Published in final edited form as:

Langmuir. 2009 February 3; 25(3): 1459–1465. doi:10.1021/la802723r.

Separation of Peptides with Polyionic Nanosponges for MALDI-MS Analysis

Ven Ney Wong[†], Ganga Fernando[†], Audrey R. Wagner[†], Jianming Zhang[‡], Gary R. Kinsel[†], Stefan Zauscher[‡], and Daniel J. Dyer[†]

[†]Department of Chemistry & Biochemistry, Southern Illinois University, Carbondale, IL 62901–4409, USA

[‡]Department of Mechanical Engineering & Materials Science, Duke University, 144 Hudson Hall, Box 90300, Durham, NC 27708–09300, USA

Abstract

A polymer brush consisting of 70% poly(N-isopropylacrylamide) (PNIPAAm) and 30% polymethacrylic acid (PMAA) was synthesized from gold substrates with a grafting-from AIBN type free-radical initiator. Fractionation of two peptides, Bradykinin and Buccalin, was accomplished in less than 120 seconds by placing a 30 pM (pH~6.2) droplet onto the polymer brush substrate. The eluant containing the anionic Buccalin is pipetted away for MALDI analysis while the cationic Bradykinin adsorbed to the swollen anionic brush and was subsequently released by adding a droplet of formic acid to the substrate. This caused the brush to collapse and release the Bradykinin, much like squeezing a sponge; these nanosponge substrates exhibited very high loading capacity (>2.0 mg/ml) compared to plasma-polymer-modified MALDI substrates. Ellipsometric measurements showed that complementary peptides adsorb rapidly while those of the same charge do not and MALDI-MS analysis of the two fractions showed separation of both peptides. The adsorption of Bradykinin was monitored over time and 85% of the peptide had been adsorbed to the nanosponge in 1 minute from a 0.5 mg/ml aqueous solution.

Introduction

Proteomics represents an important area of research with the potential to provide unprecedented insight into various biological processes leading to new medical treatments.¹ In order to realize this potential new methods for the rapid separation and identification of complex peptide/protein components are required. To this end, Matrix Assisted Laser Desorption Ionization (MALDI) Mass Spectrometry (MS) offers an attractive method for the identification of peptides/proteins owing to its ability to obtain sequence specific fragmentation profiles at extremely low limits of detection.² However, a typical biological sample may contain thousands of proteins, which can complicate the mass spectra and reduce the ionization efficiency of many of the mixture components. Because of this challenge it is essential to rapidly separate these biological samples into less complex fractions prior to MALDI MS analysis.

In response to this analytical challenge, scientists are actively pursuing a host of novel methods for isolating peptides and proteins prior to MALDI MS analysis. Much of these fractionation efforts have focused on gel-electrophoresis, HPLC and/or ion-exchange methods with a typical

CORRESPONDING AUTHORS: D.J. Dyer (E-mail: ddyer@chem.siu.edu); G.R. Kinsel (E-mail: gkinsel@chem.siu.edu); S. Zauscher (E-mail: zauscher@duke.edu)..

HPLC purification protocol requiring an hour or more. Given the large number of samples needing analysis in a typical proteomics investigation there is a clear need to develop multiplexed, high-throughput separation techniques for rapid fractionation of complex protein mixtures that are quite analogous to the field of combinatorial synthesis. For this type of application we envision a robotic system where a substrate is patterned with an array of wells, each having specific capacity to fractionate a protein mixture on the basis of targeted chemical properties. Such an array could then be subjected to MALDI-MS analysis in order to rapidly determine the protein complement of each well.

One simplified version of this approach has been realized in the commercialized technology referred to as Surface-Enhanced Laser Desorption Ionization (SELDI).³ In this approach a silicon wafer is modified with various chemical functionalities having a range of properties (e.g. acidic, basic, hydrophobic, hydrophilic, etc.), which are used for on-chip fractionation of peptide/protein mixtures. SELDI has been used primarily in the search for biomarkers of various diseases and recent reports suggest that this approach may successfully serve as a diagnostic tool.⁴ Another recently developed approach involves the deposition of polymer thin films onto a stainless steel MALDI target⁵ via a radio frequency (RF) plasma polymerization process.⁶ Similar to the SELDI approach, the polymer thin films can incorporate various chemical functionalities, depending on the monomer used,⁷ or be subsequently modified to integrate specific bioaffinity capture motifs (e.g. biotin-streptavidin).⁸ The plasma polymer modified MALDI targets are similarly used to fractionate complex peptide/protein mixtures prior to MALDI MS analysis.⁹

Nevertheless, a significant limitation of most surface modified approaches to mixture fractionation lies in the fact that the surfaces are essentially two-dimensional and only adsorb molecules at the interface, which dramatically limits the sample loading capacity. In contrast, a swollen polymer brush represents a 3-dimensional structure with substantial volume, which potentially has a much higher loading capacity compared to, for example, a planar SAM. Polymer brushes are composed of polymer chains that are adsorbed or tethered to a substrate at one end.¹⁰ Thus, the polymer chains can expand out from the substrate when the grafting density is sufficiently high. Furthermore, polymer brushes may expand or contract depending on the solvent characteristics (e.g. a hydrophilic brush will collapse in a hydrophobic solvent and vice-versa).

Consider a mixture of two peptides where we desire a rapid and quantitative partitioning based on the ionic interaction of a cationic peptide with a negatively charged polymer brush containing carboxylate anions (Figure 1). Given that a densely packed SAM of mercaptohexadecanoic acid on gold contains 4.5 molecules/nm², then a 10 × 10 nm² substrate contains a maximum of 450 carboxylates.¹¹ Furthermore, the thickness of this SAM is ~2.1 nm, which yields ~2 carboxylates/nm³. In contrast, a densely packed brush of polyacrylic acid would yield ~2500 carboxylates or 12 carboxylates/nm³ for the same volume (assuming a bulk density of 1.41 g/cm³). And a 10 × 10 × 10 nm³ volume would possess nearly 12,000 carboxylates compared to 450 for a SAM over the same footprint. For separation and loading a collapsed (or dry) brush of PAA would first be swollen by immersion in a basic buffer, thus, providing a significantly larger loading capacity than a planar SAM, while also facilitating rapid transport of the peptide to the anionic brush. As Figure 1c illustrates, once the cationic species is adsorbed to the brush, the anionic-rich eluant is removed for analysis. Finally, the loaded nanosponge is squeezed by altering the pH to collapse the brush and release the cationic species (Figure 1e).

There are also several advantages for utilizing a covalently anchored polymer brush rather than a cast film to fractionate compounds. For instance, a cast film is merely adsorbed to the substrate and so it may diffuse away when exposed to a good solvent. Depending upon the solvents used,

oligomers from these cast polymer films can also be incorporated into the MALDI matrix crystals, leading to a complex background of polymer derived ion signals in the MALDI mass spectrum. Thus, the experiment in Figure 1 would not be as convenient with a cast film since the polymer might diffuse into solution and upon acidification, would form particles that would then need to be separated from the peptides; whereas a brush remains tethered to the heterogeneous substrate and is easily separated from the rinse. Another advantage of brushes is that the dry thickness is in the nanometer range, which makes them much more responsive to incompatible solvents than bulk polymers. For example, our group has shown that polystyrene brushes in the nanometer regime respond quite well to immersion in water;¹² in this case they collapse to the surface, whereas, bulk films do not seem to respond, as evidenced by water contact angles. Therefore, ultra-thin brushes can rearrange rapidly compared to cast films. Furthermore, polymer brushes can easily be patterned onto substrates for high-throughput array-based MALDI detection.

Previously it has been shown that ionic copolymer brushes have excellent response characteristics to changes in pH.¹³ In particular, Jiang and co-workers showed that a random copolymer of poly(N-isopropylacrylamide) (PNIPAAm) and 2% by weight poly(acrylic acid) (PAA) would respond to pH and temperature in order to alter the hydrophobicity of the surface.¹⁴ Our group has synthesized similar copolymer brushes, illustrated in Figure 2, with an AFM micrograph of gold pads that were modified with a random copolymer brush of 70% PNIPAAm and 30% poly(methacrylic acid) (PMAA).¹⁵ The dry height of the brush was 9 nm and the film swelled to over 110 nm after immersion into a pH 9 buffer. Subsequent immersion into a pH 4 buffer protonated the carboxylate ions and the resultant neutral brush collapsed back to its dry height; the switching is rapid and reversible. It occurred to us that the expansion of the brush would facilitate adsorption of molecules and macromolecules, such as proteins, to the swollen brush. Furthermore, collapse of the brush would then facilitate release of the adsorbed species much like squeezing a sponge, therefore we refer to these substrates as 'nanosponges'.

Studies on the adsorption of proteins to polymer brushes have mostly focused on polyethylene glycol (PEG) or other polymers in an effort to minimize adsorption (e.g. contact lenses, bio-implants, etc.).¹⁶ However, more recent studies on polyacrylic acid (PAA) brushes examined the adsorption of cationic and anionic peptides/proteins. Specifically, Ballauf and coworkers have examined the adsorption and release of bovine serum albumin (BSA) to spherical polyelectrolyte brushes (SPB), where the brush consists of PAA.¹⁷ While, the BSA has an overall negative charge at neutral pH and would not be expected to bind strongly to the negatively charged PAA brush due to electrostatic repulsion, binding is facilitated by the release of counterions. In addition, it is possible that cationic domains within the protein might bind strongly to the anionic brush.¹⁸ Release of BSA occurs when the ionic strength of the solution is increased, essentially masking the carboxylate anions of the PAA brush. Importantly, they specifically noted that changes in the pH of the solution played a minor role in protein uptake and release compared to changes in the ionic strength.

Czeslik and coworkers have shown that both lysozyme, with a net positive charge, and BSA, with a net negative charge, will adsorb to PAA brushes attached to a planar substrate at low ionic strength, while release is facilitated by an increase in the ionic strength.¹⁹ They specifically noted that even at high pH (~8) significant amounts of BSA adsorbed, even though both BSA and the brush exhibited a net negative charge; similar results have been obtained with polysulfonate brushes rather than PAA.²⁰ Recently de Vos and coworkers found that adsorption is affected by pH but that significant quantities of BSA are adsorbed at high pH.²¹ Finally, Saito and coworkers used an amine-functionalized brush to adsorb phosphinic acid moieties into the interior of hollow membranes via electrostatic interactions.²² They demonstrated that neutral and cationic species showed little intercalation into the cationic membranes, while the anionic species readily intercalated and swelled the membranes;

although these were not peptides. They also showed in similar brushes that peptide adsorption causes both cationic and anionic brushes to expand or contract depending on the conditions.²³

Herein we describe the specific adsorption of a cationic peptide (Bradykinin) to a PNIPAAm/PMAA brush in the presence of an anionic peptide (Buccalin). The fractionation of the peptides occurs rapidly and the adsorbed peptide is easily released by lowering the pH in order to collapse the polymer brush (i.e., squeeze the nanosponge). Unlike the above examples of protein adsorption, we do not detect any adsorption of the anionic peptides to the anionic polymer brush.

Experimental

Materials

All reagents were purchased from Acros, Aldrich, or Fisher Scientific and used as supplied unless otherwise noted. NMR solvents were purchased from Cambridge Isotopes. Column chromatography was performed with standard grade silica gel (63–200 Mesh) from Sorbent Technologies and TLC was performed on 250 μ m silica gel 60 polyester backed plates with F₂₅₄ fluorescent indicator from Whatman. Silicon wafers (100) were purchased from waferworld.com and were sputtered with a layer of Cr and Au by Nano Structures Inc. (Foster City, CA). The gold-coated QCM crystals were manufactured by Q-Sense. The syntheses of 1,10-dithiodecane (**2**)²⁴ and initiator **1**²⁵ have been described previously.

4-Cyano-4-(azo-[4'-cyano-(butyl)pentanoate])-(1,10-dimercaptodecyl)pentanoate (**1**)

Compound **3** (610 mg, 1.81 mmol), 1,10 dithiodecane (**2**) (377 mg, 1.80 mmol), and DMAP (44.2 mg, 0.36 mmol) were dissolved in THF (20 ml). The reaction mixture was cooled to 0 °C (ice bath) for 15 minutes. DCC (370 mg, 1.80 mmol) was then added and the resulting mixture was stirred for 5 minutes at 0 °C and then 15 hours at room temperature. The crude was concentrated by rotary evaporation and then was purified by column chromatography on silica gel (50:50 - hexanes/ethyl acetate) to yield 200 mg (20%) of a colorless oil: R_F = 0.66 (50:50 - hexanes/ethyl acetate). ¹H NMR (300 MHz, CDCl₃) δ 0.89 (t, J = 7.3, 3H), 1.22–1.34 (m, 14H), 1.53–1.68 (m, 12H), 2.35–2.71 (m, 10H), 2.84 (t, J = 6.8, 2H), 4.03–4.09 (m, 2H); ¹³C NMR (75 MHz, CDCl₃) δ 13.64, 19.03, 23.88, 23.93, 24.58, 28.27, 28.70, 28.95, 29.04, 29.07, 29.13, 29.29, 29.33, 30.48, 33.12, 33.19, 33.96, 38.32, 39.08, 64.94, 71.79, 71.86, 117.36, 117.39, 171.31, 196.79; FT-IR (neat): 2936, 2858, 1761, 1731, 1592, 1493, 1456, 1296, 1193 cm⁻¹.

1,10-Dithiodecane (**2**)

Thiourea (6.83 g, 89.7 mmol), 1,10-Dibromodecane (10.80 g, 36.0 mmol), and ethanol (40 ml) were refluxed for 5 hours with stirring. Sodium hydroxide (4.32 g, 108 mmol) and water (40 ml) was then added and the reaction mixture was refluxed for an additional 5 hours. After cooling to room temperature the solution was acidified with 20% aqueous sulfuric acid to pH 7–8 and then was extracted with benzene (3 $\times$ 70ml). The combined organic layers were dried over anhydrous magnesium sulfate. The solvent was removed by rotary evaporation and the crude was purified over silica gel by eluting with dichloromethane. Evaporation of solvent yielded 6.9g (93%) of a colorless oil: R_F 0.73 (dichloromethane); ¹H NMR (300 MHz, CDCl₃) δ 1.20–1.50 (m, 6H), 1.58 (m, 2H), 2.49 (q, J =7.08 Hz, 2H).

4-Cyano-4-(azo-[4'-cyano-(butyl)pentanoate])-pentanoic acid (**3**)

4,4'-Azobis-(4-cyanovaleric acid) (5.6 g, 20 mmol), 1-butanol (1.48 g, 20 mmol), and DMAP (488 mg, 4.0 mmol) were dissolved in THF (120 ml). The reaction mixture was cooled to 0 °C in an ice bath for 15 minutes. DCC (4.13 g, 20 mmol) was then added and the resulting mixture was stirred for 5 minutes at 0 °C and then 15 hours at room temperature. The

dicyclohexylurea precipitate was filtered off and the crude was concentrated by rotary evaporation and then was purified by column chromatography on silica gel (50:50 - hexanes/ethyl acetate) to yield 2.96 g (42%) of a colorless oil: $R_F = 0.46$ (50:50 - hexanes/ethyl acetate); ^1H NMR (300 MHz, CDCl_3) δ 0.92 (t, $J=7.3$ Hz, 2H), 1.37 (m, 2H), 1.58–1.72 (m, 8H), 2.38–2.58 (m, 8H), 4.10 (t, $J=6.6$ Hz, 2H); ^{13}C NMR (75 MHz, CDCl_3) δ 13.66, 19.06, 23.72 (2 C), 28.86, 29.17, 30.49, 32.91, 33.16, 65.05, 71.70, 71.85, 117.44, 117.48, 171.53, 176.48.

Brush synthesis

The gold-coated wafer was cut into rectangular pieces and was cleaned in Piranha solution for 120 minutes immediately prior to use. [CAUTION: *Piranha reacts violently with organic substances.*] The substrates were then rinsed with copious amounts of Millipore-filtered water. Next, the substrates were immersed into a dilute (1 mM) tetrahydrofuran (THF) solution of initiator **1** for at least 12 hours. The samples were removed from the initiator solution, rinsed thoroughly with THF, and blown dry with liquid-nitrogen boil-off. Reflection absorption infrared (RAIR) spectra were recorded following deposition of the initiator SAM and polymerizations of NIPAAM and MMA. Infrared spectra were recorded on a Nicolet-670 FTIR spectrometer equipped with a liquid-nitrogen cooled MCT-B detector and a PIKE grazing angle accessory; all spectra were collected at an 80° grazing angle. The sample chamber was purged with nitrogen gas for 20 minutes prior to data acquisition. Static contact angles of samples were measured with a CAM 100 (KSV Instruments) contact angle meter at room temperature. Contact angles were collected and averaged from the measurements at three different spots on each substrate to yield a water contact angle of 74° . Polymerization experiments were initiated immediately following monolayer deposition and confirmation.

Polymerizations were carried out in a Rayonet photochemical reactor (model RMR-600, Southern New England Ultraviolet Co., Branford, CT). The polymerization took place by first immersing the SAM coated substrate into a Schlenk tube with methacrylic acid (324 mg) and N-isopropyl acrylamide (756 mg) and Millipore-filtered water (5 ml). The Schlenk tube was purged with argon, degassed by three successive freeze-pump-thaw cycles, and was back-filled with argon prior to irradiation at 350 nm (~ 1.6 mW/cm 2). The substrate was removed after 4 hours of irradiation at room temperature, and was then rinsed with a 1:1 solution of methanol:Millipore-filtered water. The substrate was then immersed into a vial containing fresh solution overnight. The substrate was removed and rinsed with fresh solution and then was immersed again for 24 hours. The neutral brush-modified substrate had a static water contact angle of 79° .

Fractionation studies

A 2 μl droplet of Millipore-filtered water was placed onto the polymer surface and was allowed to stand for 30 minutes; the average spot diameter was 3 mm. Following this, a 1 μl aliquot of the mixture of peptides was applied onto the spot. After 30 seconds, the solution on the spot was removed with a pipette and was added onto a conventional MALDI plate surface. Three subsequent 1 μl aliquots of water were added and removed immediately by pipette and were added onto the same spot on the MALDI plate.

To release the bound proteins from the brush polymers, a solution of 10% formic acid (FA) was added onto the spot and was allowed to equilibrate for the same amount of time as the initial exposure (e.g. 30s), at which time a second droplet containing 15 mg/ml α -cyano-4-hydroxy cinnamic acid (CHCA) as a MALDI matrix in 0.1% trifluoroacetic acid (TFA) solution was added. This solution was pipetted off and placed onto a conventional MALDI plate. MALDI mass spectra were acquired from both the polymer retained sample and the washing sample. Data acquisition and analysis was performed using a Bruker Microflex MALDI mass

spectrometer with a Nitrogen laser at 355 nm. The laser intensity was kept at 33%–35% and 100 laser shots were averaged. The linear mode was used for analysis and all spectra were calibrated using internal standards. The delay time was 150 ns and ions below mass/charge 500 were deflected.

Brush capacity studies

For the brush capacity measurements a 1 μ L aliquot of the peptide Bradykinin in Millipore-filtered water was applied to a conventional stainless steel MALDI target, a gold-coated substrate modified with a thin polymer film resulting from RF plasma polymerization of vinyl acetic acid,²⁶ and a gold-coated substrate containing the 30:70 - PMAA/PNIPAAm brush polymer. After 30 seconds of exposure the sample aliquot volume was removed from the surface, deposited on a separate conventional stainless steel MALDI target, mixed with a MALDI matrix and the signal associated with the unbound Bradykinin was recorded by acquiring a MALDI mass spectrum.

Brush kinetic studies

The same procedures were used as in the brush capacity studies except that the sample aliquot was exposed to the surface for 30, 60, 90, and 120 seconds, respectively with two different initial concentrations of 0.5 and 0.05 mg/ml Bradykinin. The removed aliquots were then deposited onto a fresh spot on a conventional MALDI target. After adding the matrix solution, the mass spectrum of the unbound Bradykinin signal was recorded.

Brush ellipsometry studies

For these experiments we used a Rudolph Research thin-film null ellipsometer (Model 43603–200E). The polymer brush in the ellipsometry experiments consisted of a layer of 30% MAA, 70% NIPAAm on gold-coated QCM crystals, which was synthesized according to published procedures.¹⁵ A pH 7.0 phosphate buffer (S11M03) was used from Radiometer. The buffer was diluted 10 times to make the salt concentration less than 10 mM as a stock solution after received. The pH 7.0 stock solution was then used to make 0.01 mg/mL Bradykinin and Buccalin solutions.

Ellipsometry data with different peptide flush sequences were carried out. The polymer sample was immersed in 20 ml pH 7.0 stock solution overnight to equilibrate the swelling of the polymer brush. Ellipsometry data was first collected in pH 7.0 stock solution. After the Δ and Ψ curves leveled off, the sample cuvette was flushed with Buccalin solution. After 20 minutes, the sample cuvette was flushed and immersed with Bradykinin solution. Before the next experiment, the sample cuvette was then flushed with pH 4.0 stock solution for 5 minutes to protonate the MAA residues in order to release the adsorbed peptides on the polymer brush. Finally the polymer sample was immersed in pH 7.0 solution for 5 minutes, to deprotonate the MAA residues, at which time it was ready for another experiment.

Results and Discussion

Brush synthesis

The polymer brushes were synthesized according to similar procedures described in our previous work with patterned gold substrates.¹⁵ As described in Figure 3, a random copolymer brush was synthesized from an aqueous solution containing a 70:30 ratio by weight of NIPAAm and MMA, respectively. Thus, approximately 30% of the monomer units in the copolymer contain carboxylate functional groups at basic pH. Initiator 1 was synthesized to include an AIBN type free-radical initiating moiety containing a thiol terminus for bonding to the gold substrate.²⁵ The monolayer was deposited on gold-coated silicon wafers using standard SAM protocols. This substrate was then immersed into a solution of the monomers

and Millipore-filtered water in a Schlenk tube, which was then de-gassed and irradiated for 4 hours at 350 nm. The substrates were then cleaned to remove unreacted monomer and residual untethered polymer. RAIRS confirms the presence of MMA with a carbonyl band and 1732 cm^{-1} , which indicates hydrogen bonding (most likely with the NIPAAM);²⁷ the hydrogen bonding dimer, which is normally at $\sim 1690\text{ cm}^{-1}$, partially overlaps with a broad band at 1662 cm^{-1} due to NIPAAM. The presence of NIPAAM is confirmed with carbonyl and amide bands at 1662 and 1528 cm^{-1} , respectively.²⁸ The dry film thickness ($\sim 9\text{ nm}$) is assumed to be the same as our previous studies.¹⁵

Peptide fractionation

A droplet containing a 10:1 (mol:mol) mixture of the peptides Buccalin ($300\text{ }\mu\text{M}$) and Bradykinin ($30\text{ }\mu\text{M}$, droplet volume $\sim 1\text{ }\mu\text{L}$, spot diameter $\sim 0.2\text{ cm}$) in Millipore-filtered water was placed on a nanosponge-coated gold substrate containing copolymer 1 (Figure 4a). A droplet from the same solution was placed on a conventional stainless-steel MALDI plate, and even with a 10 fold excess of Buccalin, the MALDI signal is barely detectable compared to Bradykinin. The low Buccalin signal is due to the reduced ionization efficiency of Buccalin in the presence of the basic Bradykinin peptide and illustrates a significant limitation in the MALDI analysis of mixtures when some of the peptides are present at low concentrations compared to others. In Figure 4b, Bradykinin adsorbs rapidly to the nanosponge and the Buccalin rich (i.e. Bradykinin depleted) eluant is pipetted off after 30 seconds exposure; this is followed by two additional aliquots of water in order to thoroughly rinse the substrate. The MALDI signal from the combined rinses confirms that only the anionic Buccalin was present. Finally, the nanosponge is 'squeezed' by neutralizing the brush with a $1\text{ }\mu\text{L}$ droplet of 10 % formic acid at $\text{pH}\sim 1.9$ (Figure 4c). This collapses the brush and the solution is allowed to equilibrate for the same amount of time as the initial exposure (e.g. 30s), at which time a second droplet containing 15 mg/ml α -cyano-4-hydroxy cinnamic acid (CHCA) as a MALDI matrix in 0.1% trifluoroacetic acid (TFA) solution was added. This was then placed onto a conventional MALDI plate and analysis shows only Bradykinin, thus confirming the rapid separation of the two peptides.

The 3-dimensional structure of the nanosponge significantly increases the quantity of peptide that can be captured for subsequent MALDI analysis, compared to conventional 2-D techniques like SELDI. Furthermore, a significant advantage of our approach is that we were able to separate the two peptides in less than 1 minute as compared to commercialized HPLC protocols that take up to an hour. Finally, the brush does not adsorb significant quantities of the anionic peptide in contrast to previously discussed studies with BSA and PAA brushes.

Evaluation of nanosponge loading capacity

We performed a set of experiments in order to explore the loading capacity of the brush polymer modified substrates, particularly as it compared with the 2-D capacity of an RF-plasma polymer modified target. The Bradykinin signals observed from several different concentrations of applied Bradykinin are given in Table 1. In this case we are examining a decrease in peptide concentration as it is adsorbed by the nanosponge. The large standard deviations are typical for MALDI experiments (i.e. relative standard deviations of 25% to 40% are common) and derive from the inhomogeneous distribution of the MALDI matrix / analyte crystals on surface of the probe. Regardless, from this data it is apparent that at the highest Bradykinin concentrations the signals from the unbound Bradykinin, from both the conventional stainless steel and RF-plasma polymer-modified surfaces, are nearly identical and likely represent saturation of the target binding sites. The signal from the unbound Bradykinin eluted from the RF-plasma polymer surface only becomes measurably lower than the Bradykinin eluted from the stainless steel surface at applied Bradykinin concentrations at, or below, 0.5 mg/ml . This suggests that at Bradykinin concentrations above this value the surface binding sites of the RF-

plasma polymer are essentially saturated. Notably, however, the signal resulting from the Bradykinin eluted from the brush polymer surface is still substantially lower than that obtained from either of the other two surfaces. This result clearly suggests that the capacity of the brush polymer surface has not yet been exceeded even at Bradykinin concentrations of 2.0 mg/ml!

To put this result in perspective, consider that a 1 μ l aliquot of the 2.0 mg/ml Bradykinin solution contains 1.1×10^{15} molecules of Bradykinin. On the brush polymer this aliquot of solution interacts with 3.1 mm² of material surface area leading to a surface concentration of 3.7×10^{14} molecules/mm². In contrast, if the cross-sectional area of a Bradykinin molecule is taken to be 1.6×10^{-12} mm²/molecule, then a monolayer of Bradykinin on a microscopically smooth surface will occur at a surface concentration of ca. 6.2×10^{11} molecules/mm². Remarkably, the amount of Bradykinin deposited on the brush polymer surface for the highest concentration solution is nearly 600 times the monolayer coverage amount and still appears to be below the saturation limit of the brush polymer. While there is a possibility that this extremely high capacity of the brush polymer may simply be attributed to surface adsorption of the Bradykinin on a highly roughened surface, it seems considerably more likely that this high capacity is related to penetration of the Bradykinin into the bulk of the conformationally expanded polymer brush.

Additional evidence in support of this interpretation is found in the time dependence of the uptake of the Bradykinin. In these experiments the brush polymer surface was incubated with a 1 μ L aliquot of the Bradykinin solution for increasing amounts of time up to 120 seconds. The remaining solution was removed and analyzed with MALDI MS to determine the uptake of

Figure 5 represents the uptake of Bradykinin as the difference in intensity of the MALDI signal from the initial solution at time 0 and eluant at 30 second intervals, for initial concentrations of 0.5 and 0.05 mg/ml. The intensity of the Bradykinin signal has decreased 85% after 1 minute for the 0.5 mg/ml sample, suggesting that the majority of adsorption occurs within this time frame. Furthermore, both concentrations rapidly level off indicating that equilibrium is reached within two minutes. The results show that the uptake of Bradykinin continued to increase by approximately one order of magnitude over a period of 2 minutes. This temporal response is most likely a reflection of the time-dependence associated with the reorganization of the brush polymer and penetration of the Bradykinin within the bulk of the material: i.e., diffusion rates within the initial 1 μ l drop alone cannot explain a 2 minute uptake of Bradykinin. Importantly, this study also reveals that the capacity of the brush polymer may be an order of magnitude (or more) larger than suggested by our initial studies with a 2 minute incubation.

Rate of peptide adsorption

One of the critical parameters we are examining is the rate of uptake and release of various compounds within the brushes. For example, we monitored the conformational change of a PMAA/PNIPAAm brush by ellipsometry after the sequential addition of pH 7 buffered solutions of Buccalin and Bradykinin. Figure 6 shows that the addition of Buccalin solution at 500s had only a transient effect on brush conformation, which returned to its initial state in about two minutes. This suggests that Buccalin likely did not intercalate or bind to the anionic brush, except perhaps at the brush-solvent interface. In contrast, the addition of Bradykinin solution at 1750s caused a rapid and persistent

Figure 5 and clearly shows that selective uptake of Bradykinin begins immediately and is most

Figure 5 indicates that adsorption continues over time and depends on the concentration of peptide. Furthermore, this experiment helps to explain the results in Figure 4 where the 30 pM Bradykinin was separated in about 30 seconds. Finally, once the brush is collapsed it can be

rehydrated with an appropriate buffer and returns to the original state. However, more detailed experiments are in progress to quantify any residual peptide that may be retained by the brush.

We are continuing to study the adsorption kinetics by MALDI-MS, ellipsometry, and time-resolved fluorescence spectroscopy. Furthermore, Kinsel and coworkers have shown that thermoresponsive NIPAAm-containing plasma polymers can be triggered to adsorb proteins above the lower critical solution temperature (LCST) and then release the proteins at lower temperatures.²⁹ This offers the potential of an additional trigger that we will explore in concert with the pH response described here.

Conclusions

It is clear from our studies that stimulus-responsive polyelectrolyte brush films offer an attractive method for fractionating peptides. These 3-dimensional nanosponges are capable of adsorption of significantly more peptide than conventional 2-dimensional substrates and release of the adsorbed peptide is facilitated with a pH trigger that ‘squeezes’ the nanosponge. Future work will focus on more detailed capacity measurements, adsorption and desorption kinetics, structural effects (e.g. cationic brushes for adsorption of anionic peptides), and the fractionation of more complex mixtures. We believe that nanosponge substrates such as these will play an important role in the fractionation of peptides for proteomic applications. Finally, similar substrates could be used for a host of other applications, such as environmental remediation, drug-delivery, tissue-engineering, and microfluidics.

Acknowledgements

We thank the Materials Technology Center at SIUC, the National Science Foundation (CHE-0719426), and the National Institutes of Health (NIGMSR15GM083325) for financial support. S. Zauscher thanks the National Science Foundation (DMR-0502953) and the Army Research Office (DAADG55-98-D-0002) for financial support.

REFERENCES

1. Weston AD, Hood L. J. *Proteome Res* 2004;3:179–196. [PubMed: 15113093]
2. Hillenkamp, F.; Peter-Katalinic, J., editors. *MALDI MS: a practical guide to instrumentation, methods and applications*. Wiley-VCH Verlag GmbH; 2007.
3. Lomas, LO.; Weinberger, SR. *Handbook of Biosensors and Biochips*. Wiley & Sons Ltd.; Chichester: 2007. p. 885–894. b Bulman A. *Amer. Biotech. Lab* 2008;26:14–16. c Seibert V, Wiesner A, Buschmann T, Meuer J. *Pathol., Res. Pract* 2004;200:83–94. [PubMed: 15237917] d Hodgetts A, Levin M, Kroll JS, Langford PR. *Future Microbiol* 2007;2:35–49. [PubMed: 17661674] e Kiehnopf M, Siegmund R, Deufel T. *Clin. Chem. Lab. Med* 2007;45:1435–1449. [PubMed: 17970700] f Weinberger SR, Lomas L, Fung E, Enderwick C. *Spectral Tech. Proteomics* 2007:101–132.
4. a Mauri P, Petretto A, Cuccabita D, Basilico F, di Silvestre D, Levreri I, Melioli G. *Curr. Pharm. Anal* 2008;4:69–77. b Niwa T. *J. Chromatogr., B: Anal. Technol. Biomed. Life Sci* 2008;870:148–153. c Park JS, Oh K-J, Norwitz ER, Han J-S, Choi H-J, Seong HS, Kang YD, Park C-W, Kim BJ, Jun JK, Syn HC. *Reprod. Sci* 2008;15:457–468. [PubMed: 18579854] d Wang S, Guo X, Tan W.-h. Geng D, Deng B.-p. Wang C.-e. Qu X. *J. Bone Miner. Metab* 2008;26:385–393. [PubMed: 18600406]
5. a Li M, Fernando G, van Waasbergen LG, Cheng X, Ratner BD, Kinsel GR. *Anal. Chem* 2007;79:6840–6844. [PubMed: 17685551] b Griesser HJ, Kingshott P, McArthur SL, McLean KM, Kinsel GR, Timmons RB. *Biomaterials* 2004;25:4861–4875. [PubMed: 15109847]
6. a Foerch R, Zhang Z, Knoll W. *Plasma Proc. Polym* 2005;2:351–372. b Denes F, Manolache S. *Prog. Polym. Sci* 2004;29:815–885. c Poncin-Epaillard F, Legeay G. *J. Biomat. Sci., Polym. Ed* 2003;14:1005–1028.
7. a Friedrich J, Kuehn G, Mix R, Fritz A, Schoenhals A. *J. Adhesion Sci. Technol* 2003;17:1591–1617. b Thissen H, Johnson G, Hartley PG, Kingshott P, Griesser HJ. *Biomaterials* 2006;27:35–43. [PubMed: 15996730] c Whittle JD, Bullett NA, Short RD, Douglas CWD, Hollander AP, Davies J. *J. Mater. Chem* 2002;12:2726–2732. d Shen M, Martinson L, Wagner MS, Castner DG, Ratner BD, Horbett

- TA. J. Biomat. Sci., Polym. Ed 2002;13:367. e Wu YJ, Timmons RB, Jen JS, Molock FE. Colloids Surf. B: Bioint 2000;18:235–248.
8. Li M, Timmons RB, Kinsel GR. Anal. Chem 2005;77:350–353. [PubMed: 15623315]
 9. Walker AK, Qiu H, Wu Y, Timmons RB, Kinsel GR. Anal. Biochem 1999;271:123–130. [PubMed: 10419626]
 10. Advincula, RC.; Brittain, WJ.; Baster, KC.; Ruhe, J., editors. Polymer Brushes: Synthesis, Characterization, Applications. Wiley-VCH; Weinheim: 2004. p. 483 b Milner ST. Science 1991;251:905. [PubMed: 17847384] c Dyer DJ. Adv. Polym. Sci 2006;197:47–65. d Brittain WJ, Minko S. J. Polym. Sci., Part A: Polym. Chem 2007;45:3505–3512. e Rhe J, Ballauff M, Biesalski M, Dziezok P, Groehn F, Johannsmann D, Houbenov N, Hugenberg N, Konradi R, Minko S, Motornov M, Netz RR, Schmidt M, Seidel C, Stamm M, Stephan T, Usov D, Zhang H. Adv. Polym. Sci 2004;165:79–150. f Ballauff M, Borisov O. Curr. Opin. Colloid Interface Sci 2006;11:316–323. g Minko S. J. Macromol. Sci., Part C: Polym. Rev 2006;46:397–420. e Senaratne W, Andruzzi L, Ober CK. Biomacromolecules 2005;6:2427–2448. [PubMed: 16153077]
 11. a Love JC, Estroff LA, Kriebel JK, Nuzzo RG, Whitesides GM. Chem. Rev 2005;105:1103–1170. [PubMed: 15826011] b Travaille AM, Kaptijn L, Verwer P, Hulsken B, Elemans JAAW, Nolte RJM, van Kempen H. J. Am. Chem. Soc 2003;125:11571–11577. [PubMed: 13129360]
 12. Feng, J. Ph.D. Dissertation. 2005. Southern Illinois University.
 13. Zhou F, Huck WTS. Phys. Chem. Chem. Phys 2006;8:3815–3823.
 14. Xia F, Feng L, Wang S, Sun T, Song W, Jiang W, Jiang L. Adv. Mater 2006;18:432–436.
 15. Kaholek M, Lee W-K, Feng J, LaMattina B, Dyer DJ, Zauscher S. Chem. Mater 2006;18:3660–3664.
 16. a Czeslik C. Z. Phys. Chem 2004;218:771–801. b Bernards MT, Cheng G, Zhang Z, Chen S, Jiang S. Macromolecules 2008;41:4216–4219.
 17. Wittemann A, Ballauf M. Z. Phys. Chem 2007;221:113–126.
 18. a Biesheuvel PM, Leermakers FAM, Cohen-Stuart MA. Phys. Rev. E 2006;73:011802. b Leermakers FAM, Ballauff M, Borisov OV. Langmuir 2007;23:3937–3946. [PubMed: 17315900]
 19. Hollmann O, Czeslik C. Langmuir 2006;22:3300–3305. [PubMed: 16548592]
 20. a Anikin K, Rcker C, Wittemann A, Wiedenmann J, Ballauff M, Nienhaus GU. J. Phys. Chem. B 2005;109:5418–5420. [PubMed: 16851574] b Wittemann A, Ballauf M. Phys. Chem. Chem. Phys 2006;8:5269–5275.
 21. de Vos WM, Biesheuvel PM, de Keizer A, Kleijn JM, Cohen Stuart MA. Langmuir 2008;24(13): 6575–6584. [PubMed: 18507422]
 22. Asai S, Watanabe K, Sugo T, Saito K. Sep. Sci. Technol 2005;40:3349–3364.
 23. Kawai T, Sugita K, Saito K, Sugo T. Macromolecules 2000;33:1306–1309.
 24. Schmidt R, Zhao T, Green J-B, Dyer DJ. Langmuir 2002;18:1281–1287.
 25. Dyer, DJ.; Feng, J.; Fivelson, C.; Paul, R.; Schmidt, R.; Zhao, T. Polymer Brushes: Synthesis, Characterization, Applications. Wiley-VCH; Weinheim: 2004. p. 129-150.
 26. Walker AK, Qiu H, Wu Y, Timmons RB, Kinsel GR. Anal. Biochem 1999;271:123–130. [PubMed: 10419626]
 27. Ye M, Zhang D, Han L, Tejada J, Ortiz C. Soft Matter 2006;2:243–256.
 28. a Maeda Y, Higuchi T, Ikeda I. Langmuir 2000;16:7503–7509. b Sun B, Lin Y, Wu P. Appl. Spectrosc 2007;61:765–771. [PubMed: 17697471]
 29. Li M, Fernando G, van Waasbergen LG, Cheng X, Ratner BD, Kinsel GR. Anal. Chem 2007;79:6840–6844. [PubMed: 17685551]

