DOI: 10.1002/adma.200800567

Combinatorial Screening of Biomimetic Protein Affinity Materials**

By *Trent R. Northen, Matthew P. Greving,* and *Neal W. Woodbury**

Materials that mimic the exquisite affinity and specificity of proteins have vast potential for multiplexed assays[1] and biomaterials.[2] The critical features of affinity materials are the recognition element(s) and the scaffold upon which the recognition element is presented. This is analogous to the structure of antibodies, which are comprised of several small recognition loops supported by a large three-dimensional scaffