution on the brush affinity toward protein molecules is also described in *Chapter 7*.

**Chapter 8** and **Chapter 9** are devoted to the synthesis of molecularly imprinted polymer brushes and the study of the protein adsorption on this type of prepared surface, respectively. A molecularly imprinted surface was prepared through a multistep process, which included chemical attachment of the protein to the surface, grafting of the poly(ethylene glycol) brush onto unoccupied spaces around the protein molecules and cleavage of the protein molecule to form adsorption sites. Solvent assisted grafting at low temperatures was employed both for the grafting of protein to the surface and for
grafting of the poly(ethylene glycol) brush. AFM and Ellipsometry were used to characterize the obtained imprints and to control the process of synthesis of imprints.

In Chapter 9, a study of protein adsorption onto these molecularly imprinted polymer brushes is described. Protein adsorption was performed in two regimes: dynamic and static. In the dynamic regime, protein was adsorbed from a flowing solution while, in the static regime, protein molecules were adsorbed from the solution where motion of the molecules was Brownian only. Adsorption of a single protein, as well as a mixture of proteins, onto molecularly imprinted polymer brush surfaces was performed. Fluorescence spectroscopy and total internal reflectance fluorescence were used to evaluate the amount of protein adsorbed onto the prepared surfaces.

In conclusion, this dissertation will provide the fundamentals for fabrication and characterization of thin polymer layers with controllable properties toward protein adsorption.
CHAPTER TWO
LITERATURE REVIEW

The present research work is devoted to the preparation of a polymer brush system with controllable protein adsorption and the study of its properties. The study requires an understanding of the basics of polymer brush preparation, polymer brush behavior and polymer brush interactions with biomolecules. Specifically this chapter gives an overview of polymer brush preparation, mixed polymer brush behavior (switching properties) and the interaction of protein molecules with polymer brushes as they relate to their application. Protein imprinting techniques are presented in this chapter to demonstrate the advantages of a surface preparation method that can selectively adsorb protein molecules at the assigned sites.

2.1: Polymer brushes. Definition and general properties:

The performance of a material is determined not only by the properties of the bulk substance from which material is made, but also by the properties of its surface. Several characteristics of a material that are determined by its surface properties are adhesion, lubrication, wettability, colloidal stability, friction and biocompatibility. Polymer chain assemblies, called “polymer brushes”, are often used to alter the above mentioned properties.

Polymer brushes gained interest in the 1950s when it was discovered that aggregation of colloidal particles can be prevented by grafting polymer molecules onto
them. Van der Waarden used hydrocarbon chains to stabilize carbon black particles, and thus began a whole new direction in polymer science.

The term “polymer brush” refers to end-tethered polymer molecules that are attached to a surface in such proximity that the distance between two grafting points is less than two radii of gyration of the tethered polymer molecule. This close proximity causes an overlap between the adjacent chains and significantly alters the conformational dimensions of the chains. As a result, the polymer molecules extend in order to avoid unfavorable conformations and form a “brush”.

In his model for tethered polymer chain extension, Alexander considered a flat nonadsorbing surface with monodispersed polymer chains tethered to it. The free energy per chain includes two terms:

\[ F = F_{\text{int}} + F_{\text{el}} \]

where \( F_{\text{int}} \) refers to the interaction energy between two statistical segments and \( F_{\text{el}} \) refers to the elastic free energy. There are two approximations: i) the depth profile of statistical segments are step-like with a constant concentration within brush \( \varphi \), \( \varphi = N\alpha/d^2 L \), where \( N \) is the number of statistical segments of polymer chain with diameter = \( \alpha \), \( d \) is the distance between tethering points, and where all free ends of the tethered polymer chains are located in a single plane at a distance = \( L \) from the tethering surface; and ii) a “Flory approximation” that is used to obtain an explicit expression of free energy. The corresponding free energy per chain \((F/kT)\) is described as:

\[ F/kT \approx \nu \varphi^2 d^2 L/\alpha^3 + L^2/R_0^2 \]
where $\nu$ is a dimensionless excluded volume parameter, $R_\theta$ is the radius of unperturbed coil, and the other variables are as described above.

The first term ($F_{\text{int}}$) represents the interaction free energy between statistical segments and the second ($F_{\text{el}}$) represents the elastic free energy of Gaussian chains. The equilibrium thickness is obtained by a minimization of $F$ with respect to $L$. This idea of a balance between the interaction energy and elastic free energy can be applied to a range of situations, such as a theta solvent or a bad solvent.$^{11}$

The obtained relationship between $N$ and the dimensions of polymer chains is shown in Table 2.1.$^{12}$

<table>
<thead>
<tr>
<th></th>
<th>Polymer brush</th>
<th>Free polymer chain</th>
</tr>
</thead>
<tbody>
<tr>
<td>Good solvent</td>
<td>$L/a \approx N(a/d)^{2/3}$</td>
<td>$R_g \approx N^{3/5}$</td>
</tr>
<tr>
<td>Theta solvent</td>
<td>$L/a \approx N(a/d)$</td>
<td>$R_g \approx N^{1/2}$</td>
</tr>
<tr>
<td>Bulk state</td>
<td>$L \approx N^{2/3}$</td>
<td>$R_g \approx N^{1/2}$</td>
</tr>
</tbody>
</table>

Stretching a polymer brush film in a direction perpendicular to the grafting surface introduces novel surface properties that are distinctly different from the film’s regular surface properties. These include interfacial localization of terminal groups,$^{13}$ diffusion control,$^{14}$ regulation of steric repulsion forces,$^{15}$ control of phase segregation in response to external stimuli,$^{16}$ wetting control,$^{17}$ control of protein and cell adsorption,$^{18}$ lubrication and flocculation control.$^{19}$
2.2. Synthesis of the polymer brushes:

2.2.1. Physisorption and “grafting from” technique:

Overall, two methods for polymer brush preparation can be distinguished: physisorption and covalent attachment (Figure 2.1). Physisorption is based on the selective adsorption of one block of a diblock copolymer to a surface. This method was used in early studies on polymer brushes. The surface was chosen to maximize the preferential adsorption of one block to the surface, while the solvent was chosen to preferentially interact with the other block. The main disadvantage of this method is the low thermal and solvent stability of the prepared brush. Additionally, the surface-adsorbed block can be displaced by other adsorbents.12

Figure 2.1. Preparation of polymer brushes by physisorption, “grafting to” and “grafting from” techniques.
These disadvantages are overcome by the second method of polymer brush preparation: the covalent attachment of polymer chains. There are two techniques for the preparation of covalently bound polymer chains, referred to respectively as “grafting from” and “grafting to” techniques.

The “grafting from” technique involves the \textit{in situ} polymerization of a monomer from the surface that is functionalized by using an initiator. Living and conventional vinyl polymerizations are successfully used to prepare polymer brushes.\textsuperscript{12} This method can produce very thick brushes, as growing chains are constantly fed by the monomer solution. When a grafted layer is swollen by the addition of monomers, there is no limitation due to diffusion of the monomer unless a very high grafting density is approached. The main complications that arise from this technique are limitations in initiator surface coverage, the initiator efficiency and the rate of diffusion of the monomer to polymerization sites. Additionally, the effects of side reactions in bimolecular termination become an issue because of the high local concentration of growing polymer chains. Hence, polymer brushes prepared by using the “grafting from” technique have a broader molecular weight distribution compared to those prepared by other methods.\textsuperscript{8}

\subsection*{2.2.2. “Grafting to” technique for preparation of polymer brushes:}

The “grafting to” technique involves the chemical reaction between presynthesized polymer molecules that bear a reactive functional end group and a
complementary surface reactive group.\textsuperscript{20} The distinct advantage of this method is the simple synthesis and precisely characterized polymer chains that are used.

Liguore and coworkers have done extensive studies on the kinetics of the "grafting to" technique.\textsuperscript{21} He distinguished two successive regimes in the kinetics of adsorption. The first one (short time) is governed by the Brownian diffusion of the chains in the solution, the second regime (long time) is governed by the activation barrier, which appears as soon as the anchored chains begin to overlap strongly and to stretch. The characteristic construction time varies exponentially with the chemical affinity of the end group and the surface.

Later, Karim et al.\textsuperscript{22} studied the grafting of trimethylchlorosilane-terminated polystyrene from solution onto a polished silicon wafer. Higher amounts of polymer were adsorbed from the poor solvent cyclohexane than from good solvent toluene. Other
studies have shown that there is a clear difference in the grafting kinetics when the procedure is performed in a good solvent (tailed parabola kinetics) compared to a bad solvent (step-like kinetics). Next, studies of the tethering of monodisperse, chain-end-functionalized polystyrene to a solid surface in a diluted toluene solution have shown three kinetic regimes. Huang et al.\textsuperscript{23} has presented support for the hypothesis that the experimentally observed third regime is the transition from mushroom to brush, and that it occurs in a spatially non-uniform manner (Figure 2.2). Both time-step snapshots generated by a Monte Carlo simulation of the tethering process and atomic force microscopy images of the actual surfaces during the process show that the third regime is characterized by a non-uniform surface texture; the surface texture is uniform prior to and after the third regime.

Moreover, it was observed by Kumacheva et al.\textsuperscript{24} that a zwitterion-terminated polystyrene polymer brush will undergo replacement in the presence of smaller molecular weight polymer chains containing the same functional end group. Hence, shorter molecules will displace longer molecules from the brush even when the concentration of the smaller molecules is 1/500 that of the longer chains.\textsuperscript{24}

The successful preparation of dense polystyrene brushes through melt grafting employing the “grafting to” technique, with a density close to that of bulk material, has been published.\textsuperscript{14,25} Zdyrko et al.\textsuperscript{26} have investigated the influence of the molecular weight of carboxy-terminated poly(ethylene glycol) (PEG) on the yield of the grafting of polymer chains. It was determined that the maximum thickness of the attached PEG films was strongly dependent on the length of the polymer chains being grafted. The
maximum grafting efficiency was found for polymers with a molar weight that was close to the critical entanglement molecular weight of PEG, and efficiency decreased with an increase in the molecular weight of the polymer chains.

A wide variety of the functionalized surfaces, as well as a wide variety of functional end groups for the polymer chains, is used to perform the grafting. The most widely used surfaces are either epoxy-functionalized\textsuperscript{24} or amino-functionalized,\textsuperscript{14,27} and the most widely used chains are carboxyl-terminated.\textsuperscript{28-32} A high grafting yield was also obtained by using chlorosilane-,\textsuperscript{22} vinyl- and silanol-terminated polymer chains.\textsuperscript{33} The variety of the end-functionalities is limited only by the reactivity of the surface, hence a chemical reaction fast enough to allow a reasonable time for polymer brush formation to occur is required. Recently, the “click reaction” was successfully used to obtain brushes of polystyrene, poly(methyl methacrylate) and poly(ethylene glycol).\textsuperscript{34} “Click reaction” is the popular name of the 1,3-cyclo-addition of azides containing terminal acetylenes by using a copper catalyst at room temperature.\textsuperscript{35}

Overall, the “grafting to” method for the preparation of polymer brushes has been widely exploited and is well documented in reviews devoted to the preparation and application of polymer brushes.\textsuperscript{8,12}

\textbf{2.3. Mixed polymer brushes. Preparation and properties:}

When two or more types of polymer chains are combined into one brush, the obtained structure is called a “mixed polymer” brush. These structures are proposed as a new type of smart material that demonstrates adaptive and responsive behavior based on
the phase segregation mechanism of the two incompatible polymers. The theoretical background of phase segregation in mixed polymer brushes has been well described on the basis of SCF theory by Marko and Witten, followed by Zhulina, Balazs and Muller. The different chemical structures of the two polymer molecules increase the mixing free energy and favors phase segregation. As polymer chains are tethered to the surface, their limited mobility prevents segregation on a larger level, therefore the polymers segregate into nano-sized phase-separated domains and the size of these domains is determined by the dimensions of the participating molecules. The reduction of the interaction energy due to segregation is opposed by the loss of entropy and by the loss of stretching energy of grafted polymer chains. Phase diagrams calculated for mixed polymer brushes considered miscibility parameters of the components, solvent quality, grafting density, molecular weight of chains, and relative grafting density of the dissimilar chains.

To avoid unfavorable segregation between the immiscible parts of the polymer, brush chains can arrange themselves into two limiting types of morphology. The tethered chains segregate perpendicularly to the surface into a layered morphology (Figure 2.3a) or a two-dimensional surface structure might self-assemble laterally, with the defined lateral length determined by the degree of molecular extension (Figure 2.3b). In intermediate cases, different morphologies as ripples, checkerboards or hexagonal dimple phases have been reported. The theory of segregation is in good agreement with the experimental results reported.
Mixed polymer brushes are synthesized in the same ways as the homopolymer brushes, but with slight modifications of the procedure. Both the “grafting to” and “grafting from” techniques have been used successfully for the preparation of binary mixed polymer brushes. Initially, covalently grafted mixed polymer brushes were prepared by using the “grafting from” approach, involving two-step radical polymerization, in 1999 by Minko, Stamm et al.\textsuperscript{45} The initiator in that case was chemically bound to the epoxy surface through a carboxylic group. Since then, a variety of modifications for this method has been reported.\textsuperscript{46-48} Another approach to synthesize polymer brushes by using the “grafting from” approach is atom-transfer radical polymerization, which gives a more uniform distribution to the molecular weight of polymer chains.\textsuperscript{49-51}

Synthesis of brushes by using the “grafting to” approach is based on sequential tethering of the brush components from the melt to the functionalized surface.\textsuperscript{25,29,52} Generally, the grafting procedure is done in several steps. First, the initial layer of the

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure2.3.png}
\caption{Schematic illustration of possible morphologies of mixed brush irreversibly grafted to solid substrates (cross-section of the layer): layered disordered morphology in a solvent selective for gray chains (a), ripple morphology in a nonselective solvent (b).}
\end{figure}
polymer is deposited and grafting is conducted from the melt at elevated temperatures. After the removal of any ungrafted polymer by using a solvent, the second layer of the end-functionalized polymer is deposited onto the previous brush layer, and the polymer chains are melt-grafted to any unreacted surface functional groups. After the removal of any ungrafted second polymer by using a solvent, a mixed polymer brush is formed.

Although well established, the “grafting to” procedure for the preparation of mixed polymer brushes is still laborious. To decrease the time and labor expenses necessary for brush preparation and study, a gradient approach for preparing mixed polymer brushes was developed. Gradient polymer brushes are synthesized by varying the grafting density of one polymer and either keeping stable or varying the grafting density of the other polymer. As in the ordinary preparation of brushes, gradient polymer brushes can be fabricated either by the “grafting to” or by the “grafting from” technique. In the “grafting to” technique, temperature is often used as the factor that governs the final grafting density of the brush. When prepared by using the “grafting from” approach, the initiator surface grafting density is varied to achieve a change in the grafting density of the mixed polymer brush. Gradient brushes made from stimuli-responsive polymers exhibit measurable changes in their properties as a function of grafting density or stimuli.

### 2.4. Protein interaction with the polymer brushes:

Protein/polymer surface interactions are of great interest to researchers, as these interactions predetermine the performance of artificial organs, medical devices, and test
There are several major mechanisms by which proteins are retained on a polymer surface. These include hydrophobic interactions, electrostatic interactions or a mixture of both. Additionally, chemical bonding between a protein and the polymer surface can take place.

Proteins are very complex structures that when adsorbed onto a surface undergo spatial changes. It was shown by Soderquist and Walton\textsuperscript{63} that plasma proteins (including albumin, globulin and fibrinogen) desorbed from copolypeptide and silicone surfaces had a different ellipticity than native protein, indicating that the secondary structure of the protein changed. Desroches and coworkers\textsuperscript{64} have done extensive studies on the conformational changes of fibrinogen adsorbed onto a biomedical grade stainless steel surface. Adsorption of fibrinogen on this surface resulted in significant changes to the protein’s secondary structure that occurs predominantly within the first minute of adsorption. Among the investigated structures, the alpha-helix undergoes the smallest changes, while the beta-sheet and beta-turns undergo more significant changes. It was shown that lateral interactions between the adsorbed molecules do not play a role in controlling the changes in secondary structure. An increase in temperature induced changes in the secondary structure of the protein, characterized by a loss of the alpha-helical content and its transformation into the beta-turn structure. AFM studies of fibrinogen adsorbed on a silica-based surface have shown that a bound protein molecule may have different conformations than that of the native protein.\textsuperscript{65}

While the adsorption of proteins to a flat surface often results in conformational changes of the protein and probably denaturation,\textsuperscript{66} the majority of the executive
functions of the protein remains intact when adsorbed onto the surface, especially those surfaces that contain long polymeric chains. Ballauff has shown that immobilized glucoamylase and glucosidase, adsorbed onto a poly acrylic acid brush, retain nearly their full activity in terms of Michaelis-Menten parameters. The same conclusion was drawn from studies of the fluorescence activity of the fluorescent protein mEosFP.

Due to the complexity of protein adsorption and the variety of factors that influence the process, there is not one single model that perfectly describes protein adsorption in all situations. Nevertheless, there are a few mechanisms proposed by several authors to describe the way proteins adsorb to a surface. Generally, the theories of protein adsorption can be divided into thermodynamic statistical models and transport kinetics models.

Statistical thermodynamic models have been developed by Su et al. for the nonlinear multicomponent protein adsorption equilibrium on an ion exchanger. This model takes into account the electrostatic interactions between the adsorbent surface and the protein molecules, as well as the lateral interactions between adsorbed protein molecules. There are two categories of model parameters: one corresponds to the adsorption affinity of the protein and the other is descriptive of the interactions between the adsorbed molecules. Therefore, all of the model parameters have definite physical meanings, and for the adsorption equilibrium of a single protein, there are only two model parameters. Szollosi et al. modeled three adsorption stages consisting of adsorption/desorption (stage 1), conformation changes (stage 2) and further stabilization (stage 3).
The models fit the experimental data of the adsorption of bovine serum albumin (BSA) well. The effects of buffer type, pH and ionic strength on the model parameters are reasonably well interpreted by the electrostatic and thermodynamic theories. Thermodynamic statistical models give a good explanation of the molecular-level mechanism of adsorption. However, there is no quantifiable image of the process.

Transport kinetic models describe the process by using the mass and charge conservation in the near vicinity of the surface. Observations made by Clark et al. indicate that the interaction of bound protein molecules with those free in solution contribute to a prolonged change in the surface energy. This has been used to define a new model for the kinetics of globular protein adsorption to a solid-liquid interface. It provides a mechanism by which the molecules in the bulk material can facilitate the desorption of an adsorbed molecule or change the energetic states of adsorbed molecules and, thus, the overall surface energy. The model includes the unique features of protein adsorption kinetics, such as i) fast mass loading; ii) the much more gradual change in surface energy that does not stop until the protein is removed from the bulk material; iii) the rapid desorption of an incubation-time-dependent fraction of bound protein when the protein is removed from the bulk material; and iv) the fixing of the residual surface concentration and surface energy at constant values once the removal of the reversibly bound protein and the free protein is complete. Generally, developed kinetic models correlate well with the quantitative experimental results.
2.4.1. Interaction of the proteins with non-ionic polymer chains, PEG:

Up to now, long non-ionic water-soluble polymeric chains, particularly PEG attached to surfaces, have been used to prevent non-specific protein adsorption.\textsuperscript{77,78} The mechanism of protein repelling by PEG has been thoroughly investigated. Three main factors contribute to the protein repelling properties of PEG: i) the low interfacial free energy in water; ii) the steric stabilization effect; and iii) the structure of PEG in water. As interfacial free energy diminishes, the driving force for protein adsorption decreases as well, so the proteins will not experience interactions other than in the bulk material. Hence, non-specific protein adsorption should not occur or should be greatly diminished at surfaces with low interfacial energies.\textsuperscript{79} At the same time, other polymer molecules with low interfacial energy are not as efficient as PEG in protein repelling.\textsuperscript{80}

There are additional factors involved in PEG protein repellency, such as steric stabilization effect. The steric stabilization effect may be classified into two main categories. The first is the volume restriction effect, associated with the reduction in the total number of conformations available to the polymer on the approach of a second surface (e.g. protein). Here, it is assumed that a second surface approaching the polymer layer is impenetrable. Thus, the polymer layer is compressed and the polymer segments contained in the interaction region lose configurational entropy. This causes the polymer segments to occupy fewer possible configurations in the compressed state than in the uncompressed state. This reduction in entropy increases Gibbs energy, producing the net effect of repulsion between the surfaces. The second category, excluded-volume (osmotic pressure) repulsion, assumes that the layers of two surfaces can overlap each
other when they approach. The polymer segments are in contact, and this contact is reduced as a result of the contact between the segments in the overlapped region. This results in an change in the enthalpy of mixing, and a reduction in the configurational entropy of the adsorbed molecules.\textsuperscript{81}

The third factor that causes high protein repellency of PEG chains is the unique structure of the PEG. Polymer chains fit into the water lattice structure without disturbing it and minimize the tendency for hydrophobic interactions. Thus, PEG chains form liquid-like surfaces in the water that exhibit a high degree of flexibility. These well hydrated chains with large excluded volume tend to repel the proteins that approach the surface.\textsuperscript{81}

\textbf{2.4.2. Interaction of the protein molecules with charged polymer chains:}

A totally different picture is observed when a charged polymer brush is used for protein adsorption. It has been shown that charged polyelectrolyte brushes absorb a vast amount of the proteins, even those of the same charge. While one would expect that there should be an electrostatic repulsion effect, Witteman and coworkers have shown that BSA is strongly attracted to the brush on spherical as well as planar electrolyte surfaces of polyacrylic acid when ionic strength is low.\textsuperscript{82} With an increase in the ionic strength of the solution, desorption occurs and virtually all of the protein can be recovered. The data demonstrate that raising the pH to well above the isoelectric point of BSA (5.1) diminishes attraction while much stronger adsorption is seen when approaching the isoelectric point. Analysis by small-angle X-ray scattering has
demonstrated that proteins such as bovine serum albumin enter deeply into the brush layer and are strongly correlated to the polyelectrolyte chains.\textsuperscript{83} Hence, more than one monolayer of the protein can be adsorbed onto the polymer brush.

Besides ionic strength and pH, additional factors such as temperature play an important role in protein adsorption to polyelectrolyte brushes. It has been found that BSA adsorption was enhanced with increasing temperature, which indicated an entropic driving force for protein adsorption.\textsuperscript{84} Moreover, the addition of salt at 40 or 20 °C to the solution had an adverse effect and resulted in an increase in protein adsorption. The authors of that study concluded that there is a difference in the mechanism of protein adsorption at 20 °C and 40 °C, but that unfolding of the biological molecule may also have been the cause.

2.4.3. Adsorption of the protein molecules on the mixed polymer brushes:

Protein adsorption to mixed polymer brushes has not been well studied. The main consideration for the adsorption of the proteins onto the mixed brushes is that they may exhibit a different affinity for the protein upon different external stimuli. To date only a few studies exist for cases where more than one polymer chain type is used for the preparation of the surface. Overall, the properties of mixed brushes are thought to be similar to those of homobrushes. For example, protein adsorption decreased with an increase in the ionic strength of the solution.\textsuperscript{85} However, Uhlmann et al.\textsuperscript{86} have done extensive studies of the adsorption of chymotrypsin (isoelectric point 8.1) and lactalbumin (isoelectric point 4.3) on the oppositely charged poly(vinyl pyridine) and
polyacrylic acid mixed brush (isoelectric point 4.9). The amount of adsorbed protein was evaluated using ellipsometry. Authors show that for chymotrypsin the amount adsorbed onto the surface at low salt concentrations increased considerably with an increase in pH. In a high-salt buffer, electrostatic interactions are screened and the amount of adsorbed protein was lower than what was seen in a low-salt buffer. There was practically no difference in the amount of adsorbed protein at different pH values, but the same trend was observed.

When poly tert-butyl acrylate was used as a component of the mixed brush, and therefore there were no charged anionic groups present, the brush adsorbed much less protein at low salt concentrations, but the adsorbed amount was practically the same as what was seen at higher salt concentrations.

A completely different behavior was observed when adsorbing lactalbumin from the same buffers. The adsorbed amount decreased with an increase in pH in both the high-salt and the low-salt buffers. Increasing the salt concentration decreased the adsorbed amount at all investigated pH values. Almost no differences were seen in the adsorption capacity of mixed brushes containing poly tert-butyl acrylate or poly acrylic acid. The adsorbed amounts were very low in both cases.

It was concluded that the adsorption of the negatively charged and conformationally stable chymotrypsin\textsuperscript{87} onto negatively charged brushes was due to counterion release and a very strong entropic driving force for protein adsorption. In this case, the proteins were acting as multivalent counterions. A decrease in the adsorption of lactalbumin was attributed to the high electrostatic repulsion between the brush and
protein, due to very close isoelectric points of both. However, lactalbumin is known as a “soft” protein and is able to adsorb to the “wrong” side of isoelectric point due to entropically based contributions that appear in response to adsorption-induced conformational changes.

When a brush was in a salted regime, the polymer chains were partially collapsed and electrostatic interactions between the proteins and the brush were screened. Partial collapse of the brush resulted in protein repellency by steric interactions. This is why the adsorbed amount of protein generally decreased when additional salt was added to the charged polyelectrolyte brush media. In the case of chymotrypsin, added salt decreased the adsorbed amount considerably because the counterion release force contributed to adsorption. When lactalbumin was adsorbed, higher salt concentrations generally decreased the adsorbed amount. This is a known behavior of polyelectrolyte brushes caused by molecule shrinkage. The higher adsorbed amount of lactalbumin, compared to that of chymotrypsin, at pH 4 was due to the higher conformational flexibility of lactalbumin. This led to higher entropically based contributions (due to protein unfolding) to the driving force, which is very likely because the shrunken brush and the hydrophobic surface behave similarly.

Moreover, chymotrypsin exhibits reversible adsorption when pH is changed from a higher to a lower value, while the amount of lactalbumin adsorbed stays practically the same regardless of a change in the media pH. Finally, other authors have concluded that adsorption at high salt concentrations proceeds by a mechanism very similar to adsorption to hydrophobic surfaces, while at low salt concentrations adsorption happens
due to the entropic driving force and the counterion release mechanism. Coulomb interactions play minor role.

In summary, it may be concluded that the interactions of protein molecules with polymer brushes are very complex procedures which depend on a variety of factors. Among these factors are the nature of the polymer brush, the nature of the protein, temperature, pH and the ionic strength of the media. While theoretical work fits well with experimental results, usually models are developed precisely for the particular case studied and do not consider a broader variety of surfaces and protein molecules.

2.5. Molecular imprinting of the protein molecules with the polymers:

Another way to control protein adsorption and geometrical positioning of the protein on the surface is molecular imprinting approach. Thermodynamically it is more favorable for the molecule to adsorb into a specially prepared adsorption site with high affinity toward the protein than to the non-adsorptive or less adsorptive matrix. The special site for the protein adsorption can be prepared using a “molecular imprinting” technique.

Recognition and specificity are powerful tools used widely in natural biological systems. Cells use cytokine-based recognition to communicate with the outside world; antibodies specifically recognize their antigens. Any foreign object inserted into a living organism will soon be recognized and encapsulated in a collagenous sac. Biological recognition mechanisms are also noted for the functioning of enzymes, lectins, integrins, DNA, RNA and saccharides.
This natural phenomenon, which so faultlessly works in living beings, has been transferred by humans to lifeless systems using a technique called molecular imprinting\(^9\), a powerful technique for the preparation of robust biomaterials that can selectively recognize a specific species. Industrial interest in molecular imprinting is rapidly increasing, resulting in a demand for commercially relevant applications. This, in turn, creates new challenges for research on imprinting.

The basic principals of molecular imprinting are explained in Figure 2.4 and can be referred as 2D and 3D imprinting procedures\(^2\). Particular procedures create special recognition sites for a template molecule. They can selectively recognize and extract the template molecule from a mixture of species similar in geometry and properties. The 3D procedure creates recognition sites inside a polymer matrix. The 2D procedure creates recognition sites on the surface. The excellent stability, durability, easy preparation and low cost of these materials have led to their application in the fields of chromatography, sensor technique, catalysis and separation\(^9\).
Procedures for the imprint template preparation include formation of reversible interactions between the template and polymerizable functionality that may involve one or more of the following interactions: reversible covalent bond(s)\(^9\), covalently attached polymerizable binding groups activated for non-covalent interaction by template

Figure 2.4. Schematic representation of the molecular imprinting process. Two types of imprinting are presented: 2D (b, c) and 3D (a). In case of 3D recognition sites are located all over the volume of polymer matrix. 2D imprinting results in the recognition sites located on the surface of template. While the representation here is specific to vinyl polymerization, the same basic scheme can equally be applied to sol-gel or polycondensation. Redrawn after Ratner B.\(^2\)

Procedures for the imprint template preparation include formation of reversible interactions between the template and polymerizable functionality that may involve one or more of the following interactions: reversible covalent bond(s)\(^9\), covalently attached polymerizable binding groups activated for non-covalent interaction by template
cleavage, electrostatic interactions, hydrophobic or van der Waals interactions or coordination with a metal center\textsuperscript{92-94}.

Each interaction is formed with complementary functional groups or structural elements of the template. A subsequent polymerization in the presence of crosslinker(s), a cross-linking reaction or other process results in the formation of an insoluble matrix (which itself can contribute to recognition through steric, van der Waals and even electrostatic interactions) in which the template sites reside. The template molecule is then removed from the polymer through disruption of the polymer–template interactions and extraction from the matrix\textsuperscript{95}. The template molecule or analogues thereof may be selectively rebound by the polymer in the sites vacated by template, the “imprints”.

Although creating an MIP (molecularly imprinted polymer) for recognition of small molecules is straightforward now, imprinting of large structures, such as proteins and other biomacromolecules, is still a challenge. Small molecules are stable, and because they are quite mobile, they can easily diffuse inside the polymer matrix and reach the recognition sites. Preparation of small-molecule templates is well investigated and undemanding. High molecular–weight proteins, because of their intrinsic properties, are unstable and can easily denature during the imprinting procedure. The viscosity of a protein solution is much higher than that of a small-molecule solution, and that impedes protein mobility. The molecular weights of protein molecules are usually hundreds of thousands of Daltons. Both mobility and size factors make it almost impossible for protein molecules to penetrate inside a crosslinked polymer matrix and approach the recognition sites.
2.5.1. 3D molecular imprinting:

The first studies related to molecular imprinted polymers take their roots from 1931 when Polyakov reported some unusual adsorption properties of silica particles prepared in the presence of additives. Silica showed a higher capacity for uptake of the additive than structurally related ligands. This study was the first in which selectivity was observed and explained in terms of a template effect.

Recently, Alexander and co-workers defined molecular imprinting as “the construction of ligand selective recognition sites in synthetic polymers where a template (atom, ion, molecule, complex or a molecular, ionic or macromolecular assembly, including micro-organisms) is employed in order to facilitate recognition site formation during the covalent assembly of the bulk phase by a polymerization or polycondensation process, with subsequent removal of some or all of the template being necessary for recognition to occur in the spaces vacated by the templating species”. This definition clearly describes the 3D molecular imprinting technique widely used to build small-molecule recognition templates.

A variety of studies have been done establishing MIP-based chromatographic systems using small molecules such as amino acids or amino acid sequences. Results showing specific affinity for lysosomes and bovine serum albumin molecules were obtained for an amphoteric copolymer network as described above using a chromatographic column. Studies with bovine hemoglobin imprinted into polyacrylamide hydrogel also showed that such a network can be used for many cycles without losing its specific affinity.
To avoid a destructive grinding procedure, Lei and co-workers developed a method that obtains molecularly imprinted microspheres using a precipitation polymerization method. Controlling the phase separation point during the polymerization obtains uniform MIP microspheres in good yield. Such microspheres display characteristics similar to the particles obtained by grinding but with improved binding kinetics.

Bulk imprinted templates have some disadvantages for large molecules, such as proteins, when recognition sites are buried within a solid structure. The print molecule will take much longer to diffuse through the rather inflexible polymeric chains, for example. Despite the laborious procedure, polymer grinding also can destroy affinity sites. Surface grafting seems to be the most promising way to overcome such problems because the binding of the print molecule will occur much more rapidly when the recognition sites are placed at the liquid–solid interface.

2.5.2. 2D molecular imprinting with thin films:

Few approaches used to expose the binding sites of an analyte molecule to the surface gave successful results. In the case of the microcontact approach, a covered glass slide was used as the support on which a protein molecule and a functional monomer were deposited. They were then connected to the glass support carrying the crosslinking agent and UV polymerized. The cover glass was then easily removed, and the polymer was washed. Polyethylene glycol-400 dimethacrylate was used as a functional monomer in the study. This method has the advantage of trapping little or no
template under the polymer film surface. It can thus be used when very little or no template material is available.

A similar approach that gives fair selectivity results used an aluminum nanoporous membrane as a support, first for glutamic acid and then for a variety of proteins. First, the analyte molecule was immobilized on the pore walls of the nanoporous aluminum, and then the nanopores were filled with a mixture of the functional monomer (acrylamide for the case of proteins and pyrrole for the glutamic acid) and a crosslinking monomer. After polymerization, the aluminum membrane was removed with chemical dissolution, leaving behind polymer nanowires with analyte molecule binding sites situated on the surface. These sites have a physical shape complementary to the protein molecule and also might have a precisely positioned amide group.

It was also shown that a 3-aminophenylboronic acid network MIP grafted to a polystyrene surface had 100 times higher affinity than when it was free standing. The authors prepared affinity matrices for proteins based on the surface coating of polystyrene microtiter plates. They used a thin layer of a stable conjugated polymer polymerized in the presence of various protein templates. After template regeneration for 5 times, they determined that the polymer, on average, lost 20% of its efficiency, mainly due to the partial destruction of polymer binding sites.

An alternative approach for molecular recognition used an epitope (a single antigenic site on a protein against which an antibody reacts) approach. Instead of using a protein molecule for the template preparation, the authors used only its linear
epitope. As a result, they avoided embedding and bleeding of the target molecule and minimized nonspecific interaction between the MIP and the target protein. An imprint was prepared on the gold surface of a quartz crystal microbalance chip using as a monomer acrylic acid–acrylamide solution.

Studies that used particles as a template support show promising results. Porous crosslinked chitosan beads, bearing functional groups to bind a protein-imprinted polymer matrix, were used as the support for a hemoglobin-imprinted polyacrylamide matrix.\textsuperscript{108} This kind of system showed higher stability for cyclic use than a system in which polyacrylamide was just physically trapped in chitosan pores. For the chitosan beads, the authors did not chemically attach the template molecule to the support.

When silica particles were used, the surface was treated to create reactive aldehyde groups by the reaction of amonopropyl silica with glutaraldehyde.\textsuperscript{109} Hemoglobin molecules are easily attached to the aldehyde groups by their reaction with template amino groups. Molecular imprinting in this case consists of two steps: siloxane-silica complex polymerization in the presence of templates and removal of template to leave recognition sites. Results show that hemoglobin-imprinted silica using covalently immobilized hemoglobin was much superior to hemoglobin-imprinted silica using free hemoglobin. The advantages of thin layer imprinting were clearly stated in a study examining thin polymeric films grafted to the entire surface of microfiltration membranes and bulk polymeric particles. As a template, atrazine (a triazine herbicide) has been used with an acrylamide-based matrix.\textsuperscript{110}
As was shown, bulk materials have poor performance in fast SPE (solid phase extraction) because of major diffusion transport resistance. Meanwhile, a significantly higher performance has been achieved for thin-layer MIPs.

### 2.5.3. 2D molecular imprinting with ultrathin polymer films:

In all of the above studies on surface imprinting, the thickness of the imprinted polymer matrix ranged up to 70 nm. Those can be considered “thin” MIPs. A larger content of matrix material — compared with “ultrathin” films — can yield better stabilization. “Ultrathin” films have maximum accessibility, but their low binding capacity requires the lowest amount of “matrix material” per number of imprinted sites. The thickness of “ultrathin” films ranges up to a few nm.

An example of an “ultrathin” molecular imprint is a study with a novel technique for the noncovalent immobilization of biomolecules using a polymer containing azobenzene groups. The azopolymer was found to deform (plasticize) due to a trans-cis-trans isomerization cycle along the contours of nanoscale macromolecules when photoirradiated, effectively immobilizing the macromolecules without chemical modification. The authors demonstrated the possibility of immobilizing a variety of macromolecules with different surface properties and sizes, including negatively charged DNA and charged proteins from aqueous solutions without denaturation.

Another method is more complicated and includes deposition of the analyte molecule onto an atomically flat mica surface with a subsequent disaccharide coating that prevents denaturation of the protein during plasma deposition of the fluoropolymers and
also creates hydrogen bonds between the hydroxyl bonds of sugars and the surface polar residues of the protein.\textsuperscript{113} When the obtained film was glued by epoxy to a solid support, the mica was peeled off. After removal of the analyte molecule, the binding properties of the obtained surface were studied. It was shown that protein recognition for this type of imprint is evident only in competitive adsorption, which suggests that exchange is occurring between nonspecifically adsorbed non-template protein and the solution phase template protein. A non-template protein that does not fit into a pit is more likely to be displaced by the template protein. The hydrophilic crosslinked sugars on protein imprints, in contrast to hydrophobic surfaces, allow a lower protein sticking probability and a higher protein exchangeability. Both of these processes lead to “recognition of the fittest” through dynamic adsorption-exchange, which is essential to protein recognition from a protein mixture.

Of the large variety of methods for protein imprinting, most show positive results. However, there is no universal method that can be considered applicable to all possible analyte molecules. For large molecules, such as proteins, studies suggest that the most suitable methods use “thin” or “ultrathin” films mounted on a solid support. In most studies of bulk templates, chromatography was used to evaluate template efficiency. The low binding capacities of “thin” and “ultrathin” templates mean that evaluation of the efficiency of the templates requires very precise methods. Competitive adsorption appears to be the most prominent method used to evaluate the efficiency of such templates.
2.6. References:

(1) Hong, J.; Degennes, P. G. *Macromolecules* 1993, 26, 520-525.


(53) Minko, S. I., Leonid; Sydorenko, Alexander; Houbenov, Nikolay; Stamm, Manfred; Zdyrko, Bogdan; Klep, Viktor; Luzinov, Igor. ACS Symposium Series 2005, 68, 183.


(64) Desroches, M. J.; Omanovic, S. Physical Chemistry Chemical Physics 2008, 10, 2502-2512.


(71) Zhou, X. P.; Su, X. L.; Sun, Y. Biotechnology Progress 2007, 23, 1118-1123.


3.1. Chemical Reagents Used:

3.1.1: Hydrogen peroxide \( [H_2O_2] \):

*Company Identification*: Acros Organics.

*MSDS Name*: Hydrogen Peroxide (30% in Water) (Without Stabilizer), Reagent ACS.

*Catalog Numbers*: AC411880000, AC411881000, AC411885000.

3.1.2: Sulfuric acid \( [H_2SO_4] \) 98%:

*Company Identification*: Acros Organics.

*MSDS Name*: Sulfuric acid, reagent ACS.

*Catalog Numbers*: 13361-0000, 13361-0010, 13361-0025.

3.1.3: Toluene:

*Company Identification*: Acros Organics.

*MSDS Name*: Toluene, reagent ACS.

*Catalog Numbers*: 424500-0000, 42455-0010, 42455-0250, 42455-5000.

3.1.4: Methyl ethyl ketone:

*Company Identification*: Acros Organics.

*MSDS Name*: 2-Butanone, 99+%

*Catalog Numbers*: 14967-0000, 14967-0010, 14967-0025, 14967-0250.

---

1 Experimental procedures that are specific to a particular chapter are outlined in the chapter's experimental section.
3.1.5: Ethanol:

Company Identification: Mallinckrodt Baker Inc.

MSDS Name: Reagent alcohol, ACS.

Catalog Numbers: 5911, 6183, 7006, 7019.

3.1.6: N,N-Dimethylformamide:

Company Identification: Acros Organics.

MSDS Name: N,N-Dimethylformamide, 99%.

Catalog Numbers: 11622-0000, 11622-0010, 11622-0025, 11622-0250.

3.1.7: Human serum albumin [HSA]

Company Identification: Sigma-Aldrich.

MSDS Name: Albumin from human serum.

Catalog Numbers: A 9511

3.1.7: Bovine serum fibrinogen

Company Identification: Sigma-Aldrich.

MSDS Name: Fibrinogen form bovine plasma, factor I

Catalog Numbers: F4753

3.1.8: Succinic anhydride (99%):

Company Identification: Acros Organics.

MSDS Name: Succinic anhydride, 99%.

Catalog Numbers: 158760010

3.1.9: Atto 520 NHS ester (80%):

Company Identification: Sigma-Aldrich.
MSDS Name: Atto 520 NHS ester, 80%.

Catalog Numbers: 77810

3.1.10: Rhodamine-B-isothiocyanate (99%):

Company Identification: Research Organics.

MSDS Name: Rhodamine-B-Isothiocyanate.

Catalog Numbers: 0808R

3.1.11: Spectra/Por® Dialysis Sasks:

Company Identification: Spectrum.

MSDS Name: Regenerated Cellulose Dialysis Tubing.

Catalog Numbers: 132651

3.2.12 Sodium Phosphate, Dibasic Anhydrous

Company Identification: J. T. Baker

MSDS Name: Sodium Phosphate, dibasic anhydrous, power

Catalog Numbers: E04H18

3.2.13 Potassium Phosphate, Monobasic

Company Identification: J. T. Baker

MSDS Name: Potassium Phosphate, monobasic, Crystal

Catalog Numbers: E09474

3.2.14 Ovalbumin

Company Identification: Sigma

MSDS Name: Albumin from Chicken Egg White, Grade V, minimum 98% agarose, gel electrophoresis
3.2. Polymers Used for Surface Modification:

3.2.1: Poly(glycidyl methacrylate) [PGMA] (Structure 3.1):

\[
\begin{align*}
\text{CH}_3 & \quad \text{CH}_2 \\
\text{C} & \quad \text{C}_n \\
\text{C=O} & \quad \text{O} \\
\text{CH}_2 & \quad \text{CH}_2
\end{align*}
\]

(S.3.1)

PGMA (Mn=210,000 g/mol with polydispersity 2.34) was synthesized by solution radical polymerization and purified by multiple precipitations by Dr. V. Klep, School of Materials Science and Engineering, Clemson University.

3.2.2: Poly(ethylene glycol) [PEG] (Structure 3.2):

\[
\text{CH}_3\left[\text{OCH}_2\text{CH}_2\right]_n\text{-OC(O)-CH}_2\text{CH}_2\text{-COOH}
\]

(S.3.2)

Poly(ethylene glycol) monomethyl ether, with \(M_n\) ca. 5,000 (Aldrich), was modified with succinyl anhydride (Aldrich) to form carboxyl terminated derivative.
3.2.3: Carboxy terminated polystyrene [PS] (Structure 3.3):

\[
\begin{array}{c}
\text{CH} - \text{CH}_2 \\
\text{COOH}
\end{array}
\]

(S.3.3)

Carboxyl terminated polystyrene with molecular weight Mn=1,000 was obtained from Polymer Source Inc., Canada.

3.2.4: Polystyrene-block-poly(acrylic acid) [PS-block-PAA] (Structure 3.4):

PS-block-PAA with number average molecular weight of polystyrene and polyacrylic acid blocks 1,000 and 27,000, respectively, was obtained from Polymer Source Inc., Canada.

\[
\begin{array}{c}
\text{CH}_2 - \text{CH} \\
\text{O} \\
\text{H} \\
\text{CH}_2 - \text{CH}
\end{array}
\]

(S.3.4)

3.2.4: Poly(acrylic acid) (Structure 3.5):

PAA with molecular weight Mn=26,500 was obtained from Polymer Source Inc., Canada.

\[
\begin{array}{c}
\text{CH}_2 - \text{CH} \\
\text{O} \\
\text{H}
\end{array}
\]

(S.3.5)

IR and GPC characterization of the polymers obtained from Polymer Source was performed by manufacturer and included as supplementary information for each sample.
3.3. Principal Experimental and Characterization Techniques

3.3.1. Dip Coating:

Dip coating is a process for preparation of thin polymer films where the substrate is immersed in a solution and then withdrawn at a constant speed. A schematic of the dip-coating process is shown in Figure 3.1. This process can be done in air or in an inert environment. Uniformity of coating and film thickness depends on speed control and vibration of the substrate and liquid surface.\(^3\) The withdrawal speed and the viscosity of the solution determine the coating thickness.

![Figure 3.1: Procedure for coating substrate with dip-coating apparatus.](image)

If the withdrawal speed is chosen such that the shear rates keep the system in the Newtonian regime, the coating thickness can be calculated by the Landau-Levich equation (E3.1).\(^3\)

\[
h = 0.94 \times \frac{(\eta \nu)^{2/3}}{\gamma_{LV}^{1/6} (\rho g)^{1/2}}
\]  
(E3.1)
Where $h$ is the coating thickness, $\eta$ is the viscosity, $\gamma_{LV}$ is the liquid-vapor surface tension, $\rho$ is the density and $g$ is the acceleration due to gravity and $v$ is the withdrawal speed. An operating speed was adjusted to approximately 4 mm/sec. A Mayer Fientechnik D-3400 dip coater was placed in a clean room to avoid contamination of the samples with dust particles. Layers of different thickness were obtained via dip coating of the samples into solutions of different concentrations.

### 3.3.2: Scanning Probe Microscopy (SPM):

![AFM schematics](image.png)

**Figure 3.2**: AFM schematics.\(^1\)

A schematic representation of SPM is shown in **Figure 3.2**. In SPM\(^4\), a sharp probe (tip) is moved across the surface of the sample and the probe-surface interaction is monitored to produce an image. Depending on the feedback signal, SPM has three
primary modes of operation: contact mode, non-contact mode and tapping mode. Scanning probe microscopy (SPM) studies were performed using a Dimension 3100 and a MultiMode™ (Digital Instruments, Inc.) microscope. Tapping and contact modes were used to study the surface morphology of the samples in ambient air and under water. Silicon tips from MicroMasch with spring constants of 50 N/m (tapping mode) and 0.40 N/m (contact mode) were used. Imaging was done at scan rates in the range of 1 - 2 Hz. Software Nanoscope III 5.12r3 was used for images processing.

**3.3.3: Ellipsometry:**

Ellipsometry measures the change in polarization of light reflected from the surface of a sample.\(^5\) The measured values are expressed as $\Psi$ and $\Delta$, which are related to the ratio of Fresnel reflection coefficients, $R_p$ and $R_s$ for $p$ and $s$-polarized light, respectively, as follows:

\[
\tan(\Psi)e^{i\Delta} = \frac{R_p}{R_s}
\]  \(\text{(E3.2)}\)

Because ellipsometry measures the ratio of two values, it can be highly accurate and very reproducible. From Eq. \(\text{(E3.2)}\) the ratio is seen to be a complex number, thus it contains “phase” information in $\Delta$, which makes the measurement very sensitive. In Figure. 3.3, a linearly polarized input beam is converted to an elliptically polarized reflected beam. For any angle of incidence greater than 0° and less than 90°, $p$-polarized light and $s$-polarized will be reflected differently.

The coordinate system used to describe the ellipse of polarization is the $p$-$s$ coordinate system. The $s$-direction is taken to be perpendicular to the direction of
propagation and parallel to the sample surface. The $p$-direction is taken to be perpendicular to the direction of propagation and contained in the plane of incidence.

**Figure 3.3.** Schematic of the geometry of an ellipsometer. Redrawn after Ulman.\(^5\)

Ellipsometry in this work was performed with a COMPEL automatic ellipsometer (InOmTech, Inc.) at an angle of incidence of 70°. For all of the experiments in the current research, it was decided to keep the compensator on for thickness values less than 11 nm and removed for thicknesses greater than 14 nm. For thickness values in between 11 nm and 14 nm (both the limits included), the average value of the thickness with and without the compensator was used.

### 3.3.4. In situ IR-Spectroscopic Ellipsometry (IRSE):

Infrared Spectroscopic Ellipsometry (IRSE) is a relatively new technique for analyzing thin films and interfaces\(^6\). Similar to conventional Visible Spectroscopic
Ellipsometry, IRSE relies on the reflection of polarized light to determine the refractive index profile at an interface. This refractive index profile can then be used to extract information such as interfacial width or film thickness. However, because IRSE uses the whole spectrum of IR light, in addition to the thickness of the film, its chemical composition can also be determined.

Characteristic vibrations of the molecules absorb IR radiation, and absorption bands corresponding to these vibrations appear in the refractive index. Thus, chemical information about a film or interface can be extracted using IRSE. This capability is particularly important for systems where, in the visible region, the refractive indices on either side of the interface are similar, such as in the case of a swollen polymer film in a water medium. In this situation, it is much more difficult to analyze the interface using conventional visible ellipsometry as the interface is only weakly reflecting. However, in the IR, where an absorption band occurs, the indices on either side of the interface may be quite different, and a strong reflection occurs. This enables data from the interface to be obtained.

**Figure 3.4** shows a schematic of the in-situ cell used for measurements of the polymer films in liquid media. The cell is built in such a way that the angle of light reflection from the silicon wafer surface and from polymer film–water interface are separated and only the signal from the interface is detected.

The setup consists of a Bruker Vertex 70 Spectrometer as light source and the actual ellipsometer which is a self-built device. IRSE measurements of a mixed brush in water are performed through an IR transparent trapezoidal Si wedge (1.5° with (111)
surface in contact with aqueous solution). The spot size on the silicone wedge is ~50 mm² at an incident angle of 60°.

The reflected radiation is described by the complex ratio of the two mutually orthogonal polarized components, \( r_s \) and \( r_p \): \( \rho = \frac{r_p}{r_s} = \tan \psi \ e^{i\Delta} \). Here \( \tan \psi = \frac{r_p}{r_s} \) is the absolute amplitude ratio and \( \Delta \) is the phase shift between the p- and s-polarized components of reflected waves. The polarized reflectances \( R_p \) and \( R_s \) are given by \( R_p = r_p^2 \) and \( R_s = r_s^2 \). The transport of radiation in the silicon wedge is described by formulas \textbf{E3.4} and \textbf{E3.4}.\textsuperscript{7} The incidence angles on the different interfaces as used in the presented experiments are given in \textbf{Figure 3.4}.

\[
\rho_{\text{eff}} = \rho'_{\text{air/silicon}} \cdot 59.2^\circ \rho_{\text{silicon/siliconoxide/water}} \cdot 13.8^\circ \rho'_{\text{silicon/air}} \cdot 12.3 \quad (\textbf{E3.3})
\]

with

\[
\rho = \left| \frac{r_p}{r_s} \right| e^{i\Delta}, \quad \text{and} \quad \rho' = \left| \frac{t_p}{t_s} \right| e^{i\Delta}. \quad (\textbf{E3.4})
\]
where \( r_p, r_s \) and \( t_p, t_s \) are the p- and s-polarized reflection and transmission coefficients, respectively.

Formula \( E3.3 \) allows quantitative interpretation of the measured in-situ IR ellipsometric spectra. Spectra taken in water are often represented as the ratio between \( \tan \psi \) of the measured spectra and \( \tan \psi \) of reference spectra.

### 3.3.5. Contact Angle Measurements:

When a drop of liquid is placed on the surface, it either spreads to cover all of the surface or it beads up. If the surface tension of the liquid is higher than the surface tension of the solid, it makes a definite angle of contact between the liquid and the solid phases. \(^8\)

Contact angle is very sensitive to the chemical composition of the top layer and is a relatively simple, inexpensive and reliable technique for characterizing polymer surfaces. The contact angle, as defined by Young’s equation, is governed by the force balance at the three phase boundaries, as shown in Figure 3.4, and is given by Equation (E3.5):

\[
\gamma_{sv} = \gamma_{sl} + \gamma_{lv} \cos \theta
\]

(E3.5)

where \( \gamma_{lv} \) is the surface tension of the liquid-vapor interface, \( \gamma_{sv} \) is the surface tension of the solid-vapor interface and \( \gamma_{sl} \) is the interfacial tension of the solid-liquid interface.
Static contact angle measurements were made using a contact angle goniometer (Kruss, Model DSA10). Calculation of the contact angle was made using the tangent method and the measurements were made with triple distilled water (pH 7.0) and a static time of 30 seconds before the angle measurement.

![Representation of the surface tensions contributing to the contact angle.](image)

**Figure 3.5.** Representation of the surface tensions contributing to the contact angle.²

### 3.3.6. Total Internal Reflectance Fluorescence (TIRF):

TIRF⁹ was used to monitor the protein adsorption onto imprinted brushes in the dynamic adsorption experiment. A block diagram of the TIRF apparatus, custom built by Dr. Bogdan Zdyrko (Clemson University), is shown in **Figure 3.6**. A monochromatic 532 nm laser signal was generated by a DPGL Series Modulated Green Laser Modulus (Part No.: DPGL-01S-TTL). FisherFinest Premier microscope slides (Cat. No. 12-544-1), which have nearly the same refractive index as the prism, were used and were modified with polymer brushes. The fluorescence detector (Si Photodiode, diameter is 11.0 mm, from Edmund Optics) was placed perpendicular to the prism to collect fluorescence signals from the labeled proteins. Slides with brushes were placed in the flowing cell. Protein solutions were injected into the flowing cell at the rate of 1g/min.
and the fluorescence emitted was collected by the detector. The data were analyzed using LabView software to capture the time-dependent adsorption breakthrough curves.

![Diagram of TIRF instrument](image)

**Figure 3.6.** Principal scheme of TIRF instrument.

TIRF work is based on the fact that when a beam of light is incident on an interface between two transparent media (with incident refraction $n_1$ and $n_2$, and $n_2 < n_1$) at an angle $\alpha$, such that $\alpha > \sin^{-1}(n_2/n_1)$, the beam totally reflects at the interface between two media. At the point of total internal reflection, an evanescent wave penetrates into the medium of lower refractive index$^9$ (Figure 3.7).

The depth of penetration $d_p$, defined as a distance from the interface where the intensity is reduced to $e^{-1}$ of its original value, is dependent on wavelength ($\lambda$) and angle of incidence ($\alpha$) as well as difference in refractive index between the two media$^9$:

$$d_p = \frac{\lambda}{2\pi} \left( n_1^2 \sin^2 \alpha - n_2^2 \right)^{-1/2} \quad (E3.6)$$

The observed fluorescence intensity ($I_F$) at wavelength $\lambda_1$ is governed by product of adsorption and emission probabilities. For excitation with an evanescent field in an
adsorption experiment, most factors will vary with the distance from the interface, yielding:

\[
I_F = k \int_{d_{min}}^{d_{max}} f(z) \phi(z) \varepsilon(z) c(z)(E^e(z))^2 \, dz
\]  

(E3.7)

where \( f \) is the fraction of emitted light at \( \lambda \), \( \phi \) is the emission probability (or quantum yield), \( \varepsilon \) the extinction coefficient, \( c \) the fluorophore concentration, \( E^e \) is the electric field amplitude for the evanescent wave and \( k \) is a constant including instrumentation factors (e.g., fraction of emitted fluorescence detected by the detector).

Figure 3.7. Geometry of total internal reflection fluorescence.

### 3.4. Characterization of the polymer brush films:

To characterize the polymer layers, several parameters have been evaluated.\(^{10}\) The surface coverage (adsorbed amount), \( \Gamma \) (mg/m\(^2\)), was calculated from the ellipsometry thickness of the layer, \( h \) (nm), by the following equation:
\[ \Gamma = h\rho \] \hspace{1cm} (E3.7)

where \( \rho \) is the density of attached (macro)molecules. The density data for PEG (1.13 g/cm\(^3\)), PAA (1.16 g/cm\(^3\)), and PS (1.05 g/cm\(^3\)) were obtained from the *Polymer Handbook*.\(^{11}\)

The chain density, \( \sigma \) (chain/nm\(^2\)), i.e., the inverse of the average area per adsorbed chain, was determined by:

\[ \sigma = \Gamma N_A 10^{-21} / Mn = (6.023 \Gamma ^ {100}) / Mn \] \hspace{1cm} (E3.8)

where \( N_A \) is the Avogadro number and \( Mn \) (g/mol) is the number average molar mass of the grafted polymer.

The distance between grafting sites, \( D \) (nm), was calculated using the following equation:

\[ D = (4 / \pi \sigma)^{1/2} \] \hspace{1cm} (E3.9)

The radius of gyration for the PEG macromolecules was estimated by the equation:

\[ 6R_g^2 = L^2, \] \hspace{1cm} (E3.10)

where \( L = 5.44 \) nm is the end-to-end distance of PEG.\(^{11}\)

The end-to-end distance for polyacrylic acid and polystyrene was calculated using the expression:

\[ L^2 = nl^2C_\infty \] \hspace{1cm} (E3.11)
where \( n \) is the number of C-C bonds in polymer backbone, \( l \) is the bond length (0.154 nm),\(^{12}\) and \( C_\infty \) is the characteristic ratio for polymer. \( C_\infty \) was assumed to be 10 for polystyrene\(^{12}\) and 6.7 for polyacrylic acid.\(^{13}\)

### 3.5. AFM surface roughness evaluation:

The RMS (root mean square) roughness is a measure of the height profile’s roughness and is defined as:

\[
\text{RMS} = \sqrt{\frac{1}{n} \sum_{i=1}^{n} (h(x_i) - \bar{h})^2}
\]

(E3.12)

where \( n \) is the number of lattice points, \( h(x_i) \) is the height at lattice site \( x_i \), and the average height of the profile is defined as:

\[
\bar{h} = \frac{1}{n} \sum_{i=1}^{n} h(x_i)
\]

(E3.13)

The RMS roughness describes the fluctuations of surface heights around an average surface height and is the standard deviation or the square root of the second cumulant (variance) in terms of statistics\(^{14}\).

### 3.6. References:

(1) www.science.siu.edu/chemistry/zang/afm.htm. (06/08/08)


(3) http://www.solgel.com/articles/Nov00/mennig.htm. (07/15/08)
(4) www.veeco.com.(06/15/08)

(5) www.uta.edu/optics/research/ellipsometry/ellipsometry.htm.(08/23/08)


CHAPTER FOUR
SYNTHESIS OF MIXED POLYETHYLENEGLYCOL/POLYACRYLIC ACID- b – POLYSTYRENE MIXED POLYMER BRUSHES

4.1. Introduction:

When a material is brought into contact with a biofluid or biological organism, the material surface first interacts with proteins or cell exteriors. This first response often predetermines overall future performance of the device/implant.¹² In addition to the surface’s integral properties, its micro- and nano-scaled interfacial properties and ability to adapt to external stimuli are important for the response and thus for the design of biomaterial interfaces.⁴ These responsive properties can be incorporated into material surfaces by their modification with thin polymer layers. One of the most promising surface engineering approaches is the employment of polymer brushes that are densely grafted to the surface polymer layers.⁵

In general, “polymer brush” means that the distance between grafting sites of the polymer chains in a grafted layer is smaller than two radii of gyration (\(R_g\)) of the polymer coil.⁵ Usually polymer chains are chemically grafted to the surface to give polymer brushes additional stability. Two most common ways to prepare chemically grafted polymer brushes are “grafting to” and “grafting from”. “Grafting to” method is easier to conduct and has better control over the molecular weight of the prepared polymer brush.⁶ In “grafting from,” the polymer brush is grown from the surface through the polymerization process of monomers. Using the “grafting from” method one can achieve
thicker grafted layers. In the “grafting to” approach polymer chains are directly grafted to the surface through reactive end group. Polymer monobrushes and mixed polymer brushes can be successfully prepared by both methods.

Using the “grafting to” approach, mixed polymer brushes are prepared in either one step or multiple steps. In the one-step process both components are grafted to the tethering surface at the same time. The drawback of this method is non-uniformity (patchiness) of the mixed brush. The multi-step process requires more time and labor and includes grafting of the first component of the polymer brush, removal of ungrafted material, and grafting of the next polymer brush layer. More uniform brushes are reported to be obtained by this method.

The “grafting from” method for mixed polymer brushes consists of grafting of the first component monomers and adding and grafting of the second monomers to the surface. Different mechanisms may be involved in each grafting step, for example, ATRP (atom transfer radical polymerization) and nitroxide mediated radical polymerization. Brushes obtained by this method have broader molecular weight distribution than those prepared by the “grafting to” method. When grafted polymers are not miscible, “patchiness” inside the brush is also reported.

Polymer brushes first gained increased interest in 1950s and have become of great interest for protein adsorption studies. A large variety of macromolecules that can be used for brush preparation provide an arsenal of surfaces with repulsive/attractive sorptive ability toward proteins. It was found that hydrophobic polymer substrates such as a polystyrene surface are mostly highly protein attracting whereas hydrophilic non-
charged surfaces such as PEG (poly(ethylene glycol)) are highly protein repelling. Protein molecules are complex structures and consist of a number of chemical fragments/groups. This results in the presence of polar as well as nonpolar (hydrophobic) patches in the protein structure.\textsuperscript{14} Attraction of proteins to the hydrophobic surfaces in liquid media arises from the high affinity of the hydrophobic fragments of proteins to the hydrophobic surfaces. The unique protein repelling properties of the PEG molecules are explained by three major reasons: low interfacial free energy in water (diminished driving force for the protein adsorption), steric stabilization effect (increase of the Gibbs energy due to decreasing entropic factor as a result of protein adsorption on the surface), and the way in which PEG interacts with water (PEG chain segments fit into water lattices without distortion of lattices).\textsuperscript{15,16} There were also studies that showed adsorption of proteins on a variety of charged ionic surfaces\textsuperscript{17} and polyelectrolyte brushes.\textsuperscript{18,19} It is already known that proteins such as bovine serum albumin, cytochrome \textit{c}, and lysozyme adsorb to charged brushes even on the “wrong side” of the isoelectric point at low ionic strength of the solution.\textsuperscript{20,21} With the increase of the ionic strength, desorption often occurs and this desorption can be done stepwise with a stepwise change in the ionic strength.\textsuperscript{22}

The possibility of creating surfaces with responsive properties for proteins, where attraction and repulsion depend on external stimuli, became of great interest for scientists. This behavior is promising for design of new biosensors,\textsuperscript{23} protein separation devices, and biocompatible devices.\textsuperscript{24} To date, little work has been done toward creating surfaces for tuning protein adsorption. These include preparation of surfaces that consist of one
polymer component, elastin-like polypeptides (switching of protein adsorption properties occurs due to temperature change)\textsuperscript{25} or polyacrylic acid, and preparation of other ionic surfaces (adsorption is controlled by ionic strength of the solution).\textsuperscript{19,21,26-31} Another type of responsive surfaces for protein adsorption has a complex architecture of two or more polymer components often combining opposite properties in one polymer assembly.

Mixed polymer brushes that possess switching behavior are systems of (end-) tethered polymer chains of two or more polymers with different properties. Responsive behavior of the mixed polymer systems tethered to the surface is based on the phase segregation mechanism of their constituents.\textsuperscript{32} External stimuli such as temperature, pH, and solvent medium and ionic strength of the solution cause the steric rearrangement of the polymer chain fragments and create gradient distribution of the polymer chains in the transverse direction. Properties of the created surface will be dominated by the properties of the polymer chains with prevailing surface area fraction. These are represented by a mixed system of poly 2-vinyl pyridine/acrylic acid,\textsuperscript{33,34} polystyrene/polyacrylic acid,\textsuperscript{33} poly(ethylene glycol)/polyacrylic acid,\textsuperscript{35} polystyrene/poly(2-vinyl pyridine),\textsuperscript{36} and poly ethyleneimine/polyacrylic acid.\textsuperscript{37-40}

The objective of this part of the work was to identify conditions for synthesis and preparation of responsive mixed polymer brushes with different ratios between the grafting density of the components of the brushes. Prepared brushes were then subjected to the studies of their response to external stimuli, morphology, and protein adsorption.

For preparation of the responsive thin polymer films for protein adsorption, as components of system, poly(ethylene glycol) (PEG), polyacrylic acid (PAA), and
polystyrene (PS) in the form of block copolymer (PAA-PS) have been chosen. Poly(ethylene glycol) is well known for its protein repelling properties. Polyacrylic acid, which is miscible with PEG, serves as a stem for protein attractive polystyrene block. Polyacrylic acid adsorbs some proteins (e.g., albumins) on both sides of the proteins’ isoelectric point at low ionic strength of the solution and becomes protein repelling when ionic strength increases. The pH of the solution during adsorption to PAA was found to be of secondary importance for protein adsorption, but some studies indicate importance of this factor in the extent of adsorption.

Overall architecture of a mixed polymer brush assembly is shown in Figure 4.1.

![Figure 4.1](image)

**Figure 4.1.** Preparation of the stimuli responsive mixed polymer brush for the protein adsorption.

Inside a mixed PEG/PAA-PS polymer brush, geometrical positions of the brush components are expected to change with external stimuli. When subjected to appropriate conditions (thermodynamically favorable for PAA), PAA chains lift polystyrene domains to the surface (Figure 4.2 a). When the brush is treated with thermodynamically unfavorable solvents for PAA, PEG chains are brought to the surface and the surface reveals properties characteristic of PEG (Figure 4.2 b). Polystyrene in this case is hidden inside the brush.
In this dissertation poly(glycidyl methacrylate) (PGMA) is used as an anchoring layer for primary surface modification. This polymer can be attached to virtually any surface and makes it suitable for future modification. After crosslinking by heat treatment, the layer is stable and has plenty of reactive groups available for grafting. Poly(ethylene glycol) is grafted first through reaction of its end carboxylic group with epoxy group of PGMA. Then, block copolymer of polyacrylic acid (PAA-PS), polystyrene, is grafted. Grafting of the second component of the polymer brush by the “grafting to” approach may present difficulties due to hindered surface reactive groups by the first layer. The system of PEG – PAA-PS overcomes this obstacle easily, however. When deposited on top of poly(ethylene glycol), this block copolymer mixes with already tethered PEG by the polyacrylic acid part of the copolymer. Due to the miscibility, PAA
chains gain access to the surface reactive groups and block copolymer is pinned to the surface through its PAA part.

The amount of the protein adsorbed onto the mixed polymer brush will be determined by:

- Size and shape of polystyrene inclusions formed after solvent treatment (discussed in Chapter 5 and Chapter 6)
- External environment (ionic strength of the solution and presence of metal ions of different valency), (discussed in Chapter 6)
4.2. Experimental:

Highly polished single-crystal silicon wafers of (100) orientation (Semiconductor Processing Co.) were used as a substrate. The wafers were first cleaned in an ultrasonic bath for 30 minutes, placed in a hot “piranha” solution (3:1 concentrated sulfuric acid/30% hydrogen peroxide) for one hour, and then rinsed several times with deionized water.

Glycidyl methacrylate from Aldrich was polymerized radically to give PGMA, Mn=290,000, PDI=2.9 (GPC). The polymerization was carried out in methyl ethyl ketone (MEK) from VWR at 60°C. AIBN from Aldrich was used as an initiator. The obtained polymer was purified by multiple precipitations from MEK solution in diethyl ether.

Poly(ethylene glycol) monomethyl ether with Mn ca. 5,000 (Aldrich) was modified with succinyl anhydride (Aldrich) to give a carboxy end group derivative (PEG). Acylation was done by refluxing of poly(ethylene glycol) monomethyl ether with large excess (ca. 20) of succinyl anhydride in tetrahydrofuran (THF) from VWR. Carboxylated PEG, latter in text referred to as PEG was purified by multiple precipitations from THF solution in diethyl ether.

PGMA was dissolved in MEK (0.08% w/v) and thin films (3±0.5 nm) were deposited on the substrate by dip coating (Mayer Feintechnik, model D-3400) and crosslinked at 110°C for 30 minutes.\textsuperscript{43,44} The thickness of deposited PGMA films was controlled via concentration of the PGMA solution. The PEG powder was deposited onto the surface of a clean glass slide and was covered with the silicon wafer modified with
the PGMA primary layer. The specimens were placed onto a temperature gradient table (35-130°C) for 3 hours to enable the end groups to anchor to the epoxy-modified substrate. At high temperatures, carboxylic groups are able to react with the epoxy groups of the PGMA layer. Unbound PEG was removed by multiple washing with toluene at 75°C including washing in an ultrasonic bath.

A second layer of block copolymer of polyacrylic acid (Mn=27000)-b-polystyrene (Mn=1000) (Polymer Source Inc.) was then deposited onto the top of the first layer by dip coating into 1% (w/v) methanol solution and grafted on the temperature gradient table using temperature range of 70-110°C for 10 minutes. Afterwards, ungrafted polymer was removed by multiple rinsing with ethanol. Prepared samples were subjected to subsequent treatments and studies.

The temperature gradient stage is constructed from a copper plate with two opposite sides connected to the heating and cooling elements (Figure 4.3.a). The temperature of the stage is monitored by five pairs of thermocouples located along the sample. The stage is equipped with a plastic cover to assure an inert atmosphere. The gradient setup provides a linear temperature increase from the cold end to the hot end (Figure 4.3.b). Grafting was performed under a nitrogen purge in order to prevent polymer oxidation.
To characterize the polymer layers, several parameters have been evaluated. The surface coverage (adsorbed amount), \( \Gamma \) (mg/m\(^2\)), was calculated from the ellipsometry thickness of the layer, \( h \) (nm), using equation (E4.1):

\[
\Gamma = h \rho
\]  
(E4.1)

where \( \rho \) is the density of attached (macro)molecules. The density data for PEG (1.13 g/cm\(^3\)), PAA (1.16 g/cm\(^3\)), and PS (1.05g/cm\(^3\)) were obtained from the Polymer Handbook.\(^{47}\)

The chain density, \( \sigma \) (chain/nm\(^2\)), i.e., the inverse of the average area per adsorbed chain, was determined by equation (E4.2):

\[
\sigma = \Gamma N_a * 10^{-21} / Mn = (6.023*\Gamma*100)/Mn
\]  
(E4.2)
where $N_A$ is the Avogadro number and $M_n$ (g/mol) is the number average molar mass of the grafted polymer.

The distance between grafting sites, $D$ (nm), was calculated using the following equation (E4.3):

$$D = \left(\frac{4}{\pi \sigma}\right)^{1/2}$$  \hspace{1cm} (E4.3)

The radius of gyration for the PEG macromolecules was estimated by the equation (E4.4):

$$6R_g^2 = L^2,$$  \hspace{1cm} (E4.4)

where $L = 5.44$ nm is the end-to-end distance of PEG.$^{47}$

The end-to-end distance for polyacrylic acid and polystyrene was calculated using the expression (E4.5):

$$L^2 = nl^2C_\infty$$  \hspace{1cm} (E4.5)

where $n$ is the number of C-C bonds in polymer backbone, $l$ is the bond length (0.154 nm),$^{41}$ and $C_\infty$ is the characteristic ratio for polymer. $C_\infty$ was assumed to be 10 for polystyrene$^{41}$ and 6.7 for polyacrylic acid.$^{48}$

Static contact angle measurements were made using a contact angle goniometer (Kruss, Model DSA10). Contact angles were calculated using the tangent method. Contact angle measurements were made with water (pH 7.0) and a static time of 60 seconds before the measurement. Ellipsometry was performed with a COMPEL automatic ellipsometer (InOmTech, Inc.) at an angle of incidence of 70°. Refractive index of 1.525 was used for the PGMA thickness calculation and 1.5 for block copolymer calculations. Original silicon wafers from the same batch and silicon wafers with PGMA
layer were tested independently and used as reference samples for the analysis of grafted polymer layers.

**4.3. Results and discussion:**

**4.3.1. Synthesis of mixed polymer brushes:**

**4.3.1.1. PEG grafting:**

Typical multi-step preparation of polymer brushes is time and labor consuming. One way to speed up studies on optimization of brush parameters is to employ a gradient approach for preparation of the samples. In the gradient approach, a sample with gradually changing brush thickness (grafting density) is prepared so there is no need to prepare multiple samples to study the behavior of brushes with different compositions. Gradients of polymer brushes can be created by applying gradients in temperature, reactive ability of the surface, and initial surface coverage.\(^7\)

Temperature gradient approach has been applied to create gradient coverage of the first component of the mixed polymer brush, poly(ethylene glycol). Grafting was carried out under a temperature gradient from 35 to 130°C, which was above Tg (-60°C) of PEG and crossed the melting temperature, Tm (60°C).\(^{47}\) Grafting was done for 3 hours on the PGMA anchoring layer of thickness of 3±0.3 nm. Thickness results of six parallel grafting experiments are shown in **Figure 4.4**. A graph of dependency between the thickness of the layer and temperature (**Figure 4.4**) reveals a nonlinear behavior of the density against temperature.
As expected, the thickness of the PEG layer increases with temperature. A sharp increase in grafting thickness happens at and above the melting temperature of PEG. Below this temperature PEG mostly exists in a semi-crystalline form, that restricts the chains’ mobility and, consequently, grafting. The mobility of the chains is an important factor in grafting of end-functionalized polymers as it determines the supply of the reactive groups to the surface and, as a result, the brush grafting density. The diffusion coefficient is a quantitative parameter that describes the mobility of polymer chains.

![Graph showing grafting thickness of PEG (Mn=5000) measured by Ellipsometry versus temperature of grafting.](image)

**Figure 4.4.** Grafting thickness of PEG (Mn=5000) measured by Ellipsometry versus temperature of grafting.

In general, there are two major processes that determine the extent of grafting. These are the diffusivity of the polymer and the rate of chemical reaction between the
polymer and functional groups on the surface. Both dependencies are exponential and thus explain the nonlinearity of the dependence curve obtained in Figure 4.4.

The reaction rate between the carboxyl terminated poly(ethylene glycol) and epoxy groups of the anchoring polymer layer is one of the controlling factors for the PEG brush formation. Interaction between butanoic acid and 1-methyl-3,4-epoxycyclohexanecarboxylic acid was used as a model system to investigate the dependency on factors such as temperature during the grafting of PEG. From the data provided for the reaction between these two components \( T=150^\circ C, k=7.4 \times 10^{-5} \text{kg mol}^{-1} \text{s}^{-1}, E_a=90.85 \text{kJ/mol}, k_0=8.08 \times 10^5 \text{kg mol}^{-1} \text{s}^{-1} \) were calculated and plotted the Arrhenius dependency of rate of the reaction on temperature (Figure 4.5) according to the following equation (E4.6):

\[
k = k_0 \exp(-E_a/RT)
\]

(Figure 4.5) shows that reaction starts to accelerate at \( 80^\circ C \). Before that the rate of reaction is very low and so is the extent of grafting of the PEG brush.

Another factor that affects the PEG grafting extent is the mobility of polymer chains. For polymers above the critical molecular weight, whose zero shear viscosity changes from \( \eta \sim N \) to \( \eta \sim N^{3.4} \), the diffusion coefficient depends on the degree of polymerization as \( D \sim N^2 \) (Ref.51). At the given degree of polymerization (\( DP=114 \) for polymer used) temperature dependence of the diffusion coefficient follows the Vogel-Fulcher law:

\[
D_{self}=D_0\exp(-B/(T-T_0))
\]
where $B$ and $T_0$ are constants for a given polymer, and are independent of the degree of polymerization for all but the shortest chains. $T_0$ is related to the glass transition temperature as it is about 50° below $T_g$ and is equal to -110°C for PEG.

![Temperature dependency of the reaction rate between the carboxyl group and epoxy group](image)

**Figure 4.5.** Temperature dependency of the reaction rate between the carboxyl group and epoxy group on the example of butanoic acid and 1-methyl-3.4-epoxycyclohexanecarboxylic acid.

Diffusion coefficients have already been determined for poly(ethylene glycol) of molecular weight used in this work at different temperatures ($D_{413K}=5.8 \times 10^{-8}$ m$^2$s$^{-1}$; $D_{393K}=4 \times 10^{-8}$ m$^2$s$^{-1}$). D$_0$ could be found by solving a system of equations (E4.8) and was obtained to be equal to $4.311 \times 10^{-6}$ m$^2$s$^{-1}$ and the constant, B, was equal to 1076.4 m$^2$s$^{-1}$.

\[
\begin{align*}
D_{413} &= D_0 \exp\left(-\frac{B}{(413-163)}\right) \\
D_{393} &= D_0 \exp\left(-\frac{B}{(393-163)}\right)
\end{align*}
\]  

(E4.8)
The values were used to obtain the dependence between the diffusion coefficient of the PEG 5,000 and temperature (Figure 4.6). Exponential growth in the graph is not as steep as the Arrhenius dependence of the rate of reaction (Figure 4.5).

![Graph showing temperature dependence of the diffusion coefficient for PEG (Mn=5,000).](image)

**Figure 4.6.** Temperature dependence of the diffusion coefficient for PEG (Mn=5,000).

Results of PEG grafting (Figure 4.4) were fitted with an exponential growth function, taken its derivative, and built the tangent lines at the border points of the curve: 35 and 130°C for all three figures, Figure 4.4, Figure 4.5, and Figure 4.6. At the intersection of these lines there is the temperature at which tangent values undergo the most rapid changes. These temperatures are different for each graph and are 98.5°C for PEG grafting curve, 125.5°C for rate reaction constant, and 86.8°C for diffusivity constant. When Figure 4.5 and Figure 4.6 are compared we may conclude that a major cause for the rapid change of rate of grafting of PEG (Figure 4.4) results from the
diffusivity dependence. Reaction rate comes into play close to 127°C and cannot be solely responsible for overall picture of the increase of grafting density of PEG with temperature.

![Graph](image)

**Figure 4.7.** Parameters of PEG (Mn=5,000) brush. (a) – Distance between grafting sites vs. temperature. Dashed line corresponds to the border between the mushroom (above line) and brush (below line) regimes. (b) – grafting density (chains/nm²) vs. temperature.

End tethered polymer assembly is called “brush” when the distance between grafting sites is smaller than two radii of gyration. The prepared polymer assembly can be assigned to “brush” or “mushroom” regime on the basis of data shown in **Figure 4.7a**.

The dashed line on the graph in **Figure 4.7a** corresponds to the border point between “brush” and “mushroom” regimes for PEG with Mn=5,000. Radius of gyration, $R_g$, for polymer of such molecular weight is 2.2 nm, with $2R_g$ of 4.4 nm (calculated using E4.4 and E4.5). Macromolecules of the prepared end-tethered polymer layer in dry state are collapsed on the surface and aligned when in crystalline state. But when in melt or in
the solution, the prepared samples are in “brush” regime, as the distance between grafting sites for the samples was less than \(2R_g\) of polymer coil. The distance between grafting sites decreases with increasing temperature and this indicates formation of a very dense brush at higher temperatures. At the same time, grafting density increases nonlinearly with time and reaches approximately 1.2 chains per \(\text{nm}^2\) for grafting temperature of \(130^\circ\text{C}\).

According to the obtained data, PEG thickness gradient can be straightforwardly created using the temperature gradient stage. Tethered polymer chains obtained even at low grafting temperatures result in “brush” regime for grafting time of 2.5 hours. Modified by PEG, the surface still has to have active grafting sites that can be used for subsequent attachment of the next brush component.  

**4.3.1.2. Polyacrylic acid-b-polystyrene grafting:**

Grafting of the second component of the polymer brush assembly, block copolymer polyacrylic acid (Mn=27,000)-b-polystyrene (Mn=1,000), was done in a range of temperatures from 70 to 100\(^\circ\text{C}\) (Tg for polyacrylic acid: 110\(^\circ\text{C}\)).\(^{52}\) This temperature range gave better control on grafting rate because diffusivity of the polymer chains could be neglected and grafting depended only on the rate of reaction between the carboxylic group of polymer and epoxy groups of PGMA. Miscibility of the polymer chains of PEG and PAA diminishes any obstacles for the chemical attachment of the block copolymer chains to the surface and improves uniform distribution of the PAA-PS chains inside the PEG brush. Additionally, the presence of PEG chains prevents formation of the PAA
crystals so mobility of the PAA chains increase and also favors grafting.\textsuperscript{3} Thickness results of the grafting experiment are shown in Figure 4.8.

Data presented in Figure 4.8 show that the amount of the polymer grafted to the surface remains practically constant despite the temperature change. This can be explained by sufficient reactivity of the carboxyl groups present in PAA chain at the temperatures used (Figure 4.5). From E4.7 it may be concluded that the diffusion factor is not of importance in the temperature range used and is very small. The amount of grafting is predetermined by high availability of the reactive groups present on the surface and the carboxyl-epoxy reaction extent.

The radius of gyration of the block copolymer molecule was calculated to be 4.1 nm and was the sum of radii of gyration for the polycrylic acid part and the polystyrene part, which were 3.5 nm and 0.6 nm, respectively (calculated using E4.4 and E4.5).
Unlike the PEG brush, PAA-PS is not a “classical” end-grafted brush. Because there are reactive functional groups along all polymer chains, many attachment points can exist. Such a brush is termed as a “Guiselin brush”\textsuperscript{53} (Figure 4.9) where, as a brush, constituent parts of the chains located in tails and loops (pseudotails) of the adsorbed molecule are taken. The average pseudotail/tail size can be estimated by the following relationship:

\[
\text{Size} = N(1-p)/2(Np/n) = (1-p)n/(2p) \tag{E4.9}
\]

where \(N\) is the degree of polymerization of the adsorbed polymer, \(p\) is the fraction of the monomeric units in the train, and \(n\) is the number of monomeric units involved in one train section of the adsorbed macromolecule. At maximum, the train fraction of the adsorbed polymer is up to 0.25, and 3 monomeric units are involved in each train.\textsuperscript{54} Because the non-reactive polystyrene part of the block copolymer can not participate in the grafting process, only the polyacrylic part of copolymer is used for calculations. With \(M_n=27,000\) and \(N=375\), the average pseudotail/tail size with different train fractions and 3 monomeric units in one train section is shown in Figure 4.10.

\[\text{Loop of } 2n \text{ monomeric units}\]
\[\text{1 tail of } n \text{ monomeric units}\]
\[\text{2 pseudotails of } n \text{ monomeric units each}\]

Figure 4.9. Concept of Guiselin brush. Definition of loop, pseudotail and train.
By evaluation of the fraction of monomeric units of polyacrylic acid that are located in trains of the synthesized brush, it is possible to predict the degree of extension of the brush loops under the solvent as being proportional to $N^{5/6}$ (Ref.53).

The volume of the monomeric unit of polyacrylic acid may be approximated to be equal to the volume of one molecule of acrylic acid and can be calculated from the following expression, and is equal to $1.14 \times 10^{-22}$ cm$^3$ or 0.114 nm$^3$:

$$V_0 = \frac{M}{\rho N_A}$$  \hspace{1cm} (E4.10)

where $M$ is the molecular weight of acrylic acid, 72 g/mole; $\rho$ is the density of acrylic acid, 1.05 g/ml; and $N_A$ is the Avogadro number, $N_A = 6.022 \times 10^{23}$.

Assuming the polyacrylic acid monomeric unit to be in cubic shape and the length of the side of the cube to be 0.48 nm, the projected surface area is 0.23 nm$^2$. The area that is occupied by one train section of polymer (3 monomeric units) is 0.7 nm$^2$. Grafting density of the block copolymer is 0.2 chains/nm$^2$ and grafting 1 polymer chain requires 5 nm$^2$ of area. The maximum amount of trains for one grafted chain is then 5nm$^2$/0.7nm$^2$=7 trains. With each train having 3 monomeric units, there are total 21 monomeric units in trains, which is 0.056 in the train fraction of the Guiselin brush.

From Figure 4.10 we can estimate that the smallest size of the pseudotail of polymer chain for this train fraction (0.056) is 25 monomeric units and the maximum is 373 monomeric units if PAA chain is attached in one point at the end of the chain. The lower border is much smaller than the length of the PEG chains in the mixed brush, which are 114 monomeric units/chain. Because the polymer brush is prepared by the “grafting to” approach by deposition of the polymeric coil onto the surface, there is a
very small chance that polyacrylic acid will be attached by one end and the tail size will reach the upper border. Taking into account the calculations, we can expect polystyrene spheres to be totally covered by the PEG chains under appropriate conditions. These estimations are done for pure block copolymer grafted to the surface. When a mixed polymer brush is prepared, the area available for grafting of the block copolymer decreases and the train fraction of polyacrylic acid decreases, increasing pseudotail size of the brush.

![Graph](image)

**Figure 4.10.** Pseudotail size of Guiselin brush vs. train fraction. Plotted using equation (E4.9).

To more precisely calculate the size of the pseudotails of the block copolymer Guiselin brush, area which is occupied by PEG chains was taken into account. Each PEG chain is tethered to the surface through one carboxylic group. We have estimated the size
of the carboxylic group with expression E4.10 using formic acid as a model compound (HCOOH). The calculated size of carboxylic group is:

\[
46 \text{g/mole}/(1.22 \text{ g/ml}\times6.022\times10^{23}) = 6.26\times10^{-23}\text{cm}^3 \text{ or } 6.26\times10^{-2}\text{nm}^3.
\]

Assuming that the carboxylic group is a cubic shape and the length of the side of the cube being 0.40 nm, the area projected onto the surface is 0.158 nm². The area occupied by PEG chains on 1 nm² is equal to \(\sigma_{\text{PEG}}\times0.158\) nm², where \(\sigma_{\text{PEG}}\) is the grafting density of the PEG molecules. The area left for grafting is \((1-\sigma_{\text{PEG}}\times0.158)\) nm². The grafting density of the block copolymer molecules is equal to:

\[
\sigma_{\text{block}}=(6.023*h*\rho*100)/(M*(1-\sigma_{\text{PEG}}\times0.158))\text{chains/nm}^2
\]

where \(h\) is the thickness of the block copolymer (7.5 nm, Figure 4.8); \(\rho\) is the block copolymer density (1.16 g/cm²); and \(M\) is the molecular weight of block copolymer (28,000 g/mole).

The area of \((1-\sigma_{\text{PEG}})\) can accommodate the maximum amount of polyacrylic acid trains, \(N_t=(1-\sigma_{\text{PEG}}\times0.158)/0.7\), that belongs to \(\sigma_{\text{block}}\) amount of chains of block copolymer. The maximum fraction of trains in block copolymer molecule is \(p = N_t*3 / (\sigma_{\text{block}}*375)\).

Using equation E4.9 and taking into account the above obtained expression for the train fraction of block copolymer as a function of PEG grafting density, Figure 4.11 is obtained. Figure 4.11 represents the minimal size of the pseudotails of the block copolymer brush. As can be seen from the graph, the calculated minimal size of the pseudotails of polyacrylic acid increases slightly and can reach up to 39 monomeric units for 1.2 chains/nm² grafting density of PEG.
When planning the experiment for mixed polymer brush preparation, the grafting behavior of PEG and the fact that it is difficult to control the thickness of polyacrylic acid were taken into account. Samples for studying brush morphology, behavior, and protein adsorption were prepared with varying thickness of PEG and constant thickness of the block copolymer. Figure 4.12 represents average sample parameters. The grafting density of the block copolymer was kept constant and equal to 0.2 chains/nm$^2$. Poly(ethylene glycol) brush varies in its grafting density from 0.2 to 1.2 chains/nm$^2$ along the sample.

**Figure 4.11.** Dependence between the parameters of Guiselin brush of polyacrylic acid in mixed polymer brush on PEG grafting density. A) - Pseudotail size of the polyacrylic acid chains vs. poly(ethylene glycol) grafting density, b) – train fraction of polyacrylic acid vs. PEG grafting density.
There is a slight decrease in the amount of grafted block copolymer possibly due to geometrical restrictions coming from the very dense grafted poly(ethylene glycol) brush at grafting densities of PEG above 0.6 chains/nm$^2$.

Taking into account the amount of material on the surface, there are a limited number of nondestructive methods for the evaluation of chemical composition of the layers. Methods such as XPS or elemental analysis could prove the expected presence of the polymer in the assembly, but these methods often require manipulations that make subsequent use of samples impossible. Composition of the prepared polymer assembly in our case was studied qualitatively using in-situ IR Ellipsometry, which is a non-destructing method suitable for small quantity samples.$^{55,56}$ Figure 4.13 represents obtained spectra taken at a few points of the wafer. Only tan$\psi$ was measured in order to
monitor the vibrational bands. Because of the very small amount of the polymers on the substrate, only the strongest peaks are well defined in the spectra. Signals at 1730 and 1560 cm\(^{-1}\) correspond to the vibrations of the polyacrylic acid carbonyl groups in nonionized and ionized forms, respectively. The signal at 1414 cm\(^{-1}\) can be attributed to the asymmetric stretch of the carboxylate group. Due to the small amount of the polystyrene and low peak intensity of the aromatic ring, polystyrene signal was not clearly detected. These IR data confirm composition of the mixed brush obtained.

**Figure 4.13.** IR-Ellipsometry (synchrotron source of light) spectra of the mixed polymer brushes at few points of the brush at pH 2 (HCl) and pH 10 (KOH). Measurements were done at the ISAS - Institute for Analytical Sciences, Berlin, Germany in Dr. Hinrichs group by Dennis Aulich.
4.4. Conclusions:

Mixed polymer brushes were prepared using the “grafting to” technique and temperature gradient with constant grafting density of polyacrylic acid-b-polystyrene and varying grafting densities of poly(ethylene glycol). Composition of the brush assembly was confirmed by Ellipsometry and IR Ellipsometry.

The prepared grafted PEG layer exists in “brush regime,” i.e., the distance between grafting sites is smaller than $2R_g$ of polymer coil. Block copolymer may have more than one connection to the surface and exists in the form of “Guiselin brush.”

Grafting of PEG was performed above $T_g$ in temperature range, 35-130°C. PAA-PS block copolymer was grafted in temperature range of 70-110°C.

The main reason for the nonlinearity of the PEG grafting extent with temperature was found to be diffusivity of the polymer chain ends to the surface. The rate of reaction was found to affect the extent of grafting at higher temperatures starting at 125°C.

With polyacrylic acid grafting, the rate of reaction determined the extent of grafting and diffusivity of the chains played a minor role in the temperature range selected.

The minimal height of the pseudotails of polyacrylic acid was calculated to be 25 monomeric units when no PEG was present in the brush and came to the minimum of 39 units and the maximum of 373 units when there were 1.2 chains/nm$^2$ PEG grafted to the surface.

The ratio between the grafting densities of PEG and block copolymer for the prepared gradient samples of the mixed polymer brush varied from 1 to 7.5. The grafting
density of PEG varied from 0.2 to 1.1 chains/nm$^2$ and the grafting density of block copolymer was close to 0.2 chains/nm$^2$.

4.5. References:


(3) Zdyrko, B., Clemson University, 2004.


(9) Zhao, B. *Polymer* 2003, 44, 4079-4083.


CHAPTER FIVE
RESPONSIVE BEHAVIOR OF MIXED POLYMER BRUSH: ORGANIC SOLVENT TREATMENT TO FORM MIXED BRUSH STRUCTURES

5.1. Introduction:

End tethered polymer brushes possess a unique set of switchable properties, determined by the properties of their constituents. Surface properties of the brush can be tuned using external factors (temperature, pH, solvent treatment, and ionic strength).\textsuperscript{1-5} The change of the surface properties lays in the phase segregation mechanism of the polymer chains due to the change in polymer molecule chain conformation. As a result of these changes, switching of the spatial distribution of functional groups inside and on the boundary of the thin polymer film occurs.\textsuperscript{7} The change in surface properties alters its interaction with the external environment.

Extensive theoretical studies of the behavior of mixed polymer brushes have been started with Marko and Witten\textsuperscript{8} and followed by other researchers.\textsuperscript{9,10} Phase diagrams of the mixed polymer brushes were calculated by varying the Flory-Huggins parameter, difference of solvent quality, total grafting density, difference of chain length, and the relative grafting density of the dissimilar polymer chains.\textsuperscript{10} In addition to two limiting types of morphologies, ripple and dimple, theoretical calculations have predicted a variety of other well-ordered structures such as “onion,” “garlic,” “dumbbell,” and flowerlike and checkerboard types, revealing mixed polymer brushes as a very promising material for creation of patterned surfaces.\textsuperscript{11-14} Distinctive difference between the phase
segregation of multicomponent polymer mixtures in melt and in tethered polymer brushes occurs because, due to the restricted mobility of the tethered chains, segregation into macroscopic domains is impossible and the system segregates into the small microphase separated domains. Increasing solvent selectivity to one of the components of the brush promotes the dimple morphology with layered segregation where one polymer is exposed to the surface while another is hidden inside the brush bulk. Theoretical predictions of lateral segregation have been confirmed experimentally in AFM and X-ray photoemission electron microscopy studies of the mixed polymer brush of polystyrene and poly(2-vinylpyridine). A number of other studies have also shown the predicted phase segregation of the mixed polymer brushes of different compositions after treatments with various solvents. 

A distinctive characteristic of the polymer brushes is a linear dependence of the brush height, L, on the number of statistical segments, N, (it corresponds to $R_g \sim N^{3/5}$ for free polymer in good solvent). However, the prefactor to the expression depends on the solvent quality and the grafting density of the brush. In $\theta$ solvents (interaction between polymer chains is equal to interaction between the polymer and solvent), the dimension of tethered polymer chains is described by the expression, $L/a \approx N(a/d)$; in good solvents, dependency of the prefactor changes to $L/a \approx N(a/d)^{2/3}$ (Ref.22). In the expressions, $d$ is the average distance between tethering points and $a$ is diameter of one statistical segment of polymer chains. A polymer chain consists of N statistical segments. Thus, treating the mixed polymer brushes with different solvents brings different polymer chains to the brush exterior.
The objective of this part of the work was to select appropriate external stimuli (organic solvents) and study changes in the morphology of the mixed polymer brush (PEG/PAA-PS) associated with the solvent treatment. The solvent treatment of the mixed polymer brush results in formation of the polymer domains of different size and spatial position inside the brush (Figure 4.1a-d). When treated with a good solvent for polymer A, the mixed polymer brush preferentially has domains of polymer A on the surface (Figure 4.1a). The same applies to other components of the mixed polymer brush. When the brush is treated with a good solvent for a certain component, larger domains of this component are formed and are located on the surface of the brush.

![Figure 5.1. Suggested morphology of a mixed polymer brush treated with the a) – selective solvent for polymer A; b) – selective solvent for polymer B; c) – selective solvent for polymer C; d) – good solvent for all components of the brush.](image)

Polystyrene is the component of the mixed polymer brush that dominates hydrophobic interactions of the brush. When treated with different solvents, differently sized domains of polystyrene are created and located at different levels inside the brush.
Depending on the size and location of the polystyrene domains, the mixed polymer brush interacts differently with hydrophobic species.

5.2. Experimental:

Samples prepared as described in Chapter 4 were treated with organic solvents and subjected to studies of morphology, water contact angle, and protein adsorption or adhesion. First, the synthesized sample was immersed into DMF for 15 minutes at room temperature and then dried in nitrogen atmosphere overnight. Later, the sample was immersed for 15 minutes into a solvent of choice (MEK, ethanol, or toluene) at room temperature and dried in nitrogen atmosphere for 1 hour. The prepared sample was used for subsequent studies.

To ensure removal of low-volatile DMF from the samples, the thickness and water contact angle of the mixed polymer brush treated with DMF and dried overnight in nitrogen stream were measured. Then the sample was placed into a vacuum oven at 40º and left overnight. The measured thickness of the sample after additional drying as well as contact measurements did not indicate any decrease in the amount of the substance on the surface or changes in water contact angle. The sample was additionally rinsed with diethyl ether (a non-solvent for all three polymers constituting the mixed brush; miscible with DMF) and dried, and the thickness and water contact angle of the sample were measured. Again, no changes in thickness or water contact angle were noticed. Therefore, the procedure of drying the DMF treated sample overnight was assumed to be sufficient for removal of DMF.
Static contact angle measurements were made using a contact angle goniometer (Kruss, Model DSA10). Contact angle was calculated using the tangent method. Contact angle measurements were made with water (pH 7.0), and a static time of 60 seconds before the angle measurement. Scanning probe microscopy (SPM) studies were performed using a Dimension 3100 (Digital Instruments, Inc.) microscope. We used the tapping mode to study the surface morphology of the films in ambient air and under water (Millipore water of pH 5.4 was used for underwater studies). Silicon tips with spring constants of 50 N/m (tapping mode) and 0.25 N/m (contact mode) were used. Imaging was done at scan rates ranging from 1 to 2 Hz. The root mean square roughness of our samples was evaluated from the SPM images recorded.

The RMS (root mean square) roughness is a measure of the height profile’s roughness and is defined as:

\[
\text{rms} \equiv \sqrt{\frac{1}{n} \sum_{i=1}^{n} (h(x_i) - \bar{h})^2}
\]  \hspace{1cm} (E5.1)

where \( n \) is the number of lattice points, \( h(x_i) \) is the height at lattice site \( x_i \), and the average height of the profile is defined as:

\[
\bar{h} \equiv \frac{1}{n} \sum_{i=1}^{n} h(x_i)
\]  \hspace{1cm} (E5.2)

The RMS roughness describes the fluctuations of surface heights around an average surface height and is the standard deviation or the square root of the second cumulant (variance) in terms of statistics.\(^{23} \)
5.3. Results and discussion:

5.3.1. Selection of the solvent for initiation of mixed brush morphology changes:

The thermodynamic quality of a solvent toward a polymer is determined by free energy of mixing.\(^{24}\) For a polymer possessing a certain molecular weight, the free energy of mixing is related to the interaction parameter, \(\chi\), and increase as \(\chi\) increases, leading to phase separation of the solvent polymer system at \(\chi > 0.5\). The interaction parameter, \(\chi\), can be estimated by the solubility parameters of both solvent and polymer. The solubility parameter is defined as energy of evaporation of 1 cm\(^3\) of the substance and is described by the following equation (E5.3):\(^{25}\)

\[
\delta = (\Delta E/V)^{1/2} \tag{E5.3}
\]

where \(\Delta E\) is the cohesive energy of the substance, and \(V\) is the molar volume of the substance.

Table 5.1 and Table 5.2 represent solubility parameters for the solvents and polymers that composed the polymer brush studied in this work.

| Table 5.1. Solubility parameters for the selected solvents.\(^{26}\) |
|-----------------|----------------|----------------|----------------|
| Solubility parameters, MPa\(^{1/2}\) | Toluene | Ethanol | MEK | DMF |
| 18.2 | 26.0 | 19.0 | 24.8 |
Table 5.2. Solubility parameters for the selected polymers.\(^{26}\)

<table>
<thead>
<tr>
<th>Solubility parameters, MPa(^{1/2})</th>
<th>PEG</th>
<th>PAA</th>
<th>PS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>20.2±2</td>
<td>25.7±2</td>
<td>18.05</td>
</tr>
</tbody>
</table>

Using the above mentioned solubility parameters, the Flory-Huggins parameter, \(\chi\), can be determined. Thus, we can evaluate miscibility of a polymer component in a solvent. To achieve sufficient miscibility, the Flory-Huggins parameter should be below or equal to 0.5 (Ref.24). The following formula (E5.4) is used to estimate \(\chi\):\(^25\)

\[
\chi = 0.35 + \frac{V_1}{RT} (\delta_1 - \delta_2)^2
\]  

(E5.4)

where \(V_1\) is the molar volume of the solvent, and \(\delta_1\) and \(\delta_2\) are the solubility parameters of the mixture components.

Solubility of the polymer in the solvent depends on the molecular weight of the polymer molecule.\(^25\) The critical parameter, \(\chi\), at which solubility still exists for the lower molecular weight components is estimated by the formula (E5.5):

\[
\chi = 0.5 + \frac{1}{N^{0.5}}
\]  

(E5.5)

where \(N\) is a degree of polymerization. This value is equal to 0.53 for polystyrene, 0.51 for polyacrylic acid, and 0.51 for poly(ethylene glycol) used in this work.

It should be taken into account that E5.4 is valid for the systems where there are no other interactions between components of the mixture beside van der Waals and some polar interactions, and the volume of the mixture is equal to the sum of the volumes of individual components. This theory does not account for hydrogen bonding and does not provide valid results for the system where these interactions are present. Only positive \(\chi\)
values are predicted by this equation.\textsuperscript{25} Hence, the calculations will reflect interactions of
PEG and PS with toluene, DMF and MEK, but not for hydrogen bonding forming PAA
and ethanol.

Results for the calculations of the $\chi$ parameter for the mixed brush components
and solvent system are provided in \textbf{Table 5.3}. Data shown in \textbf{Table 5.3} suggest that
MEK would be a good solvent for PEG and polystyrene ($\chi<0.5$), and a non-solvent for
PAA ($\chi>>0.5$). Toluene would be a good solvent for polystyrene ($\chi<0.5$), and may swell
PEG ($\chi=0.52$). Toluene would be a non-solvent for PAA ($\chi>>0.5$). As mentioned above,
in case of possible hydrogen bonding formation for such solvent and polymer as ethanol
(\textbf{Table 5.3}) and PAA, respectively, the value of the $\chi$ parameter may not represent real
miscibility of the components and show absence of miscibility for this system. Although
it is known from the literature (\textbf{Table 5.4}) that DMF and ethanol are good solvents for
PEG due to hydrogen bonding, the interaction parameter value indicates the opposite.
\textbf{Table 5.4} has been used as an additional guide for choosing the solvent for studied
polymer system. Ethanol was used as a solvent for PEG and PAA, while being a non-
solvent for PS. DMF with its moderate ability to form hydrogen bonding was used as a
reference solvent to create a system in which all three components are evenly distributed
on the surface.

Taking into account the solubility of the brush components in the solvents
distribution of the polymer chains within the brush surface can be predicted (\textbf{Figure 5.2}).
Solvent treatment is important for the formation of the polystyrene domains. Depending
on the treatment and grafting density, domains vary in size and geometrical position
inside the brush. Later, when samples are subjected to aqueous environment, complexes of water soluble PEG and PAA are destroyed, but the polystyrene domain stays intact and is in great part responsible for the hydrophobic interaction of the mixed brush under water.

Table 5.3. Flory-Huggins parameter for the selected solvent – polymer component system.

<table>
<thead>
<tr>
<th>Hydrogen bonding (HB) tendency</th>
<th>PEG</th>
<th>PAA</th>
<th>PS</th>
</tr>
</thead>
<tbody>
<tr>
<td>MEK moderate</td>
<td>0.40 solvent</td>
<td>1.97 nonsolvent</td>
<td>0.38 solvent</td>
</tr>
<tr>
<td>Toluene poor</td>
<td>0.52 poor solvent</td>
<td>2.77 nonsolvent</td>
<td>0.35 solvent</td>
</tr>
<tr>
<td>Ethanol strong</td>
<td>1.14 solvent due to HB</td>
<td>0.35 solvent</td>
<td>1.84 nonsolvent</td>
</tr>
<tr>
<td>DMF moderate</td>
<td>1.01 solvent due to HB</td>
<td>0.38 solvent</td>
<td>1.77 solvent*</td>
</tr>
</tbody>
</table>

*Determined experimentally.