Figure 1.

Smart nanosponges for high-throughput peptide separations: (a) an anionic polymer brush is immersed into a solution of acidic and basic peptides; (b) the cationic peptides disperse into the anionic brush; (c) the anionic peptides are rinsed away leaving (d) the cationic peptides entrapped in the brush; and (e) the brush substrate is triggered to collapse and release the cationic peptides.

Figure 2. Expansion and contraction of a nanosponge with copolymer **1** as a function of pH: (a) 9 nm in the dry film; (b) 110 nm in pH 9 buffer; and (c) 9 nm in pH 5 buffer solution.

Scheme 1.
Synthesis of AIBN type thiol initiator 1.

Figure 3.
Synthesis of a nanosponge containing random copolymer 1 with 70:30 NIPAAM:MAA.

Figure 4.

(a) A mixture of Bradykinin (+ charge@pH 7) and Buccalin (– charge@pH 7) is placed on a brush nanosponge-coated gold surface (w/copolymer **1**) and on a conventional MALDI plate. Since Buccalin has reduced ionization efficiency in the presence of Bradykinin it has a weak MALDI signal even though there is a 10-fold excess of Buccalin to Bradykinin in solution. (b) After 30 seconds exposure, the Bradykinin has been adsorbed by the nanosponge brush and the eluant is removed and placed onto a conventional MALDI plate; only Buccalin is detected. (c) The nanosponge is then ‘squeezed’ by treating with a drop of 10% formic acid, which collapses and neutralizes the brush and releases the Bradykinin for subsequent MALDI analysis.

Figure 5. Uptake of Bradykinin from solution at 0.5 and 0.05 mg/ml concentrations Milli-Q grade (18 MΩ/cm) water. The y-axis represents the relative intensity difference for the MALDI signal between the original solution and the solution at the specified time. Error bars represent one standard deviation and are omitted for clarity at time zero.

Figure 6.

Adsorption of Buccalin and Bradykinin on a PNIPAAm/PMAA brush as measured by liquid mode ellipsometry. Initially the brush was in a pH 7.0 buffer, the cell was then flushed with a 0.01 mg/mL Buccalin solution (at 500s), and then a 0.01mg/mL Bradykinin solution in the same pH 7.0 buffer (at 1750s). The left axis represents $\delta(\Psi)$ and the bottom line, while the right axis represents $\delta(\Delta)$ and the top line.

Table 1

Peptide adsorption is confirmed by analyzing the Bradykinin remaining in solution after a 30 second exposure to various substrates as measured by MALDI-TOF: (a) original peptide concentration; (b) conventional stainless steel plate; (c) plasma polymer modified substrate; and (d) a 70:30 PNIPAAm:PMAA polymer brush nanosponge. Errors represent one standard deviation.

(a) Original	(b) Conventional	(c) Plasma	(d) Polymer
[Bradykinin]	S.S. Plate	Polymer	Brush
2.0 mg/ml	3136 ± 815	2903 ± 1151	870 ± 157
1.0 mg/ml	1667 ± 656	1556 ± 748	877 ± 209
0.5 mg/ml	1221 ± 280	814 ± 257	453 ± 107
0.1 mg/ml	626 ± 291	550 ± 564	190 ± 65
0.05 mg/ml	852 ± 495	623 ± 262	222 ± 143
0.01 mg/ml	763 ± 302	650 ± 238	175 ± 28