Table 5.4. Common solvents and non-solvents for certain polymers.

<table>
<thead>
<tr>
<th>Polymer</th>
<th>Solvent</th>
<th>Nonsolvent</th>
</tr>
</thead>
<tbody>
<tr>
<td>PEG</td>
<td>alcohols, benzene, chloroform, DMF, water (cold), toluene</td>
<td>aliphatic hydrocarbons, ethers, dioxane</td>
</tr>
<tr>
<td>PS</td>
<td>benzene, chloroform, toluene, MEK, THF, DMF</td>
<td>acetic acid, alcohols, diethyl ether, acetone</td>
</tr>
<tr>
<td>PAA</td>
<td>Alkohols, Formamide, DMF, Alkali Slns.</td>
<td>dioxane, esters, hydrocarbons, ketones</td>
</tr>
</tbody>
</table>
When the PEG/PAA-PS mixed brush is exposed to toluene, polyacrylic acid chains have limited mobility while the polystyrene part is extracted and brought to the surface. MEK at the same time has to bring PEG to the surface while PAA chains still are restricted in motion. Ethanol is a non-solvent for polystyrene but it is a good solvent for PEG and PAA due to its formation of hydrogen bonds with these polymers, and PAA chains bring polystyrene to the surface. Because no irreversible change is done to the polymer brush, all morphologies that form due to the solvent treatments can be changed by a treatment with an appropriate solvent. Each of the polymers has its own affinity to the protein used in the subsequent study so the amount of the protein absorbed is determined by the polymer that dominates the surface after a solvent treatment. **Figure 5.2** shows the expected morphologies of the complex polymer brush.

![Figure 5.2](image)

**Figure 5.2.** Schematic representation of the mixed tri component polymer brush after treatment with different solvents; a)-MEK, b)-ethanol, c)-toluene, d) – DMF.
5.3.2. Morphology studies of the mixed polymer brush after organic solvent treatment:

5.3.2.1. Morphology of the separate components of mixed brush:

Morphological studies of the gradient mixed polymer brush were done using AFM. All of the samples were first conditioned in DMF and then exposed to the solvent and dried. Dry morphologies of the brush were studied in tapping mode. An advantage of such measurements is that samples are not destroyed during the measurements and can be used for future studies. Topography and phase images were taken in order to better understand morphological changes. While topological image shows geometrical roughness of the surface, phase image gives an idea about the distribution of different polymers on the surface.

To evaluate the changes in the morphology of the polymer mixed brush due to solvent treatment the behavior of the individual components of the brush under similar conditions has to be evaluated. AFM images of the polyacrylic acid monolayer as well as polyacrylic acid-b-polystyrene are shown in Figure 5.3.

Polyacrylic acid homopolymer brush with grafting density of 0.18 chains/nm$^2$ is a smooth plain film with roughness of 0.2 nm for topography image and 0.6° for root mean square deviation (RMSD) of phase shift in the image area that indicates a very small variation in brush density. AFM topography and phase images of the block copolymer brush prove different domain formation during solvent treatment.
Figure 5.3. 1x1 um AFM images of polyacrylic acid – polystyrene (a-d) and e) – polyacrylic acid treated with: a) - DMF, b) – MEK, c) – ethanol, d) – toluene, e) - DMF. Upper row – topography (scale 3nm), lower row – phase (scale 5°).
Indeed, (Figure 5.3, a-d) shows formation of polystyrene domains in a polyacrylic acid matrix. The size of the domains and their concentration depend on the solvent used. Specifically, toluene treatment results in the largest domains and their high concentration. Smaller domains with lesser amount are formed when the brush is treated with DMF. Domains formed by MEK are small and hardly visible on topography image. The ethanol treated sample shows presence of the domains that are intermediate in size between the DMF and MEK treated sample domains.

When polystyrene block is introduced as a brush component, RMSD of the phase shift in the images changes from $0.8^\circ$ for the brush treated with ethanol to $1.1^\circ$ with MEK, $1.8^\circ$ with DMF, and $2.6^\circ$ with toluene. Initial treatment of the block copolymer brush with grafting density of 0.13 chains/nm$^2$ with DMF results in formation of the surface with topography roughness of 0.3 nm and phase RMSD of $1.8^\circ$ and presence of all brush components on the surface. The increase in RMSD of phase shift of images indicates a change in the surface composition. Among all the solvents, toluene affects the morphology of the layer the most. Toluene can extract polystyrene and favor formation of the hydrophobic polystyrene islands closer to the boundary, that lead to the higher RMSD of the phase shift values as well as higher roughness of topography images. MEK, while being a non-solvent for polyacrylic acid and at the same time restricting the mobility of polyacrylic chains, limits the size of the polystyrene agglomerates that can be formed from closely located polystyrene chains only. Ethanol, being a good solvent for polyacrylic acid, may cause polyacrylic acid chains to cover the polystyrene formations, decreasing by this means RMSD phase shift of the image. Topography roughness varies
less and corresponds to 0.3 nm for MEK, 0.4 nm for ethanol, 0.9 nm for toluene, and 0.4 nm for DMF.

As the brush consists of only two components, using the phase RMS data obtained from images the block copolymer surfaces after treatments with different solvents may be positioned in order of polystyrene surface faction change. Polystyrene content on the surface, based on the visual interpretation of the images in Figure 5.3, has the following ordering: MEK<ethanol< DMF<toluene.

PEG brush and its comparison with PEG-PAA mixed brush AFM studies are shown in Figure 5.4. Because the melting temperature of poly(ethylene glycol) is 60°C (Ref.26) and the measurements were performed at room temperature (25°C), the PEG brush contains crystalline parts. The amount of the crystallized fraction increases with the increase of grafting density of the polymer chains. In the presence of polyacrylic acid, no crystallization of PEG was observed as these two polymers are miscible and crystallization of PEG is disrupted by the presence of another polymer (Figure 5.4).

The morphology of the different grafting density PEG brushes used in this work is represented in Figure 5.5. AFM RMS roughness profile for the sample shown in Figure 5.5 is represented in Figure 5.6. With the increase of PEG grafting density, the polymer chains are located closer and crystallize more easily. This is observed in Figure 5.5. With the increase of the fraction of crystalline regions on the surface, roughness and RMDS of phase shift also increases (Figure 5.6).
Figure 5.4. AFM topography images. Upper row – PAA (100,000)/PEG (5,000) mixed brush in the next composition (chains/nm\(^2\)/chains/nm\(^2\)): a) – 0.05/0.95, b) – 0.07/0.49, c) – 0.02/0.2. Lower row – PEG (5,000) brush, chains/nm\(^2\): d) – 0.21, e) – 0.49, f) – 1.32. Reprinted with permission from B. Zdyrko.\(^6\)

Figure 5.5. 1x1 um, 5nm/10\(^6\) AFM image of Poly(ethylene glycol) brushes. Upper row – topography images. Lower row – phase images. Numbers show grafting density of the chains (chains/nm\(^2\)).
5.3.2.2. **Morphology studies of the mixed brush:**

**Figures 5.7-5.10** show the morphology of the samples after treatment with toluene, ethanol, MEK, and DMF. All images have vertical scale of 5 nm for the topography image and 10° for the phase image. Changes in morphology after the solvent treatments may be interpreted using the roughness values and RMSD of phase shift of the images. First, there are no visible crystalline zones observed on the images. This is consistent with the fact that PEG and PAA are miscible and form one phase in dry conditions. The presence of PAA chains disrupts PEG crystal formation.

As the amount of PEG decreases, as can be seen from **Figure 5.7-5.10**, polystyrene domains increase in size and less domains appear to be formed on the surface. Polystyrene domains, while being facilitated for formation on the surface by
toluene (Figure 5.7), are still limited in size as polystyrene chains are pinned to the surface and their mobility is restricted by the mobility of polyacrylic chains. With the increase of the grafting density of PEG, the separation between the polystyrene chains increases, so the size of the formations decreases. This trend can be seen in all solvent treated systems. At the same time, proximity of the polystyrene formations to the surface depends on the type of solvent used. Toluene, being a good solvent for polystyrene, extracts polystyrene to the surface so the domains are formed on the surface and are clearly visible on the AFM images. Ethanol (Figure 5.8) is good for hydrogen bonding forming PEG and PAA. But the size of the polystyrene domains is observed to be the same as after DMF treatment since ethanol is not a solvent for polystyrene and ethanol does not have ability to reshape PS. Domains appear to be smaller than in toluene case. DMF treatment results in smooth surface (Figure 5.10).

MEK treatment (Figure 5.9) shows a smoother morphology, probably due to polystyrene domains being covered by PEG and their smaller size. In MEK, PAA is not soluble and polystyrene domains can be formed only from the nearest polystyrene chains. As in other cases, the amount of the domains and their size decrease with the increase of PEG grafting density.

AFM images studies have shown that structures formed during each solvent treatment could be reversibly reformed when DMF was used as an intermediate solvent for bringing the sample into reference conditions.
Figure 5.7. AFM image of dry mixed polymer brush after toluene treatment, 1x1 um. Upper row – topography images, lower row – corresponding phase images. Numbers under the images correspond to chain/nm$^2$ ratio between PEG and PS-b-PAA. RMS roughness for topography images (from the left to the right): 1.1; 0.8; 0.9; 0.7. RMSD of phase shift of images (from the left to the right): 0.8; 0.9; 1.7; 1.1.

Figure 5.8. AFM image of dry mixed polymer brush after ethanol treatment, 1x1 um. Upper row – topography images, lower row – corresponding phase images. Numbers under the images correspond to chain/nm$^2$ ratio between PEG and PS-b-PAA. RMS roughness for topography images (from the left to the right): 1.0; 0.9; 1.0; 0.2. RMSD of phase shift of images (from the left to the right): 1.1; 0.9; 1.0; 1.0.
Figure 5.9. AFM image of dry mixed polymer brush after MEK treatment, 1x1 um. Upper row – topography images, lower row – corresponding phase images. Numbers under the images correspond to chain/nm$^2$ ratio between PEG and PS-b-PAA. RMS roughness for topography images (from the left to the right): 0.9; 0.9; 1.0; 0.8. RMSD phase shift of images (from the left to the right): 2.4; 0.8; 0.8; 0.5.

Figure 5.10. AFM image of dry mixed polymer brush after DMF treatment, 1x1 um. Upper row – topography images, lower row – corresponding phase images. Numbers under the images correspond to chain/nm$^2$ ratio between PEG and PS-b-PAA. RMS roughness for topography images (from the left to the right): 1.1; 0.6; 0.5; 0.4. RMSD shift of phase images (from the left to the right): 2.5; 0.3; 0.3; 0.2.
A specific function of the PEG chains, that favors stretching of the polyacrylic acid chains in case of ethanol treatment, should be noticed. Roughness for the ethanol treated samples stays high enough, indicating the presence of the polystyrene formations on the surface while the bare block copolymer brush treated with the same solvent exhibits low roughness values as can be seen from Figure 5.3. Polyacrylic acid chains supported by the poly(ethylene glycol) chains extend and reveal to the surface polystyrene agglomerates formed during the DMF treatment.

5.3.3. Water contact angle on mixed polymer brush after solvent treatment:

The contact angle of the water droplet on the surface serves as measurement of surface energy. The water contact angle value can also be used to evaluate the presence of the hydrophobic and hydrophilic components on the surface. The measurements were performed on the solvent treated mixed polymer brush samples. Results are represented in Figure 5.11.

The data obtained experimentally do not agree with the values calculated with the following equation for water contact angle for this system (Figure 5.11).

$$\cos(\theta) = \phi_1 \cos(\theta_1) + \phi_2 \cos(\theta_2) + \phi_3 \cos(\theta_3)$$  \hspace{2cm} (E5.5)

where $\theta$ is water contact angle of each individual component of the brush and $\phi$ is the surface area fraction of the respective component of the brush. For the calculation, the surface area fractions were assumed to be the same as the volume fractions of the components in the brush.
The volume fractions were calculated from the Ellipsometry measured thickness. Bulk polystyrene itself exhibits a water contact angle of 90° (Ref.32). PAA is water soluble and will dramatically decrease the contact angle when it is a component of the surface. PEG in bulk has a water contact angle of 30° (Ref.33). Contact angle of each component of the brush depends also on the grafting density of the chains. The grafting density for PEG in Figure 5.11 increases to the right and coincides with the increase of water contact angle of the mixed brush. It can be explained that the PEG brush with

Figure 5.11. Water contact angle on the mixed PEG / PS-b-PAA brush treated with (□)-DMF, (▽) – toluene, (Δ) – MEK, (○) – ethanol. Line on the graph corresponds to calculated contact angle.
higher grafting density causes the PS-b-PAA chains to “extend,” bringing more polystyrene to the surface and increasing water contact angle.

The calculated water contact angle increases with the increase of PEG fraction, but reaches the maximum value of 26° at the studied grafting density of PEG. The predominant presence of PS chains on the surface increases water contact angle. All over the range, the brush treated with toluene exhibits a slightly higher contact angle than the brushes treated with other solvents due to polystyrene extracted to the surface of the polymer brush. DMF treated brush exhibits very low contact angle due to the large amount of polyacrylic acid brought to the surface by the solvent. Ethanol treatment resulted in contact angles slightly lower than those for the toluene treatment despite ethanol being a good solvent for polyacrylic acid and poly(ethylene glycol). This supports the idea that the poly(ethylene glycol) brush causes stretching and aligning of the block copolymer, which brings polystyrene formations to the surface and results in high contact angle. MEK treatment extracts PEG to the surface and results in contact angle slightly higher than that for DMF treatment.

To evaluate the ratio between the PEG and polystyrene chains, that may result in contact angles that are the same as or close to the measured values we have calculated water contact angle as a function of the ratio between the PEG and PS chains. Calculations were done for two component system in the same way as in equation E5.5. The amount of polystyrene was kept constant and equal in the mixed brush and the PEG amount was varied. Results of the calculations are presented in Figure 5.12. To reach the measured values of water contact angle that are close to 45°, the ratio between the
amounts of PEG and polystyrene chains should be equal or close to 0.8 chains/nm$^2$.

According to the calculations, the contact angle decreases as the PEG grafting density increases. In experimentally measured samples the opposite trend is observed: contact angle of the mixed brush increases with the increase of PEG grafted. This may happen only if the dense PEG chains favor stretching of the polyacrylic chains that bring polystyrene to the surface. The more densely grafted the PEG chains are, the more stretched the PAA chains are and the more polystyrene is brought to the surface.

![Figure 5.12](image.png)

**Figure 5.12.** Calculated water contact angle of the bicomponent system of PEG and PS. PS grafting density was kept constant and PEG was varied.

The effect of the presence of poly(ethylene glycol) becomes more prominent when the data presented in **Table 5.5** is analyzed. The table shows wettability of the dense PEG brush, PAA brush, and PAA-PS brush after treatments with different solvents.
Table 5.5. Water contact angle measurements for the polyacrylic acid and polyacrylic acid-b-polystyrene after treatment with selected solvents.

<table>
<thead>
<tr>
<th>Number of chains per nm²</th>
<th>Water contact angle</th>
</tr>
</thead>
<tbody>
<tr>
<td>PEG brush</td>
<td>1.0</td>
</tr>
<tr>
<td>AA brush</td>
<td>0.18</td>
</tr>
<tr>
<td>PAA-b-PS, MEK</td>
<td>0.13</td>
</tr>
<tr>
<td>PAA-b-PS, ethanol</td>
<td>0.13</td>
</tr>
<tr>
<td>PAA-b-PS, toluene</td>
<td>0.13</td>
</tr>
<tr>
<td>PAA-b-PS, DMF</td>
<td>0.13</td>
</tr>
</tbody>
</table>

All solvent treatments result in practically the same contact angle for the block copolymer brush with a slightly lower value for DMF treatment. MEK and toluene are non-solvents for polyacrylic acid, but are good solvents for polystyrene and favor extraction of polystyrene to the surface and formation of the polystyrene domains. Polystyrene on the surface results in a higher contact angle. DMF evidently results in the covering of polystyrene by PAA chains that cause a lower water contact angle. But when PEG chains are present in the system, the brush exhibits a large range of values from 20 to 50° (Figure 5.11). The data in Table 5.5 support the hypothesis that the presence of PEG chains results in the stretch of block copolymer chains and exposure of polystyrene to the surface. It also should be mentioned that measured water contact angle f the PEG brush used in this work did not change with increase of PEG grafting density after brush regime was reached. Hence, methoxy end groups of the PEG do not contribute to the
increase of water contact angle of mixed polymer brush system with increase of PEG grafting density.

5.4. Conclusions:

The studied mixed polymer brushes exhibit a switching behavior and their surface properties can be tuned using solvents such as toluene, MEK, ethanol, and DMF. Switching of the brushes was confirmed by studying the surface morphology with AFM and measurement of the surface contact angle. Roughness of the samples as well as RMSD of their phase shift depends on the solvent used for treatment and on the composition of the brush.

Treatment of the PAA-PS brush with organic solvents resulted in formation of the polystyrene domains of different size and concentration. Based on visual evaluation of the images the following ordering among the size of the domains based on the solvent used for treatment was found: MEK<ethanol<DMF<toluene.

In the mixed brush, the PAA chains formed a complex with PEG and altered crystallization of PEG. No crystalline regions of PEG were observed in the mixed polymer brush even at high grafting density of PEG. At the same time the PEG fraction of crystalline regions increased with the increase of PEG grafting density in homo PEG brush.

Measured water contact angles for the solvent treated mixed polymer brushes were significantly higher than the calculated values, and this discrepancy was explained by uneven distribution of the brush components on the surface after solvent treatments.
Toluene is a good solvent for polystyrene and resulted in the highest water contact angle. The brush treated with DMF and ethanol, good solvents for hydrophilic polyacrylic acid, showed the lowest water contact angle.

While calculations of water contact angle of PEG/PS mixture state that with the increase of PEG amount contact angle should decrease, the opposite behavior was observed. Poly(ethylene glycol) played an exceptional role for the switching behavior of the mixed polymer brush causing additional stretching of the polyacrylic acid chains and bringing polystyrene formations to the surface.

5.5. References:


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CHAPTER SIX
MORPHOLOGY AND EXTENSION OF THE MIXED POLYMER BRUSHES UNDER THE WATER

6.1 Introduction:
Water, like any other solvent, interacts with the mixed polymer brush by interacting with brush components. When these interactions are thermodynamically favorable, swelling of the brush or extension of the polymer chains occurs. In the case of unfavorable interactions, the polymer chains will remain in a non-swollen state. Studies of the extension of end-tethered polymer chains, $L$ (brush height), supported by theoretical modeling, revealed that polymer molecules in the brush regime are disturbed more than in the bulk state, and follow different dependencies for polymer coil size.\(^3\) If water is a thermodynamically good solvent for a polymer, this relationship is expressed by the equation:

$$L/a = N(a/d)^{2/3} \quad (E6.1)$$

where $N$ is the number of statistical segments, $a$ is the diameter of the statistical segment and $d$ is the distance between grafting points.

$L$ at $\theta$ – conditions for the tethered polymer molecules in the brush regime is determined by the next relationship:

$$L/a = N(a/d) \quad (E6.2)$$

The distinct difference between these polymer brushes is the linear dependency of the conformation ($L$) on the number of statistical segments/degree of polymerization.
For polyelectrolytes, the dependency of the polymer brush extension in the water environment is more complicated, as now it depends on the pH of the solution and on its ionic strength. Additionally, interactions between the charged polymer chains are introduced and strongly modified by Coulomb forces.\footnote{4} The charge density of the polymer chain depends on the degree of dissociation of the ionizable groups. If the ionizable groups are strongly acidic or basic, the degree of dissociation is equal to 1 and does not depend on the external environment.\footnote{4,5} These brushes are not sensitive to local pH or salt concentration, until the ionic strength of the solution approaches the level inside the brush.\footnote{5} Weak polyelectrolyte brushes exhibit behavior that is more interesting. They are highly responsive to changes in pH, as well as slight changes in ionic strength.\footnote{6} Overall, three major regimes for ionized brushes are distinguished: osmotic, salted and neutral\footnote{2} (\textbf{Figure 6.1}).

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{fig6_1.png}
\caption{Dependence of brush thickness (H) reduced by number of monomeric units (N) on external salt concentration ($\phi_s$) for strong (solid line) and weak (dashed line) polyelectrolyte brush. Redrawn after Wu et.al.\footnote{2}.}
\end{figure}
At high salt concentrations, the concentration inside and outside the brush is about the same, and interactions are largely screened. In such conditions, an electrolyte brush behaves the same way as a neutral brush (NB regime). With a decrease in the salt concentration, there is an imbalance between the concentrations of ions outside and inside the brush, and system can be classified as in the salted brush regime (SB). Because of electrostatic interactions inside the brush, this brush is more extended than a neutral one. If the salt concentration is further decreased, the co-ions are expelled from the brush and the brush enters an osmotic regime (OB). For strong electrolytes, the brush height is independent of salt concentration in this regime.

However, when a weak electrolyte brush enters an OB regime, a significant electric potential difference develops between the brush and the bulk solution. Additionally, the concentration of $\text{H}^+$ is considerably higher inside the brush. To avoid unfavorable interactions, weakly charged groups discharge themselves. This response is impossible for strong electrolytes. Because of the discharging process, a weak polyelectrolyte brush is less expanded in the OB regime and the dependence of brush height on salt concentration exhibits the maximum.

The following theoretical model predicts a brush height relationship for NB and SB regimes:

$$L \sim N \sigma^{1/3} \left( f^2 / \varphi_s \right)$$

(E6.3)

where $N$ is the degree of polymerization, $\sigma$ is the grafting density, $f$ is the degree of ionization, and $\varphi_s$ is external salt concentration.
For weak polyelectrolytes in the OB regime, this relationship takes the following form:\(^2\)

\[ L \sim N \sigma^{1/3} (f/(1-f))^{1/3} ([H^+] + \varphi_s)^{1/3} \]  \((E6.4)\)

The degree of ionization for strong polyelectrolytes is fixed and equal to 1, while for weak polyelectrolytes, it is determined by the expression:\(^8\)

\[ f = 1/(1 + 10^{pK - pH}) \]  \((E6.5)\)

where \(pK\) is the acid dissociation constant.

The mechanism of the response of the brush height to changes in the environmental conditions is somewhat different for mixed brushes than for homobrushes.\(^4\) Polymers in the mixed brush segregate into nanophases which scale with \(<r>^{1/2}\) (end-to-end distance) value.\(^4\) In selective solvents, mixed brushes may segregate with a lateral or layered mechanism.\(^9\) In the layered mechanism, one polymer preferentially segregates to the top of the brush while the other forms clusters segregated to the bottom. Therefore, the most important characteristic of the mixed brush is that not only height and density profile, but also composition profile, depend on solvent quality.\(^4\)

Polymer brushes in solvent may demonstrate a range of interactions between the polymer chains and surrounding molecules/objects. The following major intermolecular forces/interactions can be distinguished: Coulomb interactions, polar interactions and polarization forces, van der Waals interactions or London forces, hydrogen bonding and hydrophobic interactions\(^10\).

Coulomb forces include interactions between the charged particles (atoms or ions), and act as a physical force between molecules; they are also long range forces (up
to 70 nm distance)\textsuperscript{11}. The screening effect of neighbor ions results in a shorter range than expected.

Polar interactions include molecules that have no net charge but possess an electric dipole with another dipole or charged particle. These interactions are responsible for the so-called solvation zone around the molecule surrounded by solvent\textsuperscript{10}. Polarization forces arise when polar and polarizable molecules come into close proximity. The electric field from the polar molecule induces dipole moments in the non-polar molecule. This temporarily induced dipole interacts with other charged species. The forces are medium dependent.

Oscillation of the electrons around the nucleus at a given time results in the distortion of the electron arrangement around the nucleus. The distortion is sufficient to cause a temporary dipole effect, with an electric field that induces dipoles in surrounding molecules. The result of this induction is attraction between the non-polar or dipolar molecules; this is referred to as London or van der Waals forces. Dispersion attractive forces are always present and proportional to the distance between the species as $1/r^6$. These interactions are long-ranged (0.2-15.0 nm) and may become repulsive at very short distance.\textsuperscript{11} When two interacting molecules are dissolved in the medium, van der Waals forces between them are reduced because of the dielectric screening of the medium.

A hydrogen bond is formed if an H atom covalently bound to an electronegative atom is exposed to another strongly electronegative atom from another molecule. This bond varies in length (0.12-0.32 nm) and energy, and may be classified as strong (14-40 kJ/mol), moderate (4-15 kJ/mol) or weak (<4 kJ/mol).\textsuperscript{11} The perfect example of a
substance with hydrogen bonding is water. The water molecule has a tetrahedral coordination. Each water molecule can form a maximum of four hydrogen bonds with other molecules. When a water molecule is exposed to a non-polar molecule, one or more water molecules lose their connection toward the inner solute molecule and no H-bond is formed. This is unfavorable thermodynamically, and if the molecule is small enough, water packs around it without losing its H-bonding points, forming a cage. The “caged” water molecules are more ordered and their entropy decreases. This unfavorable entropy resulting from the caging of the non-polar molecules provides a driving force toward agglomeration of the non-polar species and the reduction of total area of contact with water molecules. Water’s ability to push hydrophobic molecules together is called the hydrophobic effect. Hydrophobic interactions between the non-polar molecules are much higher in water than in free space, and act in the range of 0-10 nm.\textsuperscript{11}

At the same time, the hydrophilic group is usually polar and capable of H-bonding. This group/molecule prefers to be in contact with water rather than with like groups/molecules.\textsuperscript{11}

Because proteins are predominantly water medium soluble, adsorption of the protein in the water environment is an important subject. The objective of this part of the research was to study the structure of the mixed polymer brush when subjected to the water medium. An additional issue considered in this chapter was studying of the hydrophobic interactions of the mixed polymer brush, as these interactions are not affected by the dielectric constant of the medium.\textsuperscript{10}
6.2. Experimental:

Mixed polymer brushes prepared as described in Experimental, Chapter 4 and treated with organic solvents – DMF, toluene, ethanol and MEK – were subjected to the following studies. The pH of the environment was adjusted with a solution of 0.1M HCl or KOH in Millipore water (initial pH of water was 5.3). Different ionic strength solutions of the calcium chloride and sodium chloride were prepared using the appropriate amounts of salt and Millipore water. The ionic strength of the solution was calculated according to the formula:

\[ I = \sum_{i=1}^{n} c_i z_i \]  

(E6.6)

where \( c_i \) is the molar concentration of the ion, \( z \) is the ion charge.

Null Ellipsometry was used to measure the thickness of the polymer brush in the aqueous environment. The measurements were carried out with a computer-controlled null ellipsometer in a vertical polarizer-compensator-sample analyzer (PCSA). A He-Ne laser (\( \lambda = 632.8 \) nm) was used as a light source. The angle of incidence was fixed at 70°. For the data interpretation, a multilayer model of the coating was assumed. This model considers the coating to be a sandwich-like structure with the following layers: Si wafer with a top silica layer, PGMA layer, and a grafted polymer layers. In this layer model, the substrate is characterized by means of Ellipsometry (the thickness of the SiO\(_2\) layer was measured for every specimen, as well as the PGMA layer).

Two parameters of the grafted polymer layer, refractive index \( n_1 \) and thickness \( d_1 \), can be obtained from the following relationship:
\[ e^{i \Delta} \tan \psi = \frac{R_p}{R_s} = F(n_k, d_k, \lambda, \phi) \]  \hspace{1cm} (E6.7)

where \( R_p \) and \( R_s \) represent the overall reflection coefficients for the basis p- and s-waves. They are a function of \( n_k \) and \( d_k \), which are the refractive indices and thickness of each layer, respectively. \( \lambda \) is the wavelength and \( \phi \) is the incident angle. Measurements of a pair of the Ellipsometric angles \( \Delta \) and \( \psi \) allow for the evaluation of the abovementioned two unknown parameters.\(^\text{12}\)

XPS measurements were performed using a "Kratos" setup with a spherical multichannel analyzer and Mg Ka radiation (maximum at \( E = 1253.6 \) eV).

The surface morphology was investigated with AFM (Nanoscope IIIa-Multimode in a tapping mode). Adhesion measurements were taken using an Si$_3$N$_4$ cantilever (0.43 N/m typical spring constant), probe NP type from Veeco, NY. Glass beads (radius 6-7um) from Potters Industries Inc. Valley Forge, PA were used. Beads modified with (heptadecafluoro-1,1,2,2-tetrahydrodecyl)dimethylchlorosilane were obtained from Gelest. Modified glass beads were glued to AFM tips with high strength “2 Ton Epoxy” epoxy glue with curing time of 30 min from Devcon.

### 6.3. Results and discussion:

#### 6.3.1. AFM studies of the mixed polymer brushes treated with different solvents in the water medium:

Two of the three constituents of the mixed polymer brush are water soluble. These are poly(ethylene glycol) and polyacrylic acid. Polystyrene agglomerates formed during the solvent treatment are not affected by the presence of water, so their shape and
size will not change under the influence of water. At the same time, any complexes created between PEG and PAA will be destroyed and, due to solubility of the components in water, the extension of polymer chains grafted to the surface will occur.

**Figure 6.2** represents the results of “under water” AFM scanning of the mixed polymer brushes treated with a number of solvents.

![AFM images of mixed polymer brush taken under water, 1x1 µm for (a), 2x2 µm (b, c and d). Upper row – topography images, vertical scale 15 nm. Lower row – phase images, vertical scale 5°. a)-DMF treated sample (roughness 0.3/1.7 for topography/phase image), b) – MEK treated sample (roughness 1.8/0.5 for topography/phase image), c)-ethanol treated sample (roughness 1.9/0.5 for topography/phase image), d)-toluene treated sample (roughness 1.2/0.4 for topography/phase image).](image-url)
The grafted chain densities for the MEK, ethanol, toluene and DMF treated samples were 0.63, 0.63, 0.53 and 0.66 chains/nm$^2$ for PEG respectively, and 0.19, 0.24, 0.17 and 0.21 chains/nm$^2$ for the block copolymer.

In the aqueous medium, water-soluble polyacrylic acid and poly(ethylene glycol) brushes have very low stiffness and flex easily under the AFM tip. These chains are observed as one phase. At the same time, non water-soluble hard polystyrene spheres are clearly seen on the images shown in Figure 6.2.

The highest roughness in the underwater images can be seen in the ethanol and MEK treated samples. The DMF sample shows almost no variations in height. Nevertheless, the RMSD of the phase shift of the image shows the opposite picture – it is low and very similar for ethanol, toluene and MEK, while it is very high for DMF. Such a uniform topographical image with the high RMSD of the phase shift may be evidence of the presence of a large amount of uniformly lifted polystyrene spheres on the interface between the water and the brush. Images of the brushes after treatment with other solvents, with higher topography roughness and low RMSD of the phase shift serves as additional evidence of the changes in morphology initiated by solvent treatment.

**6.3.2. Calculations of the size and shape of the polystyrene domains formed during the solvent treatment:**

Polystyrene plays a very important role in the development of the hydrophobic interactions of the mixed polymer brush. As was mentioned above, hydrophobic interactions do not depend on the pH or ionic strength of the solution, and develop
between the non-polar component of the brush and the non-polar external species\(^{10}\). The size of the polystyrene domains formed after solvent treatment, and their representation on the surface, may affect the extent of these interactions.

We have used AFM images (Figure 6.2) to calculate the size and number of the polystyrene spheres. The data were obtained by manual calculation of the domain population in a 500x500nm part of the image; the size of the spheres was calculated using Nanoscope III and WS&M software. The average radius of the polystyrene domain was calculated using a population of 20 randomly selected domains for each solvent used. An example of the images used for calculation after modification with WS&M software (radius of the tip was intentionally set to 100nm to increase the size of the inclusions on the surface) is shown in Figure 6.3.

![Figure 6.3](image)

**Figure 6.3.** Example of the image processed with the WS&M software. 500x500 nm sized image of the toluene treated mixed brush.

One should also be aware that the dimensions of the features measured by AFM differ from the actual dimensions because of the tip shape. Knowing the AFM tip radius,
we can recalculate the real dimensions of the objects on the surface. A tip with radius of 20 nm was used in our studies, and all measurements were recalculated and adjusted to reflect real distance values according to formula:

\[ R_{adj} = R - (2T*h - (h)^2)^{0.5} \]  \hspace{1cm} (E6.8)

where \( R_{adj} \) is the adjusted radius; \( R \) is the radius of the domains measured by AFM; \( T \) is the tip radius; and \( h \) is the height of the domains measured from the image (they were measured at 2.6\( \pm \)0.3nm for the toluene treated sample, 3.6\( \pm \)0.8nm for MEK, 5.3\( \pm \)0.8nm for DMF and 4.1\( \pm \)0.9nm) for ethanol. The results of calculating the number and size of polystyrene domains formed after treatment of the mixed polymer brush with organic solvents are presented in Table 6.1.

The polystyrene spheres so visible on AFM images in (Figure 6.2) are formed during the deposition (from ethanol) of the block copolymer for grafting and reformed during treatment of the mixed brush with the solvents. The block copolymer dissolves due to the polyacrylic acid solubility in ethanol. Polystyrene blocks are not soluble, so in the solution they aggregate to minimize the unfavorable interaction with the solvent and forms micelles.\(^{13,14}\) When deposited onto the surface and dried, block copolymer micelles formed in solution do not disintegrate, and grafting of the block copolymer is performed from the micellar structure. An evaluation of the extent of “reforming” of the polystyrene micelles/domains due to solvent treatment and the evaluation of the possible sizes and geometry of the domains at the moment of deposition onto the PEG brush may give us an understanding of the morphology formation process.
Table 6.1. Calculation for the polystyrene domains in mixed polymer brush treated with solvents of choice.

<table>
<thead>
<tr>
<th>Number of row</th>
<th>Parameter</th>
<th>Ethanol</th>
<th>TOL</th>
<th>MEK</th>
<th>DMF</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td># of domains per micron$^2$</td>
<td>968</td>
<td>1149</td>
<td>1194</td>
<td>1017</td>
</tr>
<tr>
<td>2</td>
<td>Chains/ micron$^2$ (by ellipsometry)</td>
<td>190,000</td>
<td>170,000</td>
<td>240,000</td>
<td>240,000</td>
</tr>
<tr>
<td>3</td>
<td>PS chains/ per domain</td>
<td>196</td>
<td>147</td>
<td>201</td>
<td>236</td>
</tr>
<tr>
<td>4</td>
<td>Volume of domain calculated using data, nm$^3$</td>
<td>307.8</td>
<td>231.2</td>
<td>315.2</td>
<td>370.0</td>
</tr>
<tr>
<td>5</td>
<td>Radii of domain, nm</td>
<td>4.2</td>
<td>3.8</td>
<td>4.3</td>
<td>4.4</td>
</tr>
<tr>
<td>6</td>
<td>Radii of domain, by AFM, nm</td>
<td>23.6+2.5</td>
<td>17.8+2.3</td>
<td>15.4+1.3</td>
<td>22.3+2.3</td>
</tr>
<tr>
<td>7</td>
<td>Adjusted radii, nm (tip r=20nm)</td>
<td>15.5</td>
<td>10.9</td>
<td>7.3</td>
<td>13.8</td>
</tr>
</tbody>
</table>
Radii of PS in the micelle that can be formed during phase separation in the bulk of the block copolymer are calculated using the formula\(^\text{15}\) (see Figure 6.4):

\[
R = 1.33\alpha KM^{0.5}
\]  
(E6.9)

where, \(\alpha\) measures the chain perturbation existing in the block copolymer morphology. The value of \(\alpha\) varies between 1 and 1.5. A value of 1.25 is selected for the estimations. \(K\) represents the ratio of the unperturbed root-mean-square end-to-end distance to the square root of molecular weight, \(K=0.067\). \(M\) is the molecular mass of the polymer (g/mole). \(M=1,000\)g/mole.

For a block copolymer with a polystyrene block molecular weight equal to 1,000 g/mole, the radius of the domain sphere employing formula (E6.9) is determined to be 3.52 nm. The volume of such a sphere is equal to 183 nm\(^3\).

The density of the bulk polystyrene is 1.05g/cm\(^3\) (see Experimental, Chapter 4), and the molecular weight of one mer is 104g/mole. The number of mers per cm\(^3\) is

\[
(1.05\text{g/cm}^3/104\text{g/mole})\times6.02\times10^{23}\text{mers/mole}=6.07\times10^{21}\text{mers/cm}^3
\]  
(E6.10)

yielding 1.63x10\(^{-1}\) nm\(^3\)/mer.

The number of mers in the domain is as follows:

\[
183\text{ nm}^3/1.63\times10^{-1}\text{ nm}^3/\text{mer}=1123
\]  
(E6.11)

For polystyrene \(M_n=1,000\)g/mole, the number of mers per chain is:

\[
1,000\text{ g/mole}/104\text{ g/mole}=9.62
\]  
(E6.12)

For polystyrene \(M_n=1,000\)g/mole, the number of chains/domain is:

\[
1,123/9.62=117
\]  
(E6.13)

For polystyrene \(M_n=1,000\)g/mole, the volume of 1 chain:
As result of the calculations, the data necessary for the evaluation of the polystyrene domain size were obtained, including: the volume of one polystyrene chain with Mn=1,000 g/mole – 1.6nm$^3$; the volume of one mer of polystyrene – 1.63x10$^{-1}$nm$^3$/mer; the size of one polystyrene micelle in bulk – 183.0nm$^2$; the number of chains per micelle/domain – 117.

For the calculation of the domains formed in the polymer brushes, data from Ellipsometry thickness measurements (the refractive index was assumed to be 1.5 for block copolymers) has been used. This led us to calculate the number of block copolymer chains per square micrometer.

Dividing the number of chains per square micrometer of the brush (Table 6.1, row 2) by the number of domains in the same area (Table 6.1, row 1) results in the number of polystyrene chains per domain (Table 6.1, row 3).

Knowing the number of polystyrene chains per domain and the volume of one chain, the volume of the domains as well as the domain radii (for the spherical shape) were calculated (Table 6.1, rows 4 and 5).

Knowing from AFM imaging that the lateral shape of the polystyrene domains is circular (Figure 6.3), a model for the possible polystyrene domain shapes has been calculated. Next, the possible shapes that fit this requirement were checked: sphere, cylinder and ellipsoid. The shape of the domains formed during the solvent treatment will depend on numerous factors, such as the solvent’s quality in relation to polystyrene, the volume percentage of polystyrene in the block copolymer, and the accessibility of the
neighboring polystyrene chains. The shape and dimensions of the domains are essential for the formation of hydrophobic interactions between the mixed brush and the external environment. These shapes were evaluated using measured domain volumes and radii. Based on this information, the vertical dimension (thickness) of the formation was calculated to determine which of the polystyrene shapes could be present in a mixed polymer brush after solvent treatment.

Taking volume of the polystyrene per cubic micrometer and number of the domains calculated (Table 6.1, row 1), domains of spherical shape for the mixed polymer brush treated with different solvents result in calculated radii of the spheres from 3.8 to 4.4 nm (Table 6.1, row 5).

![Parameters of the domain formed in block copolymer bulk: R =3.5 nm, V =183 nm$^3$, n=116.7 (chains per domain)](image)

Figure 6.4. Representation of the polystyrene Mn=1000 g/mole spherical formations. Data on the right correspond to dimensions of spherical polystyrene domains formed in the block copolymer when deposited onto substrate for grafting.

From the comparison of the data in Table 6.2, may be confidently stated that polystyrene domains cannot be spherical after solvent treatment. The measured radii were much larger than calculated, and reached up to 15 nm, so that there were not enough
polystyrene chains in the brush to form spheres with such a radius in the calculated number of domains.

**Table 6.2.** Radii of the imaginary spherical domains calculated using domain volume.

<table>
<thead>
<tr>
<th></th>
<th>ETOH</th>
<th>TOL</th>
<th>MEK</th>
<th>DMF</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>R</strong> in nm calculated, (Table 6.1, row 5)</td>
<td>4.2</td>
<td>3.8</td>
<td>4.3</td>
<td>4.5</td>
</tr>
<tr>
<td><strong>R</strong> adjusted in nm, measured, (Table 6.1, row 7)</td>
<td>15.4</td>
<td>10.9</td>
<td>7.3</td>
<td>13.7</td>
</tr>
</tbody>
</table>

**Figure 6.5.** Representation of the polystyrene domains formed after solvent treatment in form of cylinders.

**Figure 6.5.** Representation of the polystyrene domains formed after solvent treatment in form of cylinders. The next possible representative of the considered domain shape was cylinder (**Figure 6.5**). The height of the cylinders would be 4-18 Å. **Table 6.3** shows calculations of the cylinder height resulting from direct AFM measurements, as well as height adjusted to the AFM tip radius. The volumes and radii
of the formations from Table 6.1 were used for the calculation of the h-dimension using the formula (E6.15):

\[ h = \frac{V}{\pi R^2} \]  \hspace{1cm} (E6.15)

Table 6.3. Height of the imaginary cylindrical polystyrene domains in mixed polymer brush after different solvent treatment.

<table>
<thead>
<tr>
<th>h in nm</th>
<th>ETOH</th>
<th>TOL</th>
<th>MEK</th>
<th>DMF</th>
</tr>
</thead>
<tbody>
<tr>
<td>h_{AFM}</td>
<td>0.2</td>
<td>0.2</td>
<td>0.4</td>
<td>0.2</td>
</tr>
<tr>
<td>h_{adjusted}</td>
<td>0.4</td>
<td>0.6</td>
<td>1.9</td>
<td>0.6</td>
</tr>
</tbody>
</table>

The cylindrical shape of the domains is more probable, and gives a reasonable height for the domains. However, cylindrical domains have sharp edges and a smaller volume to surface area ratio than circular shapes.

The next possible shape for the formed agglomerates, with one of the dimensions being circular, was ellipsoid. It was assumed two of the three circular dimensions of the ellipsoid to be equal (R_1=R_2=R) (Figure 2.4). The third circular dimension was calculated using the following formula (E6.16):

\[ h = \frac{3V}{4\pi R} \]  \hspace{1cm} (E6.16)

Results of the calculations are shown in Table 6.4. The height of the ellipsoids varies between 0.6nm and 2.8nm.
Figure 6.6. Representation of the polystyrene domains formed in the mixed polymer brush after different solvent treatment.

Table 6.4. Height of the imaginary ellipsoidal polystyrene domains in mixed polymer brush after different solvent treatment.

<table>
<thead>
<tr>
<th>h in nm</th>
<th>ETOH</th>
<th>TOL</th>
<th>MEK</th>
<th>DMF</th>
</tr>
</thead>
<tbody>
<tr>
<td>h AFM</td>
<td>0.1</td>
<td>0.2</td>
<td>0.3</td>
<td>0.2</td>
</tr>
<tr>
<td>h adjusted</td>
<td>0.6</td>
<td>0.9</td>
<td>2.8</td>
<td>0.9</td>
</tr>
</tbody>
</table>

Ellipsoidal and cylindrical shapes are considered the most probable shapes of the polystyrene domains in the mixed polymer brush. However, when considered the conditions in which the domains were created, the spherical shape is most probable. In a good solvent, polystyrene chains gain mobility and may come close to each other and form clusters. With the removal of the solvent, a thermodynamically unfavorable environment surrounds the polystyrene clusters. Therefore, during solvent evaporation, the polystyrene domains will try to reduce their contact area with environment. The shapes with largest volume/area ratios are spherical and ellipsoidal shapes. Because it is already known that the spherical shape is not possible for our case, the ellipsoidal shape
is the next most favorable geometrical shape in reducing contact area between the polystyrene and the unfavorable environment.

In conclusion, it is suggested that the mixed polymer brush morphology is that represented in Figure 6.7.

![Figure 6.7. Representation of the mixed brush morphology in water.](image)

**Table 6.5** summarizes the data for the proposed architecture of the polystyrene domains after different solvent treatments, and includes data extracted from the AFM images and the calculated dimensions of the domains.
The preliminary treatment of the mixed brush with solvents results in different dimensions of the polystyrene domains, and the properties of the water environment will govern the position of the polystyrene cluster in the vertical dimension of the brush.

### 6.3.3. Measurement of the extension of the mixed polymer brushes at different pH.

**Ellipsometry and AFM measurements:**

To evaluate the extension of the mixed polymer brushes, Ellipsometry and the AFM scratch method was used. These measurements bring results that may differ in terms of absolute value, but should complement each other. Ellipsometry measures the phase shift and amplitude ratio upon reflection of polarized light at a certain incident angle. Then, because the refractive indices of the swollen polymer brush components are

---

**Table 6.5.** Data for the polystyrene domains formed after organic solvent treatment.

<table>
<thead>
<tr>
<th></th>
<th>Ethanol</th>
<th>Toluene</th>
<th>MEK</th>
<th>DMF</th>
</tr>
</thead>
<tbody>
<tr>
<td># of domains per micron$^2$</td>
<td>968</td>
<td>1149</td>
<td>1194</td>
<td>1017</td>
</tr>
<tr>
<td>Calculated volume of the domain, nm$^3$</td>
<td>307.8</td>
<td>231.2</td>
<td>315.2</td>
<td>370.0</td>
</tr>
<tr>
<td>Radii of the domain (adjusted), nm</td>
<td>15.5</td>
<td>10.9</td>
<td>7.3</td>
<td>13.8</td>
</tr>
<tr>
<td>Height of the ellipsoid, nm</td>
<td>0.6</td>
<td>0.9</td>
<td>2.8</td>
<td>0.9</td>
</tr>
</tbody>
</table>
unknown, this is determined by fitting the results with the developed mathematical models; finally, the thickness of the measured layer is obtained. Layers of the system that swell in water contribute most to the calculated brush height; in our case, these are PEG and PAA. Because the obtained results contain a number of assumptions, the results have a certain margin of error and should not be taken as absolutely accurate. Nonetheless, the ellipsometrical results should show the trend of the changes of brush height with environment change.

In AFM measurements, the results are direct and do not need any mathematical fitting. When an experiment is performed in contact mode in aqueous media, a response is given when the AFM tip interacts with a solid surface. The water-soluble polymer chains, PEG and PAA, are flexible and cannot readily be detected by the AFM tip when approached. Non-soluble polystyrene chains are segregated in the water medium and give a response when hit by the AFM tip.

Hence, the AFM measurements of the brush under water give the absolute value for height at which the polystyrene domains are located. Softer water-soluble chains are not detected. Ellipsometric measurement of the brush height under water includes all of the polymers that constitute the brush, but is not absolute, as the refractive index of the swollen polymer chains is unknown and is calculated using mathematical model fitting.
6.3.3.1. Theoretical evaluation of brush extension in the aqueous media:

Both PEG and PAA are water soluble, and their chains will extend in an aqueous environment\textsuperscript{17}. Thus, the brush height of PEG in water is governed by the equation of brush height in a good solvent (\textit{E6.1})\textsuperscript{3}. The sample brush height in $\theta$-solvent is governed by equation (\textit{E6.2})\textsuperscript{3}. The results of the PEG brush (M=5,000 g/mole) extension in good and $\theta$-solvents are shown in \textbf{Figure 6.8}.

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{image.png}
\caption{Extension of the PEG brush with degree of polymerization N=114 monomeric units in good solvent and in $\theta$-solvent vs. brush grafting density. Calculated with (\textit{E6.1}) and (\textit{E6.2}).}
\end{figure}

As a statistical segment for PEG, its monomeric unit was taken. The size of the monomeric unit was calculated from the expression:

$$V = M/(\rho N_a)$$  \hspace{1cm} (\textit{E6.17})
where $V$ is the volume of the monomeric unit ($V = \frac{1}{6}\pi D^3$); $\rho$ is the density of PEG, and $N_a$ is the Avogadro number.

The diameter $D$ of the monomeric unit of PEG was found to be 0.51nm; this was used as the size of the monomeric unit in calculations.

**Figure 6.9:** Topography images of a) dry; b) wet PEG (5,000) brush, 1.56 chains/nm$^2$ Vertical scale – 20 nm. Scratched PEG brush topography images. Vertical scale – 50 nm; c) dry brush; d) wet brush. Reprinted with permission from B. Zdyrko.$^1$

AFM scratch measurements of the dense PEG brush (1.56 chains/nm$^2$) revealed an increase in the brush height from 11.5nm in a dry state to 39nm in water (Figure 6.9).
Calculation predicts the extension for a PEG brush in good solvent with a grafting density of 1.56 chains/nm$^2$ to be 39.7 nm. This matches well with the height of the brush measured by AFM (39 nm).

Figure 6.10. Extension of the pseudotails of polyacrylic acid vs. pseudotail size in good solvent, θ-solvent and in charged state at pH 5.8. Calculated with (E6.4).

Extension of the polyacrylic acid in good and θ-solvents is also governed by equations (E6.1) and (E6.2), but only in solvents where polyacrylic acid is present in a non-ionized form. Charged weak polyelectrolytes in the OB regime are governed by equation (E6.4)$^2$. Figure 6.10 represents the calculated brush height of the polyacrylic acid pseudotails depending on the size of the pseudotails. The pseudotail grafting density for the present calculations was estimated as (375/tail size)*0.2 pseudotails/nm$^2$, where, 375 is the degree of polymerization of the PAA used in this study and 0.2chains/nm$^2$ is the grafting density of the block copolymer. The monomeric unit’s size was found to be
0.6nm in diameter (see Chapter 3). Additional data necessary for the calculations were obtained from the experimental data: the proportionality coefficient was 1.1339 and the ionization coefficient for PAA was 0.5 for pH 5.8 and a salt concentration of 0.1M.

The results of the calculation show that for the highest PEG grafting density achieved in these studies (1.2 chains/nm²), for the PAA pseudotails to be the same height as the PEG brush in good solvent, they have to consist of 80 monomeric units, and 65 units for the ionized brush at pH 5.8 and a salt concentration of 0.1M.

Because the PAA is a weak polyelectrolyte, its extension depends on the degree of ionization, which also varies with pH. Figure 6.11 represents changes in the ionization coefficient of PAA related to changes in pH. The ionization of the polyelectrolyte can be roughly calculated with the expression (E6.5); this includes the dissociation constant pKₐ, which was taken to be 4.6 (Ref.18), and the pH of the solution.

![Figure 6.11](image)

**Figure 6.11.** Degree of ionization of PAA vs. pH. Calculated using equation (E6.5).
The real values of the degree of ionization differ slightly from the one shown in Figure 6.11, and depend on the grafting density of the polymer, and the ionic strength of the solution.\(^{19}\)

### 6.3.3.2. Ellipsometric extension measurements:

Because mixed polymer brushes are designed to be effective for protein adsorption in different conditions, studies of the brush and its behavior for a whole range of pH values were conducted. Two types of measurements were performed. First, the thickness of the mixed polymer brush was measured using Ellipsometry in water cells (measurements were taken in Prof. Kilbey’s group, Clemson University). Second, an AFM scratch test was performed for direct measurements of brush height (measurements were taken by R. Lupitskyy in Prof. Minko’s group, Clarkson University, NY). Results of the Ellipsometric studies are shown in Figure 6.12.

As can be seen from Figure 6.12, mixed brushes treated with different solvents extend depending on pH and the solvent used for treatment. As should be expected, the extension of the brush increases when pH increases. This happens because of ionization and the stretching of the polyacrylic acid chains. The pK\(_a\) value of the polyacrylic acid is about 4.6 (Ref.18), and a change in the graph slope is detected for all graphs at this point. Increasing the pH results in higher ionization of the polyacrylic acid and higher stretching of the polymer chains. At a pH value close to 10, saturation occurs and there is no more increase in thickness, as all of the functional groups are ionized. Below pK\(_a\) at pH values between 2 and 4 in the medium, no significant changes in brush height are detected –
polyacrylic acid exists in the form of the non-ionized molecule, and there is no source for increasing the brush height with pH change.

It should be also noticed that there is dependency between the type of solvent used for brush treatment and the extension of the brush. The MEK treated sample exhibits the highest value of extension, while the lowest value results from the ethanol treatment. DMF and toluene occupy intermediate positions between these two. This difference can be explained in terms of the arresting of the polyacrylic acid stretching by the polystyrene domains. When looking at Table 6.1, it may be noticed that MEK treatment results in the formation of the largest amount of domains, while ethanol results in the smallest, meaning that the domain size is largest for ethanol and smallest for MEK.

Figure 6.12. Response of the mixed brush to the pH change. Ratio between initial dry thickness and thickness of the brush after pH change. A) – Thickness of the brush reduced by dry thickness. B) – actual brush thickness. Brush composition PEG/PAA-PS, nm/nm: DMF, 4.2/9.2; Ethanol, 4.5/8.7; MEK, 5.0/8.8; Toluene, 4.6/8.9.

It should be also noticed that there is dependency between the type of solvent used for brush treatment and the extension of the brush. The MEK treated sample exhibits the highest value of extension, while the lowest value results from the ethanol treatment. DMF and toluene occupy intermediate positions between these two. This difference can be explained in terms of the arresting of the polyacrylic acid stretching by the polystyrene domains. When looking at Table 6.1, it may be noticed that MEK treatment results in the formation of the largest amount of domains, while ethanol results in the smallest, meaning that the domain size is largest for ethanol and smallest for MEK.
To form large domains, it is necessary to engage higher numbers of polyacrylic acid-b-polystyrene chains, which are partially arrested after a domain has been formed. When subjected to a high pH medium, the stretching of the polyacrylic acid is again partially arrested, resulting in lower brush height. The opposite occurs in the case of MEK treatment. This solvent results in the formation of the smallest domains. The extension of the chains is not so obstructed by the polystyrene domains, and the brush has greater height gain. DMF and toluene treated samples follow the same trend, and take intermediate positions with toluene having smaller domains and a higher extension of the brush.

6.3.3.3. AFM extension studies:

6.3.3.3.1. Block copolymer studies:

First, examination of the extension of the polyacrylic acid-b-polystyrene copolymer – component of the mixed polymer brush at different pH levels has been done. A block copolymer brush was prepared on a silicon wafer and then a thin strip of the polymer layer was removed by scratching with a fine needle. The brush sample was placed into a cell and the medium was exchanged starting from a low pH. The height of the brush was measured as a step-height between the scratch and brush levels. Results of the measurements are shown in Figure 6.13.

The measured change of the height of the brush with the change in pH is schematically represented in Figure 6.14. At low pH levels, PAA chains collapse and the brush height is close to that in the dry state. With an increase of the pH of the
solution, PAA is gradually ionized and at pH=pK_a, 50% of the groups are in an ionized state. The PAA chains extend, lifting the polystyrene domains to the surface. At high pH, PAA is fully ionized and maximally stretched. PAA chains start to bend and cover the polystyrene domains as PAA chains are linked together by polystyrene, and the domain cannot extend more than shortest PAA chain that links the domain to the surface.

![Figure 6.13](image)

**Figure 6.13.** pH dependency of the polyacrylic acid – b – polystyrene height. AFM scratch measurements of brush treated with DMF. Dry thickness of the brush 6.4nm.

As can be can see from the scratch test, block copolymer brush height increases with an increase in the pH of the medium (**Figure 6.13**) due to acrylic acid ionization. The brush behavior is similar to that of the mixed polymer brush (**Figure 6.15**), and supports Ellipsometric extension measurements (**Figure 6.12**). From these, we can conclude that the extension of the mixed polymer brush at different pH levels is predominantly predetermined by the extension of the block copolymer of polyacrylic acid-b-polystyrene.
Using the calculated data and the results of the block copolymer brush extension measurements, the pseudotail size of the polyacrylic acid in the block copolymer brush can be estimated. At pH 5.8, the mixed polymer brush reaches 23 nm in height (Figure 6.13). Assuming that the extension of the brush is predetermined by the extension of the polyacrylic acid, from Figure 6.10, pseudotail size for a polyelectrolyte consists of 55 monomeric units \( (p=0.023, \text{Figure 4.9}) \).

**6.3.3.3.2. Mixed brush extension studies:**

At the same time, the trend of the extension is different for the AFM measured sample of the mixed brush and the sample measured by ellipsometry (Figure 6.15 and Figure 6.12). This difference arises from the methods of the measurements. Ellipsometry is not a direct measurement, and measures the extension of all the layers of the brush. AFM shows the position of the solid substrate (polystyrene domains in our case). The difference in the trend of the position change of the polystyrene domains inside the mixed polymer brush can be explained by the influence of PEG chains in the system.
The pseudotail size of the polyacrylic acid in the mixed polymer brush may differ from that in the block copolymer brush due to the mixed brush preparation method. PEG present during the block copolymer grafting distorts the block copolymer coil and favors the formation of longer pseudotails. At pH 5.8, the mixed polymer brush reaches ~29nm in height (Figure 6.15). Assuming that the extension of the brush is predetermined by the extension of the polyacrylic acid, from Figure 6.9, the pseudotail size for polyelectrolyte consists of 66 monomeric units (p=0.022, Figure 4.10). This is 10 monomeric units longer than in the block copolymer brush. From Figure 6.2, it is observed that polystyrene spheres were exposed to the surface of the brush, so the height of the PAA pseudotails in the water was comparable to the height of the PEG chains. For a PEG brush to reach a height of 23nm in water, the PEG grafting density has to equal 0.3chains/nm² (Figure 6.8). The maximum grafting density of PEG used in this work.
was 1.2chains/nm² (extension in water - 36nm, **Figure 6.8**). For PAA pseudotails to be of the same height, they need to be 75 monomeric units high (p=0.02, **Figure 4.10**).

Ellipsometric measurement data (**Figure 6.12**) show the ratio of the extended height/dry thickness of the brush to be 1.65 for a mixed polymer brush at pH 9.5. The AFM scratch data coefficient of extension at pH 9.5 is 2.5. This difference in measured extensions originates from above-mentioned fact that Ellipsometry requires the refractive index for thickness calculation, and this index is determined using a mathematical model, while AFM offers direct measurements of the height.

### 6.3.3.4. IR Ellipsometry measurements:

To complete this study of mixed polymer brush behavior in different pH conditions, IR Ellipsometry measurements were taken under water. In-situ IR Ellipsometry studies were conducted in Germany, ISAS, Berlin by Dennis Aulich in Dr. Hinrich’s group. The wavelength of carbonyl adsorption is easy to monitor due to its high intensity. This wavelength was essential in these in-situ studies of the brush behavior at different pH levels. The results can be seen in **Figure 6.16**.
Figure 6.16. IR Ellipsometry measurements of the a) – polyacrylic acid, b) – block copolymer, c) - mixed polymer brush.
In Figure 6.16, the high intensity signal at 1725-1730 cm\(^{-1}\) is attributed to the carbonyl stretch band. Because of a strong water signal in the wavelength range, it is difficult to obtain results from the bare spectrum. The spectrum at pH 6 was used as a reference. The ratio between \(\tan \psi\) for the reference spectrum and \(\tan \psi\) for the studied spectra are efficient in evaluating the measurement results. With an increase in pH, the peak in spectrum disappears, and appears again as a strong signal at pH 9. This band, which has shifted to 1560 cm\(^{-1}\), belongs to the carboxylate asymmetric stretch. The mixed polymer brush exhibits a response to the pH of the medium that is to that of polyacrylic acid. The results obtained underline the previous measurements of the extension of the mixed polymer brush. We can conclude from the IR Ellipsometry measurements that the clear transformation of the carbonyl to carboxylate ions, with the ionization constant being close to 1, happens at pH levels above 8.

**6.3.3.5. Treatment of the brush with bi-valent Ca\(^{2+}\) ion:**

The properties of the mixed polymer brush containing a polyelectrolyte component may also be changed using different ions in the medium. Metal ions may react with the carboxylic groups of the PAA and affect overall properties of the brush. To observe these properties, mixed polymer brush was treated with a CaCl\(_2\) solution, expecting the calcium ions to react with the polyacrylic acid and affect the total mixed brush properties. The prepared polymer brushes were treated with DMF to bring about the reference conditions. Then, the brushes were treated with a 1% water solution of CaCl\(_2\), after one of the brushes was treated with a 2% water solution of HCl. Samples
were rinsed with deionized water and dried. AFM images of the surfaces obtained are shown in Figure 6.17.

![AFM images](image)

**Figure 6.17.** 1x1 um AFM image of mixed polymer brush. 1)– HCl treated assembly. 2)- CaCl treated assembly. 1a,2a – topology images. Vertical scale – 5nm. 1b, 2b – phase images. Vertical scale - 10^0. Number on the image corresponds to roughness of the surface.

Surfaces treated with calcium chloride exhibited lower topography and phase roughness, while the roughness of the surface treated with hydrochloric acid was much greater. The increased roughness of the HCl treated samples could result from the presence of polystyrene blocks on the surface, whereas, when they were hidden in miscible PAA-PEG, no increase in roughness was observed. Polystyrene blocks may be trapped and arrested inside a polyacrylic acid net crosslinked with calcium ions.

To investigate the behavior of the brush under the influence of the bivalent calcium ions further, we conducted AFM measurements of the component of the mixed
polymer brush – block copolymer polyacrylic acid-b-polystyrene. Studies were done using the previously described scratch test. The results are shown in Figure 6.18.

![Figure 6.18](image-url)

**Figure 6.18.** Extension of the PAA-PS brush height at different ionic strength in presence of bivalent calcium ion. AFM scratch test. Dry brush thickness 6.3nm.

As can be seen from Figure 6.18, an increase in the ionic strength increases the extension of polyacrylic chains. When the concentration of the electrolyte reached approximately 1M, the extension of the brush reached its peak, and then brush height decreased. The increase in the thickness of the polymer brush is explained by the calcium ions reacting with the polyacrylic acid chains and being retained inside the brush. When the concentration of the ions increases, polyacrylic acid negative charges are screened and the osmotic pressure that was previously driving extension decreases. These factors cause the decrease in the brush extension.⁶
To determine whether the extension of the polyacrylic acid block is the main reason for the extension of the mixed brush, the behavior of the poly(ethylene glycol) chains in the presence of the calcium chloride electrolyte with different ionic strengths was studied. Studies were performed using phase-modulated Ellipsometry. For this reason, gradient sample of the range of thicknesses was prepared. The dry thickness of the sample varied from 0.5 nm (left) to the 8.8 nm (right). Results are shown in Figure 6.19.

![Figure 6.19](image)

**Figure 6.19.** Extension of the poly(ethylene glycol) brush in the presence of the electrolyte of different ionic strength. Dry thickness of the sample increases from the 0.5nm (left) to the 8.8 nm (right).

To determine whether the extension of the polyacrylic acid block is the main reason for the extension of the mixed brush, the behavior of the poly(ethylene glycol) chains in the presence of the calcium chloride electrolyte with different ionic strengths was studied. Studies were performed using phase-modulated Ellipsometry. For this reason, gradient sample of the range of thicknesses was prepared. The dry thickness of the sample varied from 0.5 to 8.8 nm. Results are shown in Figure 6.19.

Because poly(ethylene glycol) is a non-ionic polymer, the increase in the thickness with the increase of the ionic strength of the electrolyte can be explained by the penetration of the Ca$^{2+}$ ions inside the brush. However, as we can see from Figure 6.19, at a grafting density of 0.5 chains/nm$^2$ and lower there is no more extension with the increase of grafting density for all salt concentrations, however there is a slight decrease in extension with higher grafting densities. This is probably due to the exclusion effect of the polymer chains. Calcium ions will penetrate inside the brush until either the chemical
potential is equal outside and inside the brush, or the point when there is no more free space left for the bulky calcium ion inside the brush. Then, there will be no more increase in the thickness of the brush. With an increase in the salt concentration, salt ions will displace the water from inside the brush and, because they lose their interaction with water, the PEG chains will lose solubility and start to shrink.\textsuperscript{11} At low salt concentrations, ions present inside the brush will decrease the volume available for polymer chains, causing polymer chain stretch. An additional driving force for the polymer chains’ stretch is the difference in osmotic pressure between that inside the brush and that outside in the medium. However, while the increase in thickness for the polyacrylic acid is dramatic, the increase in thickness for poly(ethylene glycol) is minor. So, the overall extension of the mixed polymer brush can be attributed mostly to the extension of the block copolymer.

The AFM scratch test has shown practically no increase in the mixed brush height when it is exposed to the calcium chloride solution. Results are shown in Table 6.6. Before treatment with calcium chloride solution, the mixed polymer brush was brought to its highest extension – pH 9.5 to check effect of bivalent ion on brush height.

To determine if the calcium ions are permanently trapped inside the mixed polymer brush after treatment with CaCl\textsubscript{2}, we measured dry thickness of the mixed polymer brush sample after thoroughly rinsing it with water. It was determined in the experiment with pure polyacrylic acid (thickness 8.0nm) and block copolymer (thickness 7.2nm) that polyacrylic acid and polyacrylic acid-b-polystyrene demonstrate an increase in dry thickness after CaCl\textsubscript{2} treatment of 1.9 and 1.4 nm respectively. For the mixed
polymer brush with the different PEG thickness, the increase of the dry layer thickness after CaCl$_2$ treatment varied between 1-3nm. This increase in thickness was taken into consideration when evaluating the extent of protein adsorption.

**Table 6.6.** Results of the AFM scratch measurements of the extension of mixed polymer brush in the calcium chloride solution.

<table>
<thead>
<tr>
<th>Brush composition, PEG/PAA-PS, nm/nm</th>
<th>Brush height, nm. Measure by AFM scratch method</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pH 9.5</td>
</tr>
<tr>
<td>4.6/7.8</td>
<td>29.1</td>
</tr>
<tr>
<td>4.5/7.4</td>
<td>22.3</td>
</tr>
</tbody>
</table>

It was also determined that the increase in the swelling of the mixed polymer brush stays constant after treatment with calcium chloride solution. By the AFM scratch test, it was found that the brush thickness increases from 6.5 nm in a dry state to 13.8 nm in the presence of water, and then to 16.7 nm in the presence of calcium chloride (1% solution, 15 min). After the brush was rinsed with water, dried and then placed under water, the thickness of the brush was measured to be the same.

XPS studies have supported the idea that increase in brush thickness is the result of calcium ions incorporation into polyelectrolyte brush. XPS have shown ~4 atomic percentage of the calcium inside the mixed polymer along the gradient sample (Figure 6.20). The obtained dependency on PEG grafting density of the number of carboxylic groups that reacted with calcium ions is shown in Figure 6.21. There is an increase in
the number of carboxyl groups that are bound with calcium that corresponds to the increase in PEG grafting density. This is probably due to the fact that PEG favors PAA stretching and pushes polyelectrolyte to the surface. With an increase of PEG grafting density, more PAA is exposed to the surface and, therefore, to interaction with calcium ions.

Figure 6.20. Example of XPS spectrum of mixed polymer brush treated with calcium chloride.
6.3.3.6. AFM studies of hydrophobic interactions of the mixed polymer brush*

The polystyrene part of the mixed polymer brush possesses an important property that does not depend significantly on the environment\textsuperscript{11} – the ability to demonstrate hydrophobic interactions. These properties are very important for the regulation of protein adsorption. When in the presence of a high salt concentration\textsuperscript{22} or certain pH levels,\textsuperscript{23} there is practically no adsorption on the polyelectrolyte brushes, hydrophobic interactions between the protein and brush are not affected by salt, and will therefore be highly effective. The presence of hydrophobic polystyrene fragments in the mixed polymer brush results in the possibility of hydrophobic interactions between the mixed polymer brush and the surrounding environment. Because of the responsive properties of the prepared mixed polymer brush, these interactions may be a function of the external stimuli present in the system, such as pH and ionic strength. Both of these parameters

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{image.png}
\caption{Dependency of the amount of carboxylic groups reacted with calcium ion on PEG grafting density.}
\end{figure}
may affect the geometric position of the polystyrene fragments, which are responsible for hydrophobic interactions and will affect the extent of these interactions as a result.

Three types of architecture that the prepared system that will generate with pH change are proposed. **Figure 6.22** reflects these three possible states.

![Figure 6.22](image)

**Figure 6.22.** Representation of the three different states mixed polymer brush goes through with increase of pH. a) – low pH, PAA collapsed; b) – isoelectric point, PAA chains start to stretch; c) – high pH, PAA chains highly extended.

In state (a) at low pH, polyacrylic acid chains are collapsed and polystyrene domains are brought closer to the surface of the wafer. As PEG is not greatly influenced by pH, its chains are extended in water and cover the collapsed block copolymer. In this case, hydrophobic interactions should not be observed to a significant extent. With an increase in pH, polyacrylic acid chains began to stretch and bring polystyrene domains to the brush-water border. At a certain point, the maximum possible number of polystyrene domains will be exposed to the surface, and hydrophobic interactions will have the highest value at this point (**Figure 6.22, b**). With the increase of the pH value of the medium, polyacrylic acid will continue to stretch, continuing the trend of moving of polystyrene domains to the surface. However, because of the different lengths of the polyacrylic acid chains by which polystyrene domains are anchored to the surface, the
height to which polystyrene may be elevated is limited to the maximum extension of the shortest PAA anchoring chain. When the shortest PAA chain anchoring polystyrene domain is extended to the maximum, no more elevation of the polystyrene will occur; the remaining PAA chains will extend further and cover the polystyrene with a PAA web (Figure 6.22, c).

To investigate the hydrophobic interactions of the mixed polymer brush at different pH levels, AFM studies of the adhesion between the modified AFM tip and the brush surface were conducted. Glass beads (6-7µm) covered with heptadecafluoro-1,1,2,2-tetrahydrodecyl)dimethylchlorosilane were glued to the AFM tip, and then the tip was used to perform adhesion studies in liquid media.

The results of the studies of the dependence of the hydrophobic interaction of mixed polymer brush on the pH of the medium are shown in Figure 6.23.

![Figure 6.23](image.png)

**Figure 6.23.** Dependence of the adhesion force reduced by AFM tip radius between the mixed polymer brush and hydrophobic glass bead of AMF tip on pH of liquid medium.
Indeed, as can be observed from Figure 6.23, forces acting between the highly hydrophobic glass beads and mixed polymer brushes vary with pH. First, small interactions develop between the AFM tip and mixed polymer brush, but when pH comes to 5, which is very close to the pK\textsubscript{a} of the polyelectrolyte brush, adhesion between the glass bead and mixed brush rapidly increases. With the increase of the pH, the adhesive force declines, and exhibits values close to those obtained at a low pH state. When the pH of the system is again lowered to 5, strong interactions develop and, with the decrease of the pH, the adhesive force declines to its initial value. These results are consistent with the states described in Figure 6.22, and are reversible.

Extension of the polyelectrolyte brush not only depends on the pH of the medium, but also on the ionic strength of the solution. The results of these studies of the hydrophobic interactions between the modified glass bead and the mixed polymer brush are shown in (Figure 6.24 a, b). Studies were conducted at different pH values with different ionic strengths. Sodium chloride was used to adjust the ionic strength of the medium. At low pH, when the polyacrylic acid chains collapsed\textsuperscript{8}, there were practically no change in adhesion with changes in the ionic strength of the solution, and adhesion was low. Meanwhile, when pH was increased to the value of the pK\textsubscript{a} of polyacrylic acid, the adhesive forces first increased with the addition of the salt, and then adhesion decreased and practically disappeared at a concentration of salt of 1M (Figure 6.24, b).
When comparing the outcome from (Figure 6.24, a) with the results in Figure 6.18, which describes the extension of the brush according to the ionic strength of the solution, it can be noticed that the lowest force of adhesion occurs in the region with the highest extension of the polyacrylic brush (ionic strength of the solution 0.1-1M, Figure 6.18). This supports the idea that the brush’s ability to exhibit hydrophobic interactions

Figure 6.24. AFM measurements of the interactions between the modified with the hydrophobic glass beads and mixed polymer brush. a) Dependence of the hydrophobic interactions on salt concentration at pH 3. b) Dependence of the hydrophobic interactions on salt concentration at pH 5.
depends on the conformational state of the polyacrylic acid polymer chains. In the collapsed state of polyacrylic acid, very limited interactions between the polystyrene domains and the modified AFM tip are present. With the increase in the extension of the PAA chains, the polystyrene domains are lifted to the surface and hydrophobic interactions between the brush surface and external environment start to develop. The addition of salt in small amounts to the brush in an ionized state is known to increase brush height\(^6\). Thus, the addition of sodium chloride to the medium at pH 5 caused a rapid increase in brush extension and adhesive forces. With further addition of salt and continuing extension, PAA chains started to cover the PS domains and the adhesive forces measured by AFM decreased and then practically disappeared. This is exactly the picture seen in Figure 6.18 and Figure 6.24, b.

With increased valence of the metal ions in the salt, the effect of adding salt is the same, and adhesion decreases with the increase of the salt concentration (Figure 6.25).

A crucial difference in the salt effect between monovalent ions and multivalent ions is that in case of sodium chloride salt, hydrophobic interactions are reversible; when the electrolyte solution is replaced with a medium that does not contain ions, interactions revert to the state of the media. This was not the case in the experiment with calcium chloride. After the brush was treated with the calcium chloride salt solution, when the medium was exchanged with the Millipore water (pH=5.0), interactions were not restored, i.e. no increase in adhesion between the AFM tip and the brush surface was observed. Calcium ions interact with the polyacrylic acid, creating a chemical bond and locking the brush architecture in place when the polystyrene domains are arrested inside
the brush. Treatment of the brush with a low pH medium removes calcium ions from the brush structure and when interactions are then measured at pH 5, they are of the same value as previously measured.

These properties make the studied system unique, as the system may develop tunable hydrophobic interactions in the aqueous hydrophilic medium. While these interactions are not affected by external stimuli such as the ionic strength of the medium at low pH, when adjusted to certain values, hydrophobic interactions become tunable and reach opposite values, i.e. high hydrophobicity and very low hydrophobicity. It should be also stressed that all of these transformations occur in aqueous media. While being reversible, the hydrophobicity of the mixed brush may be locked and become relatively independent of external conditions, as in the case with calcium chloride salt.

*Measurements of the hydrophobic interactions were taken by Roman Sheparovych, Prof. Minko’s group, Clarkson University.

**Figure 6.25.** Dependence of the adhesion forces between the hydrophobic substance modified glass bead and surface of mixed brush at different concentration of bivalent metal salt. Millipore water used was of pH 5.
6.4. Conclusions:

In conclusion, it can be stated that the behavior of the mixed polymer brush in the water medium depends on the solvent with which it is treated. Treatment of the brush with MEK gives the highest brush mobility/extension. Ethanol treatment results in the obstruction of the brush extension in the water medium. The size of the polystyrene inclusion also depends on the type of solvent with which the brush was treated. Treatment with MEK gives the smallest polystyrene domain size, while ethanol results in the largest polystyrene domains.

Extension of the mixed polymer brush in water is very dependent on the pH of the medium, and can mainly be seen in relation to the extension of the block copolymer. According to the IR Ellipsometry measurements, extensive ionization of the carboxyl group occurs at pH levels of close to 8 and above. These results are confirmed with Ellipsometry measurements of the extension of the mixed polymer brush and the AFM scratch test of the block copolymer.

As well as treating of the mixed polymer brushes with organic solvents, treatment with electrolytes also affects its properties. The presence of Ca ions in the system results in the increase of dry thickness of the mixed polymer brush. It was determined that calcium ions are chemically attached to the polyacrylic acid, and cannot easily be removed from the brush. Approximately 25% of the carboxyl groups are bound to the calcium ions. On average, the dry thickness of the mixed polymer brush after calcium chloride treatment increases by 2.5 nm.
Poly(ethylene glycol) chain extension in the presence of electrolytes has a minor impact on the overall mixed polymer brush extension, and the increase in the height of the brush should be attributed mainly to the block copolymer.

Adhesion measurements of the hydrophobic interactions reveal the unique properties of the developed system. Hydrophobic interactions in the brush are tuned by changing pH and the ionic strength of the medium. The uniqueness of the system is that the hydrophobicity of the brush surface changes in the water medium and has an island-like pattern. The properties of the tunable system may be reversibly locked with bivalent salt.

6.5. References:

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CHAPTER SEVEN

STUDIES OF FIBRINOGEN ADSORPTION ON THE MIXED POLYMER BRUSHES

7.1. Introduction:

Adsorption of a protein onto a surface is one of the most important processes in the interaction of a material with a biological system. Adsorption of the biomolecule on the surface and the interaction between the two are essential for the performance of a biosystem, the biocompatibility of the artificial organs, medical devices, and test-systems. Forces that drive protein molecules to adsorb on surfaces include hydrophobic, Coulomb, London, and polar interactions, and are described in more detail in Chapter 6. Different proteins will adsorb to different surfaces according to the influence of different forces; hence, on charged surfaces Coulomb interactions may be essential, while on non-charged surfaces, hydrophobic interaction may be more important. The creation of chemical bonding between the surface and protein in the case of a reactive surface will result in the irreversible attachment of the protein to the surface.

The question of whether protein adsorbed to the surface retains its biological functions has not yet been fully answered. Studies of the adsorption of fibrinogen onto biomedical grade stainless steel indicated that there were significant changes in the protein secondary structure that occurred within the first minutes of adsorption. The conformational changes of the protein may inevitably change its ability to perform biological functions. At the same time, studies have also shown that essential properties of the protein remain intact when the biomolecule is adsorbed onto hydrophilic or
hydrophobic surfaces. For instance, adsorption of glucoseamylase and glucosidase onto a polyacrylic brush did not interfere with the enzyme functions, and both proteins exhibited nearly their full activity after adsorption.

Globular proteins are very good candidates for the study of protein adsorption onto surfaces because of their relative geometrical simplicity and wide abundance in living organisms. Fibrinogen, being one of blood protein representatives, is synthesized by the liver (Figure 7.1). Fibrinogen plays an essential role in the blood clotting.

![Figure 7.1](image-url) Representation of the fibrinogen molecule. From site: www.rcsb.org (06/28/08).

The molecular weight of fibrinogen is 330 kDa. In a dry state, the molecule has a linear structure of three nodules held together by a very thin thread. The diameter of the terminal modules is 6.5 nm; it is 5.0 nm for the central module. Connecting threads were estimated to be between 0.8 and 1.5 nm thick. The fibrinogen molecule is chemically a dimer, as it is composed of a twin set of 3 polypeptide chains, called α, β, and γ, of approximately equal molecular weight (50,000-65,000 Da). The N-terminals of the chains are held together with disulphide bonds. The molecule has an isoelectric point of 5.2. The cleavage of the end sequences of the Aα and βB peptides by trombin results in
the formation of fibrin – the activated state of fibrinogen, able to associate and polymerize. The protein molecule has a height of 3.5-4 nm and, thus, can easily be detected by Ellipsometry.

Regulation of the amount of the protein adsorbed to the surface is often conducted through modification of the surface with non-ionic poly(ethylene glycol) of a different molecular weight. For the charged surfaces (e.g. PAA), the amount of protein adsorption is regulated by the addition of salt to the system.

The objective of this part of the work was to study the extent of adsorption of bovine fibrinogen onto a mixed PEG/PAA-PS polymer brush. As was already shown in Chapter 6, treating the mixed brushes with different solvents and a solution of bivalent calcium ion changes the morphology of the brushes, as well as their potential for hydrophobic interactions. The responsive properties of the brushes were used to control the amount of the protein adsorbed onto the surface. In this chapter, it is demonstrated that a mixed polymer brush treated with MEK, which is a good solvent for the protein repelling component of the brush, PEG, exhibits lower protein adsorption, while toluene results in higher adsorption. It is also shown that treatment of the mixed polymer brush with calcium chloride results in virtually no fibrinogen adsorption on the brush surface.

7.2. Experimental:

A phosphate saline buffer (pH 7.4) was prepared from phosphate saline obtained from Aldrich and deionized water, as directed by manufacturer. Bovine fibrinogen from
Sigma-Aldrich was dissolved in 0.1M phosphate saline buffer at a concentration of 0.6mg/ml.

Wafers with mixed polymer brushes treated as described in Chapter 5 were immersed into the protein solution for 30 min. The samples were put on a shaker for gentle agitation. Then wafers were rinsed with buffer and deionized water, and dried in a nitrogen box at ambient temperature. The thickness of the adsorbed protein was later measured by Ellipsometry, assuming the refractive index of the protein to be 1.5.

For adhesion measurements, hollow glass beads of diameter of 20-30 μm were washed with a mixture of hydrogen peroxide and sulfuric acid. After this, the beads were rinsed a few times with deionized water and dried in a nitrogen box. Then, the glass beads were immersed into 0.1% (w/v) MEK solution of PGMA. The beads were then washed with MEK, dried and immersed into the bovine fibrinogen solution. After removing unbound fibrinogen with the buffer and rinsing the beads in water, the beads were dried in nitrogen. The beads were later glued to the AFM tip with epoxy glue.

Adhesion measurements were carried out using Dimension 3100 AFM (Veeco Inc.), equipped with a Nanoscope IIIa controller by Dr. R. Burtovyy (Clemson University). Several force-distance curves were collected over the wafers’ areas, which had the same ratio between PAA-PS/PEG components in the saline buffer solution. The maximum deflection threshold, ramp size and scan rate were set to 100 nm, 1000 nm and 1 Hz, respectively. The results obtained for each area were averaged. A region where the approaching curve had a constant slope was used to determine the sensitivity coefficient.
7.3. Results and discussion:

7.3.1. Adsorption of the protein onto PEG, block copolymer and polyacrylic acid:

A few possible factors will affect the protein adsorption on the prepared brush surfaces. These are the composition of the brush surface, the size of polystyrene inclusions, the ionic strength of the solution, the length of the exposure, protein concentration, and temperature. Protein concentration, the ionic strength of the solution, temperature, and the length of the exposure were kept constant for all experiments so that the influences of the brush structure/composition on the adsorption were revealed.

To evaluate the role of each brush component in the sorptive properties of the mixed polymer brush, studies of the interactions between the protein and bare PEG, polyacrylic acid, and polyacrylic acid-b-polystyrene were conducted. Figure 7.2 a,b shows the summarized results of the adsorption of fibrinogen onto the brushes. All polyacrylic acid-b-polystyrene brushes had a grafting density of 0.13±0.02 chains/nm$^2$; for the PAA brush, it was 0.21 chains/nm$^2$.

The amount of the protein adsorbed onto the PEG brush starting from a grafting density of 0.5 chains/nm$^2$ of the polymer was low and approaching zero. Thus, the majority of the grafting densities of PEG in the mixed polymer brushes studied in this work lay in the region of low/negligible fibrinogen adsorption. Indeed, poly(ethylene glycol) is the component of the mixed polymer brush responsible for the reduction of the protein adsorption on the brush surface. There are three main reasons that PEG decreases protein adsorption on the surface. First, PEG is soluble in water and has a steric structure that does not disturb the water’s structure by its presence. Adsorption on the
surface of the poly(ethylene glycol) chains result in the perturbation of the water – PEG lattices architecture, and that is thermodynamically unfavorable. The second reason is the excluded volume effect. Adsorption of the protein on the surface decreases the polymer excluded volume, decreasing its entropy and leading to the steric instability of such a formation. Finally, the third reason is that the fast mobility of the polymer chains in the water prevents the approach of the protein molecule to the surface, which is required for adsorption.

A polyacrylic acid brush with a grafting density of 0.21 chains/nm$^2$ adsorbed, on average, 2.3nm of the fibrinogen. When a polystyrene block was introduced into the brush, the adsorption of the fibrinogen on the brush surface increased. The increase of

Figure 7.2. Fibrinogen adsorption onto the a) PEG brush, Mn=5,000. Reprinted with permission from B. Zdyrko; b) – polyacrylic acid (0.21 chains/nm$^2$) and block copolymer of polyacrylic acid (0.13 chains/nm$^2$) after different solvent treatment. Ellipsometry measurements. 1 nm of fibrinogen corresponds to $3 \times 10^{-9}$ mol/m$^2$, or 21% of monolayer of protein.

A polyacrylic acid brush with a grafting density of 0.21 chains/nm$^2$ adsorbed, on average, 2.3nm of the fibrinogen. When a polystyrene block was introduced into the brush, the adsorption of the fibrinogen on the brush surface increased. The increase of
the adsorption compared to bare polyacrylic acid can be clearly attributed to the presence of the polystyrene component in the system. Electrostatic interactions that govern the adsorption of the protein to the polyacrylic acid were constant throughout the experiment with all brushes, as the ionic strength of the solution was kept constant.

Data from Figure 7.2b reveal that the block copolymer brush treated with toluene demonstrated the prevalent adsorption of the protein molecule on its surface. Possible brush morphologies after the solvent treatment are shown in Figure 7.3. Toluene dissolves only the polystyrene part of the block copolymer, so high adsorption compared to other treated samples can be explained by the selective extraction of the polystyrene to the brush exterior. Ethanol and DMF reveal the same extent of adsorption. Both solvents will result in the presence of polystyrene parts as well as polyacrylic acid parts on the surface, both adsorbing protein. It should be noticed that MEK results in the smallest amount of the protein being adsorbed. MEK is a good solvent for polystyrene, but not polyacrylic acid. The low protein adsorption on the surface compared to the adsorption after DMF treatment may be explained by the smaller lateral size of the polystyrene domains formed during treatment. The immobility of the polyacrylic acid chains may result in the small size of the polystyrene domains, because the domains that can be formed are restricted by the extension of the polyacrylic acid chains.
7.3.2. Protein adsorption on the solvent treated mixed polymer brushes:

The extent of the adsorption was measured for the mixed brushes under study by Ellipsometry, and is presented in Figure 7.4. There is a clear dependency between the solvent used for the mixed brush treatment and the amount of the protein adsorbed on the surface. Treatment of the brush with MEK resulted in the smallest amount of the protein.
being adsorbed. DMF and toluene treated brushes presented the highest degrees of fibrinogen adsorption. We may also notice influence of the presence of PEG component of the brush on the extent of protein adsorption. While we may position protein adsorption for the block copolymer solvent treated surfaces in the next order: toluene > DMF ≈ ethanol > MEK, mixed polymer brush shows next order: DMF ≥ toluene > ethanol > MEK.

It is well known that in polymer brushes, the polystyrene and polyacrylic acid elements are responsible for two major protein adsorption interactions – hydrophobic interactions and Coulomb interactions. Any protein molecule is represented as a set of hydrophobic (non-charged) and positively and negatively charged patches distributed on the surface of the molecule.\textsuperscript{6} Polystyrene adsorbs proteins through the hydrophobic interaction with the hydrophobic patches of protein. Polyacrylic acid will adsorb protein through an electrostatic mechanism, due to interaction between the charged patch of the protein and charged polymer molecules.\textsuperscript{14} In the mixed brush, however, long-range Coulomb interactions did not change in this experiment since the pH and ionic strength of the solution were kept constant. Thus, variation in the position and shape of PS fragments are responsible for different levels of protein adsorption.

A schematic representation of the brush treated with different solvents is shown in Figure 7.5. Low adsorption of the protein on the surface can be explained by the predominant presence on the upper layer of the brush protein repellent PEG and the low number of small sized polystyrene fragments on the surface. Because MEK is a good solvent for PEG, we can expect this situation (Figure 7.5a). Ethanol dissolves both
polyacrylic acid and poly(ethylene glycol). By bringing polyacrylic acid to the surface, we simultaneously bring polystyrene that is attached to the end of the polyacrylic acid chains to the surface. The presence of both the protein attractive parts on the surface, together with the alcohol soluble protein repellent part, results in average protein adsorption (Figure 7.5 b). With the toluene treatment, polystyrene chains are dissolved and extracted to the surface. The polyacrylic acid architecture will stay intact when treated with toluene, and PEG will swell. The large amount of the protein sorbed on the toluene-treated surface can be attributed to the polystyrene locked on the surface after treatment (Figure 7.5c). High protein adsorption on the sample treated with DMF, because this is a good solvent for all three components, may be related to the predominant presence of the two protein adsorptive components, polyacrylic acid and polystyrene, on the surface.

Figure 7.5. Schematic representation of the mixed tri component polymer brush after treatment with different solvents. A)-MEK, b)-ethanol, c)-toluene, d) - DMF.

Fibrinogen adsorption can be related to the size of the polystyrene domains formed after solvent treatment (see Chapter 6). The MEK treated sample had the
smallest lateral size of polystyrene domains, as well as the smallest, close to zero, amount of fibrinogen adsorbed. For the DMF, toluene and ethanol treated samples, the polystyrene domains formed were of comparable size, with the following order: ethanol>DMF>toluene. These samples had a much higher protein adsorption. Ethanol formed the largest polystyrene domains, but showed medium adsorption. This can be explained by the ethanol soluble PEG extracted to the surface, which reduces adsorption. Toluene and DMF formed polystyrene domains that were close in size and showed similar amounts of protein adsorbed on the surface.

However, one can also notice that despite the increase in the PEG brush grafting density, while the PA-b-PS grafting density is kept constant, protein adsorption does not diminish, as would be expected. The increase in PEG brush density may have this adverse effect, as polymer chains of the brush will expel each other from the surface even more due to the excluded volume effect. The increase in the grafting density of the PEG brush favors the ejection of the polystyrene domains and polyacrylic stems holding them to the surface.

7.3.3. Adsorption of the protein onto the bivalent ion treated polymer brush:

Treatment of the polymer brushes changes the geometrical positioning of the brush components on the surface and inside the brush, and these results in the change in protein adsorption onto the brush. Morphology studies and adhesion studies have also demonstrated that the treatment of the brush with salt results in changes of surface composition. In addition to studies of protein adsorption on organic solvent treated
brushes, studies on calcium ion treated brushes were also conducted. To remove any unbound calcium, brushes were rigorously rinsed with water. As already discussed, calcium ions are permanently bind to the mixed polymer brush. According to the XPS analysis, there is 4 atomic percent of the bound calcium through the whole brush thickness range that corresponds to approximately 25% of the carboxyl groups engaged with calcium.

To compare the difference in the performance of the polymer brushes in terms of protein adsorption, a mixed polymer brush with the same architecture, but treated with diluted hydrochloric acid (1% water solution), was examined for protein adsorption. The results of this experiment are shown in Figure 7.6.

![Figure 7.6](image-url)

**Figure 7.6.** Results of adsorption studies for poly(ethylene glycol) – polyacrylic acid-b-polystyrene mixed brush. a) – Brush treated with HCl prior to adsorption, b) – assembly was treated with Ca$^{2+}$ ions. 1 nm corresponds to 3x10$^9$ mole/m$^2$ and 21% of protein monolayer.
**Figure 7.6** indicates that treatment with calcium ions has a prominent effect on protein adsorption. Assemblies free from the presence of the calcium ions exhibit much higher adsorption that increases to the monolayer size with an increase of the PEG brush grafting density. This increase can only explained by the larger amount of polystyrene present on the surface due to the pushing effect of PEG on the block copolymer chains.

Measurements of the fibrinogen adsorption on the polyacrylic acid homobrush (0.17 chains/nm$^2$) and block copolymer brush (0.13 chains/nm$^2$) treated with calcium chloride have shown 2.7 nm adsorption to the block copolymer and 1.9 nm to polyacrylic acid. The calcium ion treated brush adsorbs practically no protein along the whole range of grafting thicknesses. The pH of the media in which adsorption occurred was 7.4, with a salt concentration of 0.1M.

Few possible causes for the protein repelling properties of the mixed polymer brush treated with calcium ions (**Figure 7.7**) can be considered. The presence of calcium ions in the system results in the system crosslinking. Polystyrene domains that are responsible for hydrophobic interactions with the proteins are trapped inside the crosslinked network. Due to crosslinking, large fibrinogen molecules cannot penetrate inside the brush, and may adsorb only on the surface of brush. This eliminates adsorption of the protein due to hydrophobic interactions. The presence of PEG in the system has an additional protein repelling effect, as while there is 2.7 nm of protein adsorbed onto the block copolymer after calcium treatment, virtually no adsorption is detected on the mixed brush. Moreover, at pH 7.4, polyacrylic acid exists in its negatively charged form (see **Chapter 6**). The positively charged calcium ion binds to the carboxylate ion with the
formation of salt that is stable to dissociation. The electrostatic adsorption of fibrinogen results from the interaction between positively charged patches of fibrinogen and negatively charged carboxylate ions. Calcium bound to the carboxylate group may screen electrostatic interactions, resulting in a decrease of protein adsorption in comparison with the DMF treated block copolymer brush (3.3 nm).

The results of the protein adsorption are consistent with the results of the adhesion measurements. The low adsorption of protein, as well as the low adhesion, results from the calcium locking the polystyrene inside the brush and freezing the system.

\textbf{Figure 7.7.} Schematic representation of the PEG/polyacrylic acid-b-polystyrene mixed brush when treated with calcium chloride ions.

The results of the protein adsorption are consistent with the results of the adhesion measurements. The low adsorption of protein, as well as the low adhesion, results from the calcium locking the polystyrene inside the brush and freezing the system.

\section*{7.3.4. AFM adhesion studies:}

From the above-mentioned results, it may be seen that protein adsorption on a mixed polymer brush is in good accord with the AFM (hydrophobic bead) adhesion measurements. Interaction between the mixed polymer brush and the protein may also be studied using the same experimental procedure. However, unlike the hydrophobically modified bead, the protein-modified bead will go through a much wider range of interactions with the mixed brush and these include, in addition to hydrophobic, Coulomb...
and polar interactions. Still, the results of the AFM adhesion measurements will represent the summed attraction between the protein and the mixed brush.

Preparation of the glass bead covered with the bovine fibrinogen layer, and the manner in which measurements were conducted, are described in Experimental part of this chapter. The schematic and image of the AFM tip with glass beads are shown in Figure 7.8.

![Figure 7.8. Adhesion measurements by AFM. Principal scheme of adhesion measurement and picture of the fibrinogen modified glass bead glued to AFM tip.](image)

**7.3.4.1. Dry contact AFM adhesion measurements:**

Dry contact adhesion measurements exclude the influence of the solvent, i.e., polarity, ionic strength, and increased flexibility of the polymer chains. **Table 7.1** represents the constitution of the mixed polymer brush used for dry adhesion studies.
Table 7.1. Geometrical parameters of the mixed polymer brush used for dry contact adhesion measurements.

<table>
<thead>
<tr>
<th>Solvent</th>
<th>PEG thickness, nm</th>
<th>PAA-PS thickness, nm</th>
<th>PEG grafting density, chains/nm²</th>
<th>PAA-PS grafting density, chains/nm²</th>
</tr>
</thead>
<tbody>
<tr>
<td>MEK</td>
<td>6.1</td>
<td>8.3</td>
<td>0.8</td>
<td>0.21</td>
</tr>
<tr>
<td>Toluene</td>
<td>5.7</td>
<td>7.5</td>
<td>0.8</td>
<td>0.19</td>
</tr>
<tr>
<td>Ethanol</td>
<td>6.5</td>
<td>6.9</td>
<td>0.9</td>
<td>0.15</td>
</tr>
</tbody>
</table>

In Figure 7.9, pull-off deflection refers to the deflection of the AFM tip before the detachment from the surface. This deflection originates from the interaction between the surface and the protein covered glass bead, and may serve as an adhesion equivalent. Residence time is the time of contact between the surface and the AFM tip before withdrawing from the surface. As can be seen from the graph (Figure 7.9), the adhesion between the brush and fibrinogen layer for the toluene treated surface is higher than for the ethanol treated one. The MEK treated sample demonstrates the lowest level of adhesion. This exhibits the same trend as the protein adsorption measurements, additionally supporting the validity of the previous results. Because the measurements were performed in the absence of a liquid medium (in dry contact), the main role in the adhesion between the protein and the mixed brush will be played by dispersion forces.
As can be noticed from Figure 7.9, the mixed polymer brush treated with toluene exhibits four times higher affinity to the protein covered glass bead than the MEK treated brush. This is consistent with the results shown in Figure 7.3. Higher interaction results in a higher extent of protein adsorption. It is not presently possible to correlate adsorption and adhesion results quantitatively, as the relation between the amount adsorbed and the pull-off deflection is unknown.

![Figure 7.9. AFM adhesion measurements between mixed polymer brush and protein covered glass bead.](image)

With the residency time increased (time during which the tip is pressed to the surface), adhesion increases. From these data, we may conclude that time of the forced interaction of the protein-mixed polymer brush plays an important role in the extent of protein-brush attraction. Forced contact time increases the probability of development of interactions between the brush component and the protein molecule if the protein and mixed brush come into a proximity comparable to the force’s acting range (see Chapter)
6 for force acting ranges). It can be expected that a longer residency time will provoke
the development of attractive forces that require the steric rearrangement of the molecule
or that develop when a sufficient distance between species is met.

7.3.4.2. Buffer mediated adhesion measurements:

To evaluate the attractive forces between the mixed polymer brush and the protein
collector in the buffer medium, adhesion measurements between the protein covered
glass bead that was glued to the AFM tip and the mixed polymer brush in 0.1M
phosphate buffer have been performed.

When fibrinogen is in contact with mixed polymer brush of PAA-b-PS and PEG
in the presence of the pH 7.4 buffer, three types of different interactions are expected.
These are:

- attractive forces between positively charged patches of the protein and
  negatively charged polyacrylic acid;
- repulsive forces between negatively charged polyacrylic acid and overall
  negatively charged fibrinogen molecules (isoelectric point 5.2) or
  negatively charged patches of the protein molecule;
- hydrophobic interactions between the polystyrene part of the mixed brush
  and hydrophobic patches of the protein molecule, together with dispersion
  forces.

Repulsive forces between poly(ethylene glycol) and protein will also be present.
Electrostatic repulsion between the polyacrylic acid and the protein molecule may be
partially screened due to the ionic strength of the buffer solution used (0.1M). Adhesion forces between the protein and mixed brush will be represented as the sum of all of the above-mentioned interactions.

**Figure 7.10** represents the adhesion measurements between the protein and mixed polymer brush, where the residence time of the AFM tip on the brush surface is 1s and 50 s for two extreme cases – toluene and MEK treated samples. It is known from the protein adsorption experiments that the amount of fibrinogen anchored to the toluene treated brushes was approximately 4 times larger than to the MEK treated ones. There is a qualitative correlation between this result and the magnitudes of the initial force and the force for 1 s residence time. Both values are much larger for the toluene treated samples. The difference in adhesion also persists at the 1 s residence time, but vanishes completely for 50 s.

An interesting tendency can be observed for relative error values. For $F_{\text{max}}$ (force developed between the tip and the surface after 50s contact) and $F_0$ (time of contact between the tip and the surface 1s), the value is always 1.5 times larger for MEK treated samples. The error indicates how the data are scattered. The pull-off force values for toluene are more consistent, whereas in case of MEK the data are more scattered (for the same residence time and the same component ratios, very large or very small adhesion was measured). With respect to adsorption, this means that the adsorption event has a more probabilistic character. Adhesion can be present or not and, thus, some amount of the protein can be adsorbed on the surface, but a fully saturated monolayer is never formed. It can be stated that AFM results for short exposures (**Figure 7.9** and **Figure
are in qualitative agreement with the observed adsorbed amounts of fibrinogen (Figure 7.4).

Another interesting result is the dependence of the pull-off force on the ratio of components in the film. Again, a similar situation to the case of average adsorption can be noticed for this dependency. At lower PEG content, the adsorbed amount decreases initially, then increases; for high values, it is almost constant. Both systems (treated with MEK and toluene) reveal the same behavior. A similar tendency is found for the initial pull-off force, though the force tends to increase further, and not saturate even at the highest ratio. The only difference observed is the actual ratio of polymers when adhesion/adsorption increases. An apparent discrepancy can arise from the fact that the conformation of the fibrinogen in solution and that attached to the bead could be

Figure 7.10. AFM adhesion measurements in “wet contact” between the mixed polymer brush and protein covered glass bead. Circles – toluene treated sample. Triangles – MEK treated sample.
different; thus, it reveals the same general behavior but does not show agreement in exact numerical values.

It is worth mentioning that the adsorbed amount of the protein as well as the adhesion at the initial state and after 1 s of residence time are larger for higher PEG contents. General logic would imply the opposite behavior, as PEG is a well-known protein-repelling polymer. The complexity of the system’s behavior can be explained by considering individual conformations of brush components and their state in the buffer solution. As discussed earlier in this work, this can be explained by the additional stretching of the PAA-PS chains and the pushing of polystyrene domains to the surface in the environment of densely grafted PEG chains.

As discussed in Chapter 6, the lateral size of the polystyrene domains formed after the toluene treatment is larger than for the MEK treatment. The domain size may play a very important role in protein adsorption, and large number of small protein attractive domains scattered inside the protein-repelling matrix may be less effective for adsorption than larger sized domains. On the other hand, large polystyrene domains may block PEG chains positioned under the domains from coming to the surface, and in this way decrease the amount of the protein-repelling component on the surface, increasing absorption and adhesion on the surface of mixed brushes. This, indeed, was confirmed by AFM adhesion measurements, where the average adhesion for the toluene treated samples is higher than for MEK.

When analyzing the dependence of an adhesive force on the relative content of components in the layer, one should keep in mind that the total grafting density in the
polymer layer in performed experiments increases together with an increase in the PEG to PAA-PS ratio (Figure 7.10). The initial decrease in adhesion up to approximately 2:1 in the PEG:PAA-PS ratio is caused by increasing the amount of PEG in the layer, leading to the growth of the repulsive components of interactions and consequently lower adhesion. The further increase at higher values of the ratio can be explained when the increasing total polymer density is taken into account. This gradual increase results in the extension of polymer chains from the surface; thus, eventually, the PS part can rise above the PEG layer. For adhesion measurements, such changes will affect the balance of attractive and repulsive forces, with attraction continuously increasing. The favorable spatial position of PS at the top of the layer will determine higher adsorption at a high ratio of grafting densities.

An additional possible explanation for high adhesion at higher grafting densities could be the influence of the PEG layer itself. PEG is known for its protein repelling properties, but if it can develop some adhesion, with time this can result in increasing adhesion and adsorption. In fact, it was found that fibrinogen could show significant adhesion to the PEG layer in the case of longer protein-brush contact (Figure 7.11). However, there is no initial adhesion and the trend for maximum force is opposite to what was observed for mixed brushes. These results demonstrate that PEG is not responsible for the observed changes in adsorption and adhesion at higher grafting densities.
Mixed polymer brushes of poly(ethylene glycol)/polyacrylic acid-b-polystyrene have proved themselves as useful tools in protein adsorption control. Treatment of the mixed polymer brush with solvents selected for each component of the brush results in changes in the extent of interaction between the polymer brush and the protein. The highest protein adsorption was achieved after the toluene treatment, which is a good solvent for polystyrene. MEK resulted in the smallest amount adsorbed. MEK is a very good solvent for poly(ethylene glycol) – a protein-repelling agent. Ethanol and DMF occupy an intermediate position between the extreme cases.

**Figure 7.11.** Dependence of pull-off force on grafting density of PEG layer and time of forced contact between the AFM tip and polymer brush.
The presence of the polystyrene part in the block copolymer results in higher adsorption as compared to the bare polyacrylic acid. Similar to the mixed polymer brush, the block copolymer brush treated with toluene exhibits the highest adsorption, while that treated with MEK exhibits the lowest. DMF and ethanol possess intermediate values.

One of the useful properties of the mixed polymer brush is its interaction with the calcium chloride solution. The treated brush obtained has well defined protein-repelling properties, while the brush not treated with bivalent ion has high attraction toward the biological macromolecules.

AFM studies of the adhesion force in dry and wet modes has revealed that the attractive forces of the protein covered surface toward the mixed polymer brush are four times higher in cases where the mixed brush was treated with toluene than when it was treated with MEK. One of the interesting findings was that the adhesive force becomes practically equal for surfaces treated with both solvents when residency time is increased to 50 s.

Results obtained in this study may be employed for the preparation of the surfaces with tunable properties for controlled protein adsorption. Figure 7.12 represents summary of the prepared and studied in this work surfaces.

Pure substances, polystyrene and polyacrylic acid, differ in the extent of protein adsorption, and this extent cannot be changed with external stimuli. Composed of two components, the block copolymer brush exhibits slight tunability when treated with an organic solvent, but the changes are small. With an addition of a third component to the system, external stimuli control the extent of the protein adsorption on the mixed polymer
brush without a doubt. The amount of protein adsorbed varies from 4.2 nm for the brush after toluene treatment to practically zero after the calcium chloride treatment.

The results obtained show that mixed polymer brushes are very successful tools for the control of protein adsorption. A mixed polymer brush of one composition may be tuned with external stimuli to be extremely protein attractive, totally protein resistive or attain morphology that will show a moderate amount of adsorption between the extremes.

**Figure 7.12.** Surfaces studied and protein adsorption achieved.

The results obtained show that mixed polymer brushes are very successful tools for the control of protein adsorption. A mixed polymer brush of one composition may be tuned with external stimuli to be extremely protein attractive, totally protein resistive or attain morphology that will show a moderate amount of adsorption between the extremes.

**7.5. References:**


CHAPTER EIGHT

CONTROL OF THE PROTEIN ADSORPTION THROUGH MOLECULAR
IMPRINTING APPROACH. IMPRINT SYNTHESIS

8.1. Introduction:

In the previous chapter, we described the synthesis and study of the properties of a surface consisting of a mixed polymer brush that has controllable protein adsorption characteristics. Adsorption sites on these mixed polymer brushes were distributed evenly all over the area of polymer brush surface and, therefore, protein molecules were adsorbed to the surface randomly. In this part of the work, a brush surface that can adsorb protein molecules to geometrically specified positions has been developed. Instead of being randomly distributed on the surface, protein molecules will be positioned in predetermined places on the surface.

Specificity is a powerful tool used widely in natural biological systems to position biological molecules into certain predetermined adsorption sites. Cells use cytokine-based recognition to communicate with the outside world; antibodies specifically adsorb their antigens. Any foreign object inserted into a living organism will soon be recognized, positioned properly and encapsulated in a collagenous sac. Biologically specific adsorption mechanisms are also noted for the functioning of enzymes, lectins, integrins, DNA, RNA and saccharides.

The molecular imprinting (MIP) technique described in Chapter 2 can mimic nature’s molecular recognition. 3-D imprinting is not an effective route for the
preparation of large organic molecules’ specific adsorption sites due to the problem of low accessibility of the imprinting sites. Specifically, the large size of the molecules and their low diffusivity do not allow them to travel successfully through an imprinted polymer matrix.

The goal of this part of the work was to synthesize specific adsorption sites using the MIP technique, in order to control the spatial positioning of protein molecules on a substrate. Here, a method of preparing the 2-D protein adsorption sites using protein-repelling PEG brushes has been developed. 2-D imprints have several advantages over 3-D imprints. First, in 2-D imprints, the adsorption sites are suitably accessible for large molecules, as they are exposed to the imprint surface. Second, little of the imprinting material is used, which is very important in the case of the costly protein-substrate. Third, the low diffusion of the protein molecules does not play such an important role for imprint performance, since 2-D imprints are highly accessible.

8.2. Experimental:

8.2.1. Synthesis of the molecular imprint:

Molecular imprints were synthesized on silicone wafers cleaned and covered with 2±0.5 nm of PGMA, as described in Chapter 4. Ovalbumin and human albumin, obtained from Sigma, were dissolved in a phosphate buffer (pH 7.4) at a concentration of 1 mg/ml. A monolayer of the protein was deposited onto the PGMA by dipping the wafer into the protein solution for 30 min. Next, the wafers were rinsed with DI water to remove unbound protein. Wafers containing deposited protein were incubated overnight
in the presence of water vapor at a temperature of 37°C. The layers obtained were characterized with Ellipsometry (the refractive index was assumed to be 1.5 for proteins) and AFM (tapping mode).

PEG 1% w/v in ethanol was deposited onto the top of protein-containing wafers by dip-coating. Solvent assisted grafting of the PEG to the PGMA was carried out in the presence of cyclohexane vapor with triethylamine as a catalyst. Grafting was conducted at 37°C overnight. Ungrafted PEG was removed by multiple washings with ethanol. Layers were characterized with Ellipsometry (the refractive index was assumed to be 1.5 for PEG) and AFM (tapping mode).

The prepared protein-polymer wafers were subjected to protease cleavage. Nonspecific protease Subtilisin A with activity of ~12 units/mg was purchased from Sigma. The enzyme was dissolved in a phosphate buffer pH 7.4 at a concentration of 0.01mg/ml. The prepared wafers were immersed into the protease solution for overnight treatment at a temperature of 37°C. Protease treated wafers were rinsed with water to remove the remaining cleaved peptides and then sonificated in a mixture of acetone/water (95:5 v/v) to remove protease attached to the imprint. The prepared imprint was characterized with Ellipsometry and AFM.

**8.2.2. Labeling of the proteins:**

Protein (human albumin or ovalbumin) was dissolved at a concentration of 2 mg/ml in a carbonate buffer (pH 8.3). Fluorescent dye was dissolved in the dimethylsulfoxide at a concentration of 500 mg/mL. A 2.5 molar excess of the dye was
used to achieve the sufficient labeling density of the protein through reaction of acylation of side amino groups of protein molecule with N-succinyl carboxylate of the dye. Solutions were mixed and the reaction was held with slight agitation of the solution at room temperature for 2.5 hrs. Yield of reaction was found to be 20-28%. Later, unreacted dye was separated from the labeled protein on the chromatographic column with Sephadex 50 as a packing and phosphate buffer pH 7.4 as an eluent. The collected solution of the labeled protein was kept at -20°C in aliquots of 1.5 mL.

The labeling densities of the protein molecules were determined using the UV-VIS adsorption of the conjugate molecules at wavelength 280 nm and 520 nm. Adsorption of the proteins in the UV region at 280 nm is due to the benzene rings of the aromatic amino acids. Adsorption in the visible region at 520 nm represents the dye molecules. The adsorption spectra at the above-mentioned wavelengths were taken using a UV-3101PC UV-VIS-NIR scanning spectrophotometer from Shimadzu. Figure 8.1 represents the adsorption intensities for the albumin and dye at different concentrations.

The concentration of the dye for the conjugate solution and the adsorption that resulted from the dye molecule at 280 nm was determined from the intensity of adsorption of the conjugate solution at 520 nm (Figure 8.1, a). The concentration of the protein was determined from the intensity of the adsorption of the conjugate solution at 280 nm, which was determined by expression \( E(8.1) \) and data from (Figure 8.1, b).

\[
I_{\text{protein}} = I_{\text{total}} - I_{\text{dye}} \tag{E(8.1)}
\]

The extent of labeling for protein molecules, \( \omega \), molecules of dye/molecules of protein was calculated using the formula \( E(8.2) \):
\[ \omega = \frac{c_{\text{dye}}}{M_{\text{dye}}} \times \frac{c_{\text{protein}}}{M_{\text{protein}}} \]

where \( c_{\text{dye}} \) is dye concentration, mg/ml; \( M_{\text{dye}} \) is the molecular weight of the dye, g/mole; \( c_{\text{protein}} \) is the protein concentration, mg/ml; \( M_{\text{protein}} \) is the molecular weight of protein, g/mole.

**8.3. Results and discussion:**

**8.3.1. Scheme for the imprint preparation:**

The approach developed for the preparation of the 2-D polymer imprint for specific protein adsorption is illustrated in Figure 8.2.

This procedure includes:
1) Preparation of the analyte template using a covalent attachment of the protein of choice to reactive groups in the anchoring layer (stages I, II; Figure 8.2), with sequential removal of the attached molecule such that the spatial position of the charged amino acids remaining will correspond to the spatial positions of the oppositely charged sites of the parent molecule (stage IV, Figure 8.2).

2) Creation of readsorption sites in the protein-repelling polymer matrix. A protein-repelling polymer brush was grafted to the remaining active groups of the primary polymer layer (stage III, Figure 8.2) around the grafted protein molecule. After the removal of the protein molecule (stage IV, Figure 8.2) the geometry (shape) of the readsorption sites obtained on the last stage of preparation should be contoured by the polymer brush and correspond to those of the template protein molecule as in stage II (Figure 8.2).

**Figure 8.2.** Procedure utilized for the preparation of template for specific protein adsorption. (I)–activation of the surface with grafted anchoring layer, (II) – protein adsorption and grafting to the reactive surface, (III) – polymer grafting to the residual surface functional groups around previously attached protein, (IV) – proteolytical treatment with enzyme, results in creation of the cavities in polymer film, which are geometrically and chemically complementary to the protein used in (II) stage.
Two types of molecular imprints were prepared following the procedure described in Figure 8.2. If stage III of the procedure was not performed, and stage II was directly followed by stage IV, a molecular imprint with only amino acid footprints was synthesized (MIPF). With all the steps of the procedure described in Figure 8.2 performed, the molecular imprint with the amino acid footprint (MIPF) and the polymer brush (MIPB) was synthesized. Both imprints were subjected to protein adsorption studies.

Figure 8.3. Adsorption points due to shape factor of the protein molecule. (1,2) – protein adsorption and grafting to the reactive surface. (3) – protein repelling polymer brush attached to the surface remaining space. (4) – shape of the readsorption site after protease treatment corresponds to this of template protein molecule.

Adsorption should be achieved with polymer imprint discrimination between protein shapes (Figure 8.3). The protein-repelling polymer brush should prevent proteins whose shapes do not correspond to the recognition sites from adsorbing on the imprinted surface. As a reactive layer for protein attachment, poly(glycidyl methacrylate) was used. As a protein-repelling matrix for protein footprints, poly(ethylene glycol) was used.
8.3.2. Activation of the surface with anchoring polymer layer:

PGMA was used to activate the initial solid support for the molecular imprint (stage I, Figure 8.2). This polymer is known to be readily deposited as a uniform layer on a large variety of organic and inorganic surfaces, such as activated silicon oxide, alumina, gold, poly(ethylene terephthalate), polyethylene, nylon, and others. Because of the chemical versatility of the epoxy group, the MIP technique developed using this approach will be universal for most solid supports. PGMA has also been chosen because of its reactivity with a majority of the chemical groups present in proteins, such as the amino, hydroxy, carboxyl and thiol groups. PGMA having one epoxy ring in each repeat unit chemically attach to the activated surface – imprint support. Parts of the macromolecule located in the “loops” and “tails” remain unused and available for further protein and polymer brush chemical attachments.

It has been shown that PGMA layers deposited by dip coating or spin coating and then annealed cause the auto-crosslinking of epoxy groups, and cannot then be removed from the silicon wafer using vigorous solvent treatment. Such layers were uniform and smooth with an AFM roughness ~0.3 nm on an area of 100 µm² (Ref. 5,6). The thickness of such a layer and the number of epoxy groups available can be varied by changing the concentration of the solution and the solvent used for layer deposition. For this study, silicon wafers were used as solid supports. Si-OH groups created by oxidation on the silica surface react with PGMA epoxy groups. A polymer layer with a thickness of 2±0.5 nm after annealing was used for the following protein-imprinted polymer constructions.
8.3.3. Protein attachment using a anchoring PGMA layer:

Two types of globular protein were used as template molecules: human albumin and chicken ovalbumin with molecular weights of 66 kDa and 44 kDa, respectively. Albumins are easily soluble in water, acetone, and ethyl alcohol, and are stable against denaturation. The two proteins are relatively close in molecular weight and shape, but they have different amino acid sequences. To bring the properties of albumins as close as possible to those in-vivo, a phosphate buffer solution with a pH of 7.4 was used as a solvent for the protein molecules with a protein concentration of 1 mg/mL.

A monolayer of the protein was deposited by dipping silicon wafers already coated with a crosslinked PGMA layer into a buffer solution of the analyte protein molecule (stage II, Figure 8.2). Protein molecules were adsorbed onto a reactive primary layer by the intermolecular interaction between the protein molecule and the polymer molecule. Only a monolayer of the protein is adsorbed onto the polymer surface, despite the protein solution concentration, because of the intrinsic repulsion of the biological molecules in the solution. Adsorption is inevitably followed by a reaction between the epoxy groups of the primary reactive layer and the free side chemical groups of the protein molecule, such as amino, alcohol, carboxyl or thiol groups, with the formation of stable chemical bonds.

After washing away the unattached protein, the wafers were kept in water vapor for 12 hrs. It is known that in the presence of a solvent vapor, a polymer’s chains gain mobility because of plasticization effects. The adsorbed protein monolayer thus undergoes water vapor post-treatment. Protein chains still chemically attached to the
polymer layer can attain their most favorable spatial configuration in the presence of water vapor. Additionally, during this treatment, chemical links between less reactive protein side groups and the polymer layer can be formed.

**Figure 8.4** represents AFM images of the monolayer of human albumin and ovalbumin. We can see from the images that proteins on the surface are present in the form of connected clusters of a few molecules. There is unoccupied space between the molecules, which can be used for PEG anchoring. The RMS roughness of the AFM image of the human albumin monolayer is 0.4nm/1.0° for topology/phase images (**Figure 8.4**). For ovalbumin, these values are 0.5nm/1.7°. The smaller roughness value for the larger protein may be due to geometrical rearrangements of the protein molecules adsorbed on the surface. The topographical roughness (describing the fluctuations of the surface heights around the average surface height) of the images and the Ellipsometrical measurements are of the same order as the measured size of the biomolecules (2.15 x 6.29 x 8.47 nm for ovalbumin\(^{10}\) and 5 x 5 x 9.5 nm for human albumin\(^{11}\)). The dry thickness of the complete ovalbumin monolayer, as measured by Ellipsometry, comes to 1.5±0.4 nm (3.41x10\(^{-23}\) mol/m\(^2\)); for human albumin, it is 1.9±0.3 nm (2.88x10\(^{-23}\) mol/m\(^2\)). The protein layer obtained appears to be uniform, with some unoccupied space between molecules that can be used for the subsequential modification of the unoccupied surface. From the data regarding the actual size of the molecules and assuming the molecule’s smallest dimension to be the height of the monolayer, we may predict that there is approximately 30% of unoccupied space between the protein molecules for ovalbumin and 62% for human albumin.
AFM images of the monolayer of proteins were used to calculate an average occupation area. Estimation was performed by averaging 5 randomly selected surface areas bearing measurements\(^{12}\) (percentage of the recovered area at a defined height) on NanoScope III 5.12.r3 software. The area covered by protein was found to be 43.2±1.6\% in case of human albumin and 42.9±2\% in case of ovalbumin. The remaining space was used to graft carboxyl terminated polyethylene glycol chains to form a protein-repelling surrounding around the adsorbed biological macromolecules.

**8.3.4. Grafting of the PEG to the PGMA surface:**

PEG chains were grafted around the protein molecules adsorbed and chemically bonded onto PGMA to fill the unoccupied space. PEG molecules will define the borders of the readsorption sites for the protein molecules. Space protected by PEG will become inaccessible for the protein molecules’ adsorption. In this way, control the spatial location of the adsorbed species can be done.

![AFM images of human albumin (a) and ovalbumin (b) monolayer, 1x1um. Topography (left) vertical scale 3nm, phase (right) vertical scale 10°.](image-url)
The PEG matrix was grafted to the unoccupied surface between protein molecules, which, as stated before, was 56.8% for human albumin and 56.1% for ovalbumin. A PEG-mono methoxyl ester with a Mn of 5,000g/mole was first modified with succinic anhydride according to the scheme shown in Figure 8.5. The solution of PEG in tetrahydrofuran was refluxed with a 5-fold excess of succinic anhydride for 7 hrs, and then the polymer was separated and purified by precipitation from ether.

The modified PEG was dissolved in methanol and then deposited onto the protein layer on the wafer by dip coating. For thermodynamic reasons, PEG tends to dewet from the PGMA surface when heated. To prevent dewetting during grafting and to denaturation or chemical alteration of the protein, solvent-assisted grafting of PEG was conducted.

The process was implemented in the presence of cyclohexane — a swelling solvent for PEG — and triethylamine (TEA) vapor. TEA was used as a catalyst to open the rings of the PGMA epoxy groups with the carboxyl groups of PEG. Using
cyclohexane to plasticize the PEG chains disturbs the crystallization of the polymer and increases its mobility. The mobility of the chains increases the possibility of interaction between the epoxy group of the primary layer and the carboxyl group of the polymer, and thus increases the grafting density of the PEG.

Ungrafted PEG was rinsed with methanol using ultrasonification. The thickness of the grafted layer for wafers with human albumin was 0.9±0.2nm; in the case of ovalbumin, it was 1.3±0.3nm. The AFM images of the monolayers of human albumin and ovalbumin with PEG grafted onto the unoccupied space between protein molecules are shown in Figure 8.6.

It was calculated that 56.8% of the surface area was available for PEG grafting after protein deposition in the case of human albumin, and 53.1% in case of ovalbumin. This increased the actual amount of the grafted PEG chains to 1.6ng/m² or 0.2chains/nm², and 2.5ng/m² or 0.3chains/nm² for human albumin and ovalbumin, respectively.

The roughnesses/RMSD of the phase shift shown in the images in Figure 8.6 was found to be 0.6nm/2.2° and 0.7nm/2.2° for human albumin and ovalbumin, respectively. The increase RMSD of the phase shift for both proteins as well as increase in roughness values can be explained by the presence on the surface of second different in intrinsic properties component - PEG. PEG chains may have been trapped between the protein molecules and, because of their thermodynamic incompatibility with protein molecules, segregated in the dry state into nanosized domains.
The change in the AFM roughness/RMDS of the phase shift of the images, together with the increase of the surface layer thickness measured by Ellipsometry, demonstrates the successful tethering of the PEG chains in between the protein molecules. After removing the protein molecules with protease, vacancies with a shape complementary to the protein are expected to be obtained in the PEG matrix (stage III, IV; Figure 8.2; Figure 8.10).

8.3.5. Labeling of the proteins with fluorescent dye:

Fluorescence is known to be an extremely sensitive method for the quantitative evaluation of biological molecules. Fluorescently labeled protein molecules to study the protease cleavage of biomolecules and for the evaluation of readsorption studies have been used.
Ovalbumin and human albumin were labeled with the fluorescent dye Atto Ester 520 (Figure 8.7), and purified using a standard procedure.\textsuperscript{14} This dye has high molecular adsorption (110,000) and quantum yield (0.90), shows little if any cis-trans isomerization and, due to its insignificant triplet formation rate, is well suited for the application of single molecule detection.\textsuperscript{15} Figure 8.7 represents the adsorption and emission spectra of the dye in ethanol. The dye molecule adsorbs and emits light at 520 and 540 nm, respectively, and the signals are well separated, making this dye suitable for protein quantification purposes.

![Figure 8.7. Chemical structure, absorption and fluorescence emission of Atto 520 in water of pH 7.0.](http://www.sigmaaldrich.com/fluka/product%20information%20sheet/77810_data_sheet_131kb.pdf (05/06/08)).

The amount of dye attached to the surface and the concentration of the obtained solutions of labeled dyes were determined using the calibration curves of dye and proteins from UV measurements (Figure 8.1). The labeled densities obtained for the protein molecules were in the range of 0.5-0.7 labels/molecule.
The dye was attached to the protein using its side amino group and formed a stable imide bond. Two amino acids that are part of the natural molecules supply the side amino groups in proteins — arginine and lysine. The overall number of side amino groups for ovalbumin is 140, and for human albumin 162. Only 0.3-0.4% of side amino groups for ovalbumin and 0.4-0.5% for human albumin react with the dye, and it can be expected that this small amount of change in the chemical structure will not affect the overall protein conformation.

8.3.6. Protease treatment of the protein monolayer:

Protein molecules have to be removed from the protein repelling polymer beds, so that the adsorption sites will be created. Protein molecules are constituted from peptide bonds that can be ruined by the harsh condition of alkali hydrolysis. Amide/peptide bonds are far more stable than the ester bonds that bind PEG molecules to the PGMA surface. This is why alkali hydrolysis cannot be used for the cleavage of protein molecules. Enzyme cleavage of peptide bonds proceeds at a wide range of pH levels and at a wide range of temperatures, depending on enzyme type.\textsuperscript{16} Subtilisin-A, a nonspecific protease,\textsuperscript{17} has been selected to remove the protein molecules from the PEG beds and for the creation of adsorption sites.

Treatment of the protein monolayer was carried out with subtilisin-A in a buffer solution with pH 7.4 at an enzyme concentration of 0.1 mg/ml and a temperature of 37 °C for 8 hrs. The Ellipsometry thickness of the remaining amino acid layer after the overnight protease treatment was 0.8±0.2nm for human albumin and 0.4±0.1nm for
ovalbumin. The percentage of the protein removal according to the fluorescence analysis was approximately 45% (Figure 8.8). The performance of the protease in the bulk solution and near the surface may vary because of steric hindrances; therefore, evaluation of enzyme activity was carried out to determine the optimal time of treatment. The active center of the enzyme is designed to hydrolyze only peptide bonds and not to be active toward the ester bonds in the polymer reactive layer and the imine, ether, or sulfide bonds between the protein and the polymer layer. This gentle treatment results in amino acids attaching to the surface of the PGMA, corresponding to their order in the protein molecule, without damaging the PGMA polymer itself.

![Graph](image.png)

**Figure 8.8.** Treatment of human albumin monolayer with protease. Dependence on time. (○)-percentage of treatment using height of fluorescent signal as input data. (□) – area of signal as input data.
Five wafers covered with a monolayer of labeled human albumin were treated for
different periods of time. To estimate the extent of the protein cleavage, the fluorescent
response from the wafers was used. As can be seen from Figure 8.8, no further decrease
in the fluorescent intensity was noticeable after 5 hrs of treatment. The remaining
fluorescent signal from the wafer can be attributed both to the incomplete cleavage of the
labeled protein, because spatial obstacles prevented the active center of the enzyme from
reaching the amide bond close to the dye attachment, and from the dye attached
chemically to the epoxy groups on the surface.

![Figure 8.8](image)

**Figure 8.8.** 1x1um images of MIPF of a) human albumin, b) ovalbumin. Left – topography image, vertical scale 10 nm, right – phase image, vertical scale 10°.

In Figure 8.9, MIPFs of the human albumin and ovalbumin are shown. Samples
were subjected to protease treatment in buffer pH 7.4 at temperature 37°C overnight. The
RMSD of the phase shift of the images decreased from 1.0° to 0.6° for the human
albumin monolayer and from 1.7° to 0.7° for ovalbumin. The roughness of the images
increased from 0.4nm to 0.9nm and from 0.5nm to 0.7nm for human albumin and
ovalbumin, respectively. The change in the characteristics of the images occurred due to
the partial disintegration of the protein layers. The partially cleaved protein molecules
resulted in the increase of surface roughness. Molecule parts no longer held together may cover areas of PGMA that were unoccupied before and decrease the RMSD of the phase shift of images. Changes of the appearance of the layer, together with the decrease of the fluorescent signal from the surface and the decrease of the layer thickness measured by Ellipsometry give evidence of the cleavage of the protein monolayer with subtilisin-A.

Figure 8.10. AFM image of the surface of 500x500 nm MIPB. a) – monolayer of human albumin, b) – human albumin monolayer with PEG brush grafted between protein molecules and treated with protease for 8hrs. Topography (left) vertical scale 3 nm, phase (right) vertical scale 1.0°.

The AFM studies of the protein surface treated with the protease show a significant decrease in the size of the grains on the surface for PEG containing MIPB (Figure 8.10). The grain size on the images, calculated with nanoscope software (five random measurements), showed a decreased from a mean area of grains of 541±15 nm² for the human albumin monolayer (agglomerate of 11 molecules) to 236±27 nm² for the molecular imprint in the PEG brush for human albumin. These numbers were 941±80 nm² (agglomerate of 17 molecules) and 311±35 nm², respectively, for the ovalbumin imprint.
The RMS roughness of the imprint after protease treatment, calculated from the AFM image, was 1.0 nm/1.1° for human albumin and 0.7 nm/0.7° for ovalbumin. At the same time, the roughness of the protein imprint inside the poly(ethylene glycol) brush was 0.7 nm/2.2° for human albumin and 0.6 nm/2.2° for ovalbumin.

Five bearing measurements of the surface area of the images revealed that 52.8% and 51.4% of the area can be attributed to the imprinting sites of the protein created for human albumin and ovalbumin, respectively. These numbers are comparable with the fraction of the area occupied by proteins at the beginning of the imprint preparation procedure.

The decrease in roughness RMSD of the phase shift as well as the decrease in the calculated average grain size is connected with the removal of the protein from the surface. PEG agglomerates not supported by neighbor protein molecules may collapse onto the surface and partially cover the surface, decreasing surface roughness and the deviation of the phase shift. All of the above-mentioned changes in the AFM image and Ellipsometric thickness of the layer suggest the successful preparation of the molecular imprint in the PEG brush.

8.4. Conclusions:

It was confirmed that the 2-D protein imprint can be synthesized following the scheme underlined in Figure 8.2. Usage of the PGMA as an anchoring layer for the imprint preparation widens the choices of substrates for the preparation of imprinted surfaces. A polymer surface, as well as textile and metals, can be used for support.
The protein monolayer is easily deposited onto the PGMA layer from the buffer solution. There is approximately 55% of unoccupied space left on the PGMA layer after protein deposition. After deposition, protein becomes covalently bound to the surface and cannot be removed without bond breakage.

Carboxyl-terminated PEG chains were grafted into the open space between the protein molecules. Solvent-assisted grafting of the polymer brush removes the necessity of applying harsh conditions to achieve the grafting of the polymer. The application of triethylamine in addition to cyclohexane vapors catalyzes the reaction of the epoxy ring opening and increases the rate of grafting. Additionally, solvent-assisted grafting decreases possibility of the denaturation of the protein molecules during the process.

Subtilisin-A has been proven an effective tool for the cleavage of protein molecules adsorbed on the surface. It was determined that after 5 hours of treatment, there is no more available protein left for cleavage. Enzyme treatment breaks highly stable amide bonds in ambient conditions, while it spares ester bonds, which are essential for imprint preparation.

Prepared protein imprints were used for the adsorption studies.

8.5. References:


(8) www.rcsb/pdb/.(05/07/08)


(15) http://www.atto-tec.com/ATTO-TEC.com/Products/documents/ATTO%20520.pdf (09/02/08)


CHAPTER NINE

STUDIES OF THE PROTEIN ADSORPTION ON THE IMPRINTED POLYMER BRUSH

9.1. Introduction:

Evaluation of the adsorption properties of a surface for protein molecules is a tedious procedure due to the small quantities of the adsorbed substance per surface area. There are a few methods that are routinely used for protein adsorption measurements. These include quartz microbalance, fluorescence, XPS, Ellipsometry, and chromatography combined with UV-VIS spectrophotometry.

Protein adsorption to 3-D MIPs is often evaluated using liquid affinity chromatography. A chromatographic column is prepared with packing from MIP, and then a solution of the proteins is flushed through the column. The concentration of the protein of interest is measured before and after the column. In this way, binding capability and efficiency of the MIP is evaluated.

For a smaller amount of MIP prepared, when there is not enough material for chromatographic column loading, researchers often incubate the imprinted material with the proteins and then measure amount of material adsorbed. For very precise measurements of the readsorption capability of the MIP, fluorescent imaging was employed by Ikawa et al.

All of the previously mentioned methods have their advantages and, in general, complement each other’s results. Chromatography analysis reveals the efficiency of the
imprint in dynamic conditions when there is rapid motion of the analyzed molecules near recognition sites. The incubation method gives a picture of readsorption when conditions are stationary and protein molecules have more time to reorient themselves toward the recognition site for more favorable interactions. Fluorescence is a very sensitive method and allows separate molecules on the surface to be captured in qualitative as well as quantitative ways.

In this work, the strategy used for the study of protein adsorption on the MIPs for the evaluation of protein adsorption on the prepared MIPF and MIPB was followed. We employed fluorescent labeled protein to determine the adsorption efficiency in stationary as well as dynamic conditions, i.e. the incubation method and TIRF (total internal reflectance fluorescence).

For stationary methods, MIPF and MIPB on microsized glass beads were prepared and incubated with the protein solutions. The solutions were then checked for decreases in the intensity of the fluorescent signal after incubation. In a static experiment, a protein molecule is given time to rearrange itself to attain the most thermodynamically favorable conformation for adsorption.

In the dynamic, TIRF method, MIP on the surface of the glass slide were prepared and adsorption of the fluorescent labeled protein molecules on the MIP surface under constant flow conditions was monitored. The protein solution was pumped through the system at a constant rate; knowing the geometry of the cell, the shear rate of the protein solution at the MIP surface was calculated. In the dynamic experiment, interactions between the protein and surface are rapid, and there is limited or no time for protein
rearrangement; hence, there less probability for the development of strong interactions between the protein molecules and the surface.

The results reported in this chapter should be considered as preliminary, since only one experiment of each kind was conducted.

9.2. Experimental:

Fluorescent spectra of the samples were taken on the fluorescent spectrophotometer Fluorolog III (Horiba Jobin Yvon). Spectra were analyzed (baseline, peak area, height) with DataMax-32 software. TIRF measurements were performed on a custom-made machine with a laser excitation wavelength of 532 nm.

Two types of surfaces for readsorption studies were prepared. The type I surface did not contain PEG chains (MIPF) and was synthesized without the PEG-grafting step. The type II imprint did contain PEG chains (MIPB) grafted to the surface.

For the stationary experiment, a molecular imprint was prepared on glass beads with a diameter of 75 um. The imprint was prepared as described previously.

Four types of solutions in phosphate buffer pH 7.4 were prepared for the experiment:

- Labeled human albumin (0.0037mg/ml)
- Labeled ovalbumin (0.0031mg/ml)
- Mixture of labeled human albumin and unlabeled ovalbumin (1:1, 0.0035mg/ml total)
• Mixture of unlabeled human albumin and labeled ovalbumin (1:1, 0.0030 mg/ml total)

For the static readsoption experiment, 2.5 g of molecularly imprinted beads of each protein (human albumin and ovalbumin) were immersed into 5 mL of each solution. To evaluate the extent of adsorption, the initial solutions’ signal ($I_0$) and the signal after readsoption ($I$) were collected on the Fluorolog III. The amount of the protein adsorbed $m$, (mg/g) was calculated according to the formula:

$$m = \frac{(I_0 - I) \times c \times 5}{I_0 \times 2.5} \quad (E9.1)$$

where $c$ is the concentration of the labeled protein in the solution, (mg/mL); 5 mL is the volume of the solution used for each experiment; 2.5 g is the weight of the molecularly imprinted glass beads used for experiment.

For the dynamic adsorption experiment (by TIRF), the following solutions were prepared in a phosphate buffer pH 7.4 (0.01M):

• Labeled human albumin, 100 ppm (0.1 mg/ml);
• Labeled ovalbumin, 70 ppm (0.07 mg/ml);
• Mixture of labeled human albumin and unlabeled ovalbumin (50:35 ppm);
• Mixture of labeled ovalbumin and unlabeled human albumin (35:50 ppm);
• Labeled fibrinogen, 250 ppm (0.25 mg/ml);
• Mixture of labeled fibrinogen and unlabeled human albumin (250:50 ppm)
9.3. Results and discussion:

9.3.1. Evaluation of the MIPF efficiency through stationary method:

Two type of proteins, human albumin and ovalbumin, were labeled with fluorescent label Atto ester 520. The label is chemically attached to the protein molecule via the formation of an amide bond. The extent of labeling was 0.5-0.7 (number of dye molecules per molecule of protein), which is 0.3-0.4% of the side amino groups for ovalbumin and 0.4-0.5% for human albumin. This amount of labeling gives a sufficient signal for detection on the Fluorolog III spectrophotometer used for these studies, and should not alter the intrinsic properties of the biological molecules\textsuperscript{15}. Fluorescent labeling is widely used to monitor vital processes and no significant changes in the performance of biomolecules is expected to be noticed, as the extent of the labeling is low and presence of one dye molecule (~0.5 kDa) attached to the biomolecules is negligible compared to the size of biomolecules itself (44/66 kDa).

Preparation of the MIP on the glass beads was achieved following the same procedure as for the silicon wafer. It was assumed that the geometry of the imprint on the glass beads was the same as for the model system with the silicon wafer. Two types of glass beads were prepared. Type I contained an amino acid footprint of protein molecules (MIPF) on the surface. In type II, the PEG brush was grafted to outline the borders of each imprint, as was described previously (MIPB).
9.3.2. Stationary experiment of the MIPF protein adsorption:

As already shown, protein molecules may undergo slight spatial changes when adsorbed to a surface\textsuperscript{16}. Hence, when allowed to adsorb slowly, the protein may attain the most favorable conformation, and MIPF may exhibit a higher affinity to the protein molecules of interest.

1 g of glass beads with a diameter of 75 μm and a density of 2.2 g/cm\textsuperscript{2} has a surface area of 0.0646 m\textsuperscript{2}. The maximum protein coverage (considering that the density of the dry protein is equal to 1 g/cm\textsuperscript{2}) is ~1.49 ng/m\textsuperscript{2} for ovalbumin and ~1.92 ng/m\textsuperscript{2} for human albumin or 9.62x10\textsuperscript{-5} g and 12.40x10\textsuperscript{-5} g to cover 1 g of beads with a dense uniform layer of 1.49 nm and 1.92 nm of ovalbumin and human albumin, respectively.

The results of the experiment with the MIPF prepared on glass beads are shown in Figure 9.1. As can be seen from the data (Figure 9.1a, b, d), there is some remaining affinity of the protein molecules to its amino acids. However, it may be noticed that ovalbumin molecules prefer to adsorb to the human albumin footprints rather than their own (Figure 9.1, c). While it is difficult to give an exact explanation for such a behavior, it may be speculated that some specific affinity of the molecule to the human albumin amino acid footprint is decreased in the presence of another protein in the mixture. For the human albumin solution, more protein is adsorbed from the pure solution compared to the mixture on both types of templates. No such dependence is noticed for ovalbumin; moreover, there was larger amount of labeled ovalbumin adsorbed from the protein mixture than from the pure solution of ovalbumin.
Table 9.1 represents the quantitative amount of the protein adsorbed compared to the initial amount of labeled protein. Ovalbumin adsorbs similarly to the human albumin and ovalbumin MIPFs. However, in the mixture with human albumin, this amount decreases. The greater extent of adsorption of the ovalbumin to the MIPFs may be explained by the smaller size of the molecule and, therefore, its higher mobility. Human

Figure 9.1. Adsorption studies of the human and ovalbumin aminoacid footprints (MIPF). Letters on the top of each graph correspond to the solution used for readsorption and labels on the x-axis of each graph – the protein used for MIP preparation. LHUA – solution consisted of labeled human albumin, LHUA/OVA – mixture of labeled human albumin and unlabeled ovalbumin, LOVA – labeled ovalbumin solution, HUA/LOVA – solution of mixture of unlabeled human albumin and labeled ovalbumin.
albumin is a larger molecule and adsorbs less. Adsorption of the protein itself to the surface is a very complex process, but adsorption from the mixture becomes even more complicated, and now depends not only on the interaction of the protein with the surface but also on the interaction of the protein with another protein in the mixture\textsuperscript{17,18}. However, it may concluded from the data in Table 9.1 that amino acid footprints could be successfully used as a substrate for protein adsorption.

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<td>0.00044</td>
<td>0.00016</td>
<td>0.00048</td>
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</tbody>
</table>

9.3.3. Stationary experiment of the MIPB protein adsorption:
When MIPB template silicone wafers with the poly(ethylene glycol) grafted to the surface were subjected to the same experiment, the results, in addition to the capacity for adsorption ability, have shown the recognition capacity of the imprint for all cases (Figure 9.2).
Figure 9.2. Readsorption studies of the human and ovalbumin amino acid MIPF templates. The letters on top of each graph correspond to the solution used for readsorption and Labels on the x-axis of each graph – to the protein on the substrate. LHUA – labeled human albumin solution, LHUA/OVA – mixture of labeled human albumin and unlabeled ovalbumin, LOVA – labeled ovalbumin solution, HUA/LOVA – mixture of unlabeled human albumin and labeled ovalbumin.
When stationary adsorption of the protein molecule is performed onto the molecular imprint containing poly(ethylene glycol) as a protein adsorption-controlling agent, a distinct selectivity of the imprint is noticed. In the case of the solution of labeled human albumin adsorbed onto the surface, 57% more protein was adsorbed onto the human albumin imprint than onto the ovalbumin imprint. This was also noticed with ovalbumin: 43% more ovalbumin was adsorbed onto its own imprint than onto the human albumin imprint. Although albumins are considered to be globulins, this does not mean that these proteins have an ideal spherical shape. Each globulin shape deviates from the spherical, and for human albumin it is more heart-shaped, as human albumin consists of two protein chains bundled together (Figure 9.3a). Ovalbumin has a geometrical form closer to the spherical (Figure 9.3b). Because of geometrical deviations, there is the possibility that proteins will fit into the imprint of another protein molecule, even though
the interactions with the imprinting site may not be as effective as for the parental protein with its imprint.

Competitive adsorption studies of the imprint surface revealed the same trend as one-component studies, but with less selectivity. The labeled ovalbumin mixed with unlabeled human albumin in a 1:1 mass proportion was adsorbed onto its parental imprint only 3% more than onto the human albumin imprint. Meanwhile, the mixture of labeled human albumin with unlabeled ovalbumin adsorbed onto the parental imprint 39% more than on the ovalbumin imprint. The difference may lay in the size difference of the proteins. The molecular mass of human albumin is 66kDa, while ovalbumin has 44kDa. The larger size of the human albumin molecule results in larger sized imprint sites. Therefore, because ovalbumin has smaller geometrical parameters, it can fit into the human albumin imprinting sites, occupying them and decreasing the available sites for human albumin adsorption. In contrast, large-sized human albumin occupies vacant sites on the ovalbumin imprint on the surface slowly, so that ovalbumin is prevalently adsorbed to the surface. Even if the proteins are close in size, it is expected that the molecule that fits best into the imprint site will bear the largest energetic gain and will be preferentially adsorbed to the surface\textsuperscript{19}.

Molecular imprints prepared using polymer brushes can be used for controlled protein adsorption on a surface, and can also exhibit a certain amount of selectivity towards the protein used for the imprint preparation.
9.3.4. Dynamic studies of MIPFs and MIPBs performance:

The main difference between the static and dynamic methods of adsorption is that in the dynamic method, adsorption is obtained from flow with a short amount of time allowed for interaction between the protein molecules and the surface. If the attractive forces do not develop between the molecule and surface in this short period of time, the protein molecule will be carried away with the flow. In this case, there is no statistical chance for the molecule to interact with the same area again, unlike in the static method. In the static method, the time of interaction between the surface and protein molecule is limited only by Brownian motion and, if attractive interactions do not develop, there is the possibility for the molecule to come into contact with the same area again.

9.3.4.1. Adsorption of the monolayer of proteins onto hydrophobic reactive surface for quantification of subsequent protein adsorption results:

First, adsorption of Rhodamine B labeled human albumin and ovalbumin onto the hydrophobic and reactive surface made of a crosslinked copolymer of poly(glycidyl methacrylate)-co-poly(butyl methacrylate) of 3.5±0.5 nm thickness was studied. A glass slide was used as a support for the polymer layer. This polymer surface has a water contact angle of 78±2°. It attracts protein molecules because of hydrophobicity. Additionally, the epoxy groups of glycidyl methacrylate permanently bind adsorbed molecules through reaction with protein side chain amino groups. The results of the adsorption are shown in Figure 9.4a, b. Proteins were dissolved in 0.01M phosphate buffer of pH 7.4. Concentrations of the protein solutions used in this part of work were...
100 ppm for human albumin and 70 ppm for ovalbumin. This ratio of concentrations results in approximately the same amount of protein molecules per certain volume, i.e. 1.52x10^{-6} \text{mol/L} for human albumin and 1.59x10^{-6} \text{mol/L} for ovalbumin.

While both proteins at first sight exhibit a transport-limited type of adsorption, the results of fitting the adsorption parameters into the transport-limited model for protein adsorption led to an amount of adsorption that was in disagreement with the Ellipsometry measurements and clearly did not represent the absolute value of protein adsorption.

Because quantification of the adsorbed protein could not be done through model fitting, we have measured the amount of the protein adsorbed onto the silicon wafer covered with the crosslinked PGMA-co-PBMA layer by Ellipsometry. These results are consistent with the monolayer thickness measured on the pure PGMA layer: 1.9 nm for

**Figure 9.4.** TIRF measurements of the adsorption of RhB labeled proteins on PGMA-PBMA surface. Human albumin (a), and ovalbumin (b). Labeling density of the human albumin 0.51 lab/molecule, ovalbumin – 0.56 lab/molecule. Concentration of the human albumin 100 ppm and ovalbumin 70 ppm. Proteins were dissolved in 0.01M phosphate buffer pH 7.4. 1-addition of buffer; 2-addition of the unlabeled protein used for adsorption study; 3-addition of another unlabeled protein.
human albumin and 1.5 nm for ovalbumin. The coefficients for the calculations of the amount of protein adsorbed onto the surface through the detector/laser ratio obtained directly from TIRF measurements were found to be 1.50 for human albumin and 1.39 for ovalbumin.

The subsequent results in this chapter were obtained by multiplying the detector/laser signal ratio obtained by these coefficients.

During the experiment, it was determined that adsorption of the albumins used in this study to the hydrophobic and reactive surface is practically irreversible under conditions employed. The first dashed line on Figure 9.4a, b corresponds to the addition of buffer without any protein. A small decrease in the fluorescent signal is observed, and then the signal levels. This decrease in the signal is due to the removal of the unbound protein present in the solution close to the surface. The second dash-dot line in both graphs corresponds to the addition of 100 ppm of unlabeled human albumin (Figure 9.4a) and 70 ppm of unlabeled ovalbumin in Figure 9.4b. The third, dash-two dots line corresponds to the addition of 70 ppm of unlabeled ovalbumin for graph (a) and 100 ppm of unlabeled ovalbumin for graph (b). As can be seen from the graphs, the protein adsorbed on the surface is replaced neither with the unlabeled protein, nor with another type of albumin for either case. Replacement would happen if the adsorbed protein was in dynamic equilibrium with the protein in the solution and protein in the solution would have higher affinity to the surface than adsorbed protein.

Adsorption of the labeled protein from the protein mixtures was also studied to clarify the behavior of the protein on the molecular imprint surface. Two mixtures were
used for the study: mixture I contained 50 ppm of labeled human albumin and 35 ppm of ovalbumin; mixture II contained 50 ppm of unlabeled human albumin and 35 ppm of labeled ovalbumin. The results of the studies are presented in **Figure 9.5 a, b**.

![Graphs showing adsorption of proteins](image)

**Figure 9.5.** Adsorption of the proteins onto the PGMA-co-PBMA from 0.01M phosphate buffer pH 7.4. A) – mixture of labeled human albumin (50 ppm) and unlabeled ovalbumin (35 ppm), b) – mixture of labeled ovalbumin (35 ppm) and unlabeled human albumin (50 ppm). Dashed line corresponds to the injection of buffer.

Overall, the total amount of protein adsorbed onto the surface decreases slightly when adsorbed from a mixture of labeled and unlabeled protein. From the graphs in **Figure 9.5** we may see that there is only a 39% decrease in the adsorption of human albumin and a 14% decrease in the adsorption of ovalbumin. It may be assumed from these results that a labeled protein may have a higher affinity to the surface than an unlabeled one. However, because the ratio in which proteins are adsorbed from the mixture depends on the protein itself, the surface used for adsorption, as well as the composition of the mixture, and because this is a very complex process, this conclusion may not be absolute. At the same time, this adsorption is finite – no more than a monolayer of each protein is adsorbed to the surface. The total amount of the labeled
human albumin adsorbed from the mixture was 1.17 mg/m$^2$, and that of labeled ovalbumin was 1.26 mg/m$^2$.

**9.3.4.2. Adsorption study of the human albumin MIPF surface:**

To determine the affinity of the protein molecules to the remaining amino acids from the human albumin, a human albumin imprint on a glass slide was prepared according to the procedure described in the previous chapter. The sequence of the amino acid in each protein type is unique, and after protease treatment, it is expected to obtain a spatial pattern that is unique for each protein on the surface. This spatial placement of amino acids may become a key factor for the molecular recognition of the parental protein molecule.

A prepared molecular imprint of human albumin was subjected to an adsorption study with 100 ppm human albumin solution and 70 ppm ovalbumin solution. The results of this experiment are given in Figure 9.6 a, b. The results show that for the case of human albumin, 0.79 mg/m$^2$ of the protein was adsorbed (41% of monolayer) and 0.59 mg/m$^2$ (40% of monolayer) ovalbumin was adsorbed.

Human albumin does not show any weakly bound protein on the surface, as there is no decrease in the signal when a buffer solution is injected into the stream (dashed line on graph (a)). Adsorbed human albumin does not exist in a dynamic equilibrium with the protein in the solution, as no decrease in the signal was noticed when the unlabeled protein was injected (dash-dotted line on graph (a)).
Ovalbumin, at the same time, shows the presence of a protein weakly bound to the surface. After the addition of the buffer to the system, a decrease in the intensity of the fluorescent signal can be seen. The protein adsorbed onto the surface cannot be exchanged with the unlabeled protein (point of injection – dash-dotted line).

**Figure 9.6.** Adsorption of the proteins from 0.01M phosphate buffer onto human albumin aminoacids remainings surface. A) – human albumin (100ppm), b) – ovalbumin (70ppm). 1-buffer injection; 2-unlabeled protein injection.

Ovalbumin, at the same time, shows the presence of a protein weakly bound to the surface. After the addition of the buffer to the system, a decrease in the intensity of the fluorescent signal can be seen. The protein adsorbed onto the surface cannot be exchanged with the unlabeled protein (point of injection – dash-dotted line).

**9.3.4.3. Adsorption study of the human albumin MIPB surface:**

The presence of poly(ethylene glycol) on the surface of the imprint resulted in a certain selectivity of the imprint in the stationary experiment. Dynamic measurements differ, as there is a much smaller amount of time in which the molecule is allowed to rearrange itself to win the free unoccupied imprinting site on the surface. If forces between the recognition site and the protein molecule do not develop during this limited period of time, the protein molecule will be repelled from the surface by the poly(ethylene glycol) chains that surround the imprinting site.
A human albumin imprint with poly(ethylene glycol) chains was prepared as described in the previous chapter. The results of the adsorption of the human albumin and ovalbumin are shown in Figure 9.7 a, b.

Only 4% of the monolayer of the human albumin (0.07 mg/m$^2$) was adsorbed onto its imprint, and 12% of monolayer of ovalbumin (0.18 mg/m$^2$) was adsorbed. Both human albumin and ovalbumin showed a decrease in the fluorescent signal with the addition of the buffer, and no decrease when the unlabeled protein was added, showing that there is no dynamic equilibrium between the protein adsorbed onto the surface and the protein in the solution.

Competitive adsorption onto the human albumin MIPB was accomplished using a mixture of labeled and unlabeled proteins. The concentration of the mixture was 50 ppm for human albumin and 35 ppm for ovalbumin. The results of the studies are shown in Figure 9.8.
The protein mixture with the labeled human albumin and unlabeled ovalbumin shows a decrease in the fluorescent signal when subjected to buffer flow. 0.17 mg/m$^2$ of labeled human albumin was adsorbed onto the surface (9% of the monolayer). Ovalbumin mixture adsorption did not show a decrease in the signal with the addition of the buffer, and resulted in 0.20 mg/m$^2$ adsorption (14% of the monolayer).

**9.3.4.4. Adsorption study of the ovalbumin MIPB surface:**

Because the size of the molecular imprinting site for ovalbumin is smaller than for human albumin, we also studied the performance of the ovalbumin MIPB. The results of the adsorption of the labeled human albumin and ovalbumin onto the surface of ovalbumin MIPB are shown in **Figure 9.9a, b**.

The amount of labeled protein adsorbed to MIPB was 0.07 mg/m$^2$ (4% of the monolayer) for human albumin and 0.24 mg/m$^2$ (16% of the monolayer) of ovalbumin.
Both experiments show a decrease in fluorescent intensity when the protein is subjected to a buffer solution (dashed line). There was no more decrease in the signal, i.e. no protein available for exchange, when the unlabeled protein was injected into the stream.

**Figure 9.9.** Adsorption of the protein in 0.01M phosphate buffer onto the ovalbumin MIPB. a) – labeled human albumin (100 ppm); b) – labeled ovalbumin (70 ppm). Dashed line corresponds to the buffer injection.

The competitive adsorption of the protein mixture is shown in **Figure 9.10 a, b.** Human albumin adsorbed from the mixture in the amount of 0.27 mg/m$^2$ (14% of the monolayer), while ovalbumin adsorbed from the mixture in the amount of 0.19 mg/m$^2$ (13% of the monolayer). In both experiments, there is a decrease in the fluorescent signal when the buffer solution is injected into the stream. After the weakly bound protein was removed with the buffer stream, no more desorption was observed. In addition, the adsorbed proteins cannot be replaced with other unlabeled proteins from the solution.
9.3.4.5. Summary of the adsorption of the human albumin and ovalbumin on their MIPFs and MIPBs:

The results of the adsorption study of the protein MIPs, summarized in Table 9.2, show that MIPF can adsorb larger amounts of the protein than MIPB. While for MIPF, the extent of the adsorption was around 40% of the monolayer, for MIPBs, these numbers ranged from 4 to 16% of the monolayer. Human albumin adsorbed in smaller amounts (4-14% of the monolayer) than ovalbumin (12-16% of the monolayer) onto MIPBs. This difference probably comes from the size and shape of the molecule. Since ovalbumin is oval-shaped and has a symmetry axis, it does not need specific rearrangements to fit into the adsorption site, while human albumin, which is heart-shaped, should approach the adsorption site in the same manner in which the initial molecules were adsorbed. Human albumin adsorbs more into adsorption sites when competitive adsorption is complete than

Figure 9.10. Competitive adsorption of the mixture of proteins from 0.01M phosphate buffer. a) – labeled human albumin (50 ppm) mixed with unlabeled ovalbumin (35 ppm), b) - labeled ovalbumin (35 ppm) mixed with unlabeled human albumin (50 ppm). 1-buffer injection, 2-unlabeled protein injection.
when it is adsorbed from a mono solution. It may happen that when positioned into the
adsorption site, the ovalbumin molecule “opens” the adsorption site covered by the PEG
brush for the attachment of another, probably larger, molecule. We should recall here
that adsorption sites are not created from a single molecule, but from molecular
agglomerates (see Chapter 8). Hence, if the small ovalbumin molecule is adsorbed in
random sites on the adsorption site, this may result in PEG chains segregating from
protein molecule due to thermodynamic incompatibility, as well as the exposure of the
adsorption site for subsequential attachment.

Table 9.2. Protein adsorption studies results for the MIPF and MIPB of human albumin
and ovalbumin.

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246
9.3.4.6. Adsorption study of the human albumin MIPB surface with large protein

(bovine fibrinogen):

Human albumin and ovalbumin are close in size and properties. To verify the performance of an imprint in the presence of the larger protein, we performed an adsorption study for the PEG containing MIP with bovine fibrinogen (330 kDa).

First, labeled bovine fibrinogen was adsorbed onto the glass substrate to evaluate the intensity of the fluorescent signal from the monolayer of protein. The results of the adsorption of fibrinogen onto glass are shown in Figure 9.11.

Fibrinogen adsorbs in the amount of 4.52mg/m$^2$ in monolayer form onto the glass surface (Figure 9.11, a). When subjected to adsorption onto the PEG-containing human albumin MIP, 2.92 mg/m$^2$ gets adsorbed onto the surface (65% of the monolayer) (Figure 9.11, b).

When mixed with unlabeled human albumin, fibrinogen adsorbs in the amount of 3.72 mg/m$^2$ (82% of the monolayer) (Figure 9.11, c). In the case of a mixture with labeled human albumin, first, human albumin is adsorbed onto the surface in the amount of 0.43 mg/m$^2$ (22% of the monolayer), and then the adsorbed protein is gradually replaced by unlabeled molecules of fibrinogen. The desorption of the human albumin molecules continues after the buffer solution is added to the stream.

The difference between the adsorption results in static and dynamic methods may be explained by the difference in how the protein molecules approach the surface.

When studied with fibrinogen, 0.2 chains/nm$^2$ adsorbed 2.8nm (56.9% of the monolayer) of fibrinogen and 1.7nm (36.2% of the monolayer) was adsorbed onto the 0.3
chains/nm$^2$ of PEG (see Chapter 7). Hence, the PEG brush that is present on the surface of MIP is not effective in the prevention of the protein adsorption of the large molecules. To improve the performance of MIPB for large molecule adsorption, a dense PEG brush should be synthesized on the surface.

Figure 9.11. Adsorption of the protein onto the surface. A) – glass, fibrinogen 250 ppm; b) – human albumin MIP, fibrinogen 250 ppm; c) – human albumin MIP, mixture of labeled fibrinogen (250 ppm) and unlabeled human albumin (50 ppm); d) – human albumin MIP, mixture of labeled human albumin (50 ppm) and unlabeled fibrinogen (250 ppm).

Additionally, instead of adsorption sites prepared from molecular agglomerates, a single molecule adsorption site with a large distance between the adsorption sites should
be synthesized for better control of protein adsorption. This may be achieved by increasing the time/temperature of the PEG grafting as well as more effective epoxy ring opening catalyst applications. The change in the distance between molecules may be achieved by decreasing the protein solution concentration used for imprint preparation and decreasing the time of the adsorption in stage II of imprint preparation\textsuperscript{20,21} (Figure 8.2, Chapter 8).

\textbf{9.4. Conclusions:}

The molecular imprinting technique can be used to regulate the extent of protein adsorption on the surface. Imprints containing protein-repelling polymer PEG on the surface exhibited lower adsorption than without PEG. Hence, the overall amount of the protein adsorbed to the surface may be regulated by the size of prepared adsorption sites and the grafting density of the PEG.

From the results of the adsorption of the proteins from the mixture, it may be suggested that there is a certain preference for the adsorption of the labeled molecules to the surface compared with the unlabeled ones; however, additional experiments have to be done to support this hypothesis.

The prepared surface was found to adsorb proteins with a large molecular weight (fibrinogen) in quantities equal to 66\% of the monolayer.

Molecularly imprinted surfaces with PEG-grafted chains have shown selective adsorption toward the parental protein molecules in the static adsorption experiment. No such effect was noticed for the dynamic experiment. This may arise from the difference
in experimental designs. Apparently, from the results obtained, it may be concluded that the selectivity of the MIPBs is present when there is enough time for the molecule to rearrange to attain the same geometrical position as the molecule used for imprint preparation. Rapid interactions that develop between the molecule and the MIP surface in a dynamic experiment do not result in any specificity.

Additional experiments have to be run to confirm all of the above statements, as only one experiment of each kind was performed.

9.5. References:


CHAPTER TEN

SUMMARY

The work presented here provided results on synthesis of mixed polymer brushes and molecularly imprinted polymer brushes. The outlined results provide a basis for future development of polymer brush modified surfaces with controllable properties toward protein adsorption.

10.1. Synthesis of mixed poly(ethylene glycol)/polyacrylic acid-b-polystyrene mixed polymer brushes:

Mixed polymer brushes were prepared using the “grafting to” technique and a temperature gradient with a constant grafting density of polyacrylic acid-b-polystyrene and varying grafting densities of poly(ethylene glycol). The composition of the final brush assembly was estimated by Ellipsometry and IR Ellipsometry. Nonlinearity was observed in the dependence between the tethered amount of poly(ethylene glycol) and temperature used for grafting.

One main reason for the nonlinearity of the extent of PEG grafting with temperature was found to be the diffusivity of the polymer chain ends to the surface. The rate of reaction was found to significantly affect the extent of grafting at higher temperatures, starting at 125°C. With polyacrylic acid grafting, the rate of reaction determined the extent of grafting, while diffusivity of the chains played a minor role across the temperature range selected.
The minimal height of the pseudotails of the grafted polyacrylic acid was calculated to be 25 monomeric units, when no PEG was present in the brush. This ranged from a minimum of 39 units to a maximum of 373 units when PEG grafting density on the surface was 1.2 chains/nm$^2$ (the maximum grafting density of PEG used in this work).

10.2. Responsive behavior of mixed polymer brush: organic solvent treatment to form mixed brush structures:

The mixed polymer brushes exhibit a switching behavior and their surface properties can be tuned using solvents such as toluene, methyl ethyl ketone (MEK), ethanol, and dimethylformamide (DMF). Switching of the brushes was confirmed by studying the surface morphology with AFM and measurement of the surface contact angle. Roughness of the samples as well as RMSD (root mean square deviation) of their phase shift depends on the solvent used for treatment and on the composition of the brush.

Measured water contact angles for solvent treated mixed polymer brushes were significantly higher than the calculated values, and this discrepancy was explained by an uneven distribution of the brush components on the surface after the solvent treatments. Toluene is a good solvent for polystyrene and resulted in the highest water contact angle. The brush treated with DMF and ethanol, good solvents for hydrophilic polyacrylic acid, showed the lowest water contact angle.

While calculations of water contact angle of PEG/PS mixtures showed that with the increase in the amount of PEG, the contact angle should decrease, the opposite behavior in the experiment was observed. Poly(ethylene glycol) played an important role
for the switching behavior of the mixed polymer brush, causing additional stretching of the polyacrylic acid chains and bringing polystyrene formations to the surface.

10.3. Morphology and extension of the mixed polymer brushes under the water:

Behavior of the mixed polymer brush in an aqueous medium depended on the solvent with which it was treated. Treatment of the brush with MEK gives the highest brush mobility/extension. Ethanol treatment results in the obstruction of the brush extension in the water medium. The size of the polystyrene inclusion also depends on the type of solvent with which the brush was treated. Treatment with MEK gives the smallest polystyrene lateral domain size, while ethanol results in the largest polystyrene domains.

Poly(ethylene glycol) chain extension in the presence of electrolytes has a minor impact on the overall mixed polymer brush extension, and the increase in the height of the brush should be attributed mainly to the block copolymer.

As well as treating mixed polymer brushes with organic solvents, treatment with electrolytes also affects its properties. The presence of calcium ions in the system results in a higher extension of the mixed polymer brush. It was determined that calcium ions are chemically attached to the polyacrylic acid, and cannot easily be removed from the brush.

Adhesion measurements of the hydrophobic interactions reveal the unique properties of the system developed in this study. Hydrophobic interactions in the brush are tuned by changing pH and the ionic strength of the medium. The uniqueness of the system is that the hydrophobicity of the brush surface changes in the water medium and
has an island-like pattern. These properties of the tunable system may be reversibly locked with a bivalent salt.

10.4. Studies of fibrinogen adsorption on the mixed polymer brushes:

Mixed polymer brushes of poly(ethylene glycol)/polyacrylic acid-b-polystyrene have shown themselves to be useful tools in the control of protein adsorption. Treatment of a mixed polymer brush with solvents selected for each component of the brush results in changes in the extent of interaction between the polymer brush and the protein. The highest protein adsorption was achieved after the toluene treatment, which is a good solvent for polystyrene. MEK, a very good solvent for poly(ethylene glycol) – a protein-repelling agent—resulted in the smallest amount being adsorbed. Ethanol and DMF occupy an intermediate position between these extreme cases. Mixed brushes pretreated with calcium chloride appeared to be highly protein repelling.

AFM studies of the adhesion force between the mixed brush and protein in dry mode has revealed that the attractive forces of the protein-covered surface toward the mixed polymer brush are four times higher in cases where the mixed brush was treated with toluene than when it was treated with MEK. One of the interesting findings was that the adhesive force becomes practically equal for surfaces treated with both solvents when residency time is increased to 50 s.
The results obtained show that the mixed polymer brushes are very useful tools for the control of protein adsorption. A mixed polymer brush of one composition may be tuned with external stimuli to be extremely protein attractive, totally protein resistive or to attain a morphology that will show a moderate amount of adsorption between the extremes (Figure 10.1).

![Figure 10.1](image.png)

**Figure 10.1.** Surfaces studied and protein adsorption achieved.

10.5. Control of the protein adsorption through molecular imprinting approach.

**Synthesis of the molecularly imprinted polymer brush:**

A 2-D protein imprinted polymer brush surface has been synthesized. Use of PGMA as an anchoring layer for the imprint preparation widens the choices of the substrates for preparation of imprinted surfaces. Polymer surfaces as well as textiles and metals can be used for support.
Carboxyl terminated PEG chains were grafted into the open space between the protein molecules adsorbed onto the primary polymer layer. Subtilisin A has been used for cleavage of protein molecules adsorbed onto the surface. As a result, cavities complementary to the protein shapes were created. Synthesis of the imprint was monitored using AFM and Ellipsometry. Prepared protein imprints were used for adsorption studies.

10.6. Studies of protein adsorption on the imprinted polymer brush:

It was determined that surfaces prepared through the polymer brush imprinting technique can be used to regulate the extent of adsorption onto the surface. Imprints containing the protein-repelling polymer PEG on the surface exhibited lower adsorption than those without PEG. Hence, the overall amount of protein adsorbed onto the surface can be regulated by both the size of the prepared adsorption sites and the grafting density of the PEG.

Molecularly imprinted surfaces with PEG grafted chains have shown selective adsorption toward the parental protein molecules in a static adsorption experiment. No such effect was noted for the dynamic experiment. Additional experiments have to be run to confirm all of the above statements, as only one experiment of each kind was performed.
10.7. Publications and presentations:

The above-mentioned research resulted in the publications and presentations listed below from May 2004 to August 2008.

10.7.1. Publications:


2. Responsive mixed polymer brush containing protein adsorbing and protein repelling components. Hoy, Olha; Zdyrko, Bogdan; Burtovyy, Ruslan; Lupitskyy, Robert; Minko, Sergiy; Aulich, Dennis; Hinrichs, Karsten; Esser, Norbert; Luzinov, Igor. PMSE Preprints (2008), 99 114-115.

3. In-situ infrared ellipsometry for the analysis of stimuli-responsive polymer brushes. Hinrichs, Karsten; Aulich, Dennis; Esser, Norbert; Minko, Sergiy; Hoy, Olha; Luzinov, Igor; Eichhorn, Klaus-Jochen; Stamm, Manfred. PMSE Preprints (2008), 99 23.

4. Three-Dimensional Analysis of Switching Mechanism of Mixed Polymer Brushes. Usov, Denys; Gruzdev, Viacheslav; Nitschke, Mirko; Stamm, Manfred; Hoy, Olha; Luzinov, Igor; Tokarev, Ihor; Minko, Sergiy. Macromolecules (Washington, DC, United States) (2007), 40(24), 8774-8783.

5. Stimuli-responsive colloidal systems from mixed brush-coated nanoparticles. Motornov, Mikhail; Sheparovych, Roman; Lupitskyy, Robert; MacWilliams,
Emily; Hoy, Olha; Luzinov, Igor; Minko, Sergiy. Advanced Functional Materials (2007), 17(14), 2307-2314.

6. Control of interfacial properties of silica nanoparticles with grafted mixed polymer shell. Motornov, Mikhail; Sheparovych, Roman; Lupitskyy, Robert; MacWilliams, Emily; Hoy, Olha; Luzinov, Igor; Minko, Sergiy. Polymer Preprints (American Chemical Society, Division of Polymer Chemistry) (2007), 48(1), 377-378.

7. Mixed hydrophilic grafted layers with imbedded hydrophobic fragments. Zdyrko, Bogdan; Hoy, Olha; Curry, Jason; Luzinov, Igor; Minko, Sergiy; Varshney, Sunil K. PMSE Preprints (2006), 94 631.


9. Protein imprinting via solvent assisted grafting of polymer brush. Zdyrko, Bogdan; Hoy, Olha; Luzinov, Igor. Polymer Preprints (American Chemical Society, Division of Polymer Chemistry) (2005), 46(2), 76-77.

10.7.2. Presentations:


2. Control of protein adsorption on the mixed polymer brushes: Tirf and ellipsometry studies. Hoy, Olha; Zdyrko, Bogdan; Burlovyy, Ruslan; Lupitskyy, Robert;


5. Control of interfacial properties of silica nanoparticles with grafted mixed polymer shell. Motornov, Mikhail; Sheparovych, Roman; Lupitskyy, Robert; MacWilliams, Emily; Hoy, Olha; Luzinov, Igor; Minko, Sergiy. 233rd ACS National Meeting, Chicago, IL, United States, March 25-29, 2007 (2007), POLY-297.


7. Protein-mixed polymer brush interactions measured by atomic force microscopy. Burtovy, Ruslan; Hoy, Olha; Zdyrko, Bogdan; Lupitskyy, Robert; Minko, Sergiy; Luzinov, Igor. 233rd ACS National Meeting, Chicago, IL, United States, March 25-29, 2007 (2007), COLL-120.

8. Mixed hydrophilic grafted layers with imbedded hydrophobic fragments. Zdyrko, Bogdan; Hoy, Olha; Luzinov, Igor; Curry, Jason; Minko, Sergiy; Varshney, Sunil K.


CHAPTER ELEVEN

FUTURE WORK

In this chapter future prospective of the performed research experiments is described.

Mixed polymer brushes synthesized and studied in this work have been shown to be an effective tool for the surface modification for controlled adsorption of proteins. By combining in a single brush both protein-attractive and protein-repelling responsive components, variations in the extent of fibrinogen adsorption on these surfaces were obtained. However, there is a number of questions that should be addressed in future work:

1) Evaluate structural integrity of the protein molecule adsorbed onto the brush.

It was shown in scientific literature that proteins adsorbed onto flat hydrophobic surfaces exhibit changes in tertiary and even secondary (α-helix and β-sheet) structures. Hydrophobic fragments of the mixed brush are not rigid but attached on a flexible portion of the brush. When protein is adsorbed this surface may conform to protein shape to maximize thermodynamic interactions. Thus, study of the conformation of the protein molecule adsorbed onto these mixed polymer brushes of different composition should be done. Similar adsorption experiments onto rigid hydrophobic surfaces, such as polystyrene should also be done.
2) Identify relationship between molecular weight (size), shape and surface charge distribution of protein and protein adsorption on the mixed polymer brush.

In this work only a single protein, bovine fibrinogen, was used for the protein adsorption studies. However, there are a large variety of protein molecules that possess different molecular weight, shape, and surface charge distribution. The intrinsic properties of the protein molecules are very likely to affect the interactions between biomolecule and the mixed polymer brush. To better understand the interactions between the protein molecules and mixed protein brush, adsorption of the biomolecules of different size and different surface charge distribution should be studied. The effect of media pH on the extent of adsorption should also be done as pH influences the overall surface charge of the biomolecule.

3) Evaluate dependence of the extent of the protein adsorption on size of hydrophobic fragments;

Protein molecules have high affinity toward the hydrophobic surfaces, such as polystyrene. However, for the interactions to be developed and be sufficient, a minimum area of contact may be required. Larger sized protein molecules may require larger contact areas for adsorption.

The amount of polystyrene in the brush controls the size and shape of hydrophobic fragments. There is a potential to match the concentration and shape of the hydrophobic fragments of the mixed brush to the shape and area of binding sites of protein molecules. This may allow design of the surface with ability to attract specific proteins.
4) Develop dependence between the molecular weight of the mixed brush structural components and activity of the brush toward protein adsorption;

It was shown that there is a dependence between the solvent used for the brush treatment and the extent of the protein adsorbed onto the mixed brush. Through change of the molecular weight of the brush it may be possible to obtain a surface with gradual change of protein adsorption along the surface. Mixed brushes with different molecular weights of PEG and PAA-PS should be investigated in order to obtain the gradient of the adsorbed protein along the sample starting from the monolayer of the protein on one end of the sample and zero adsorption on another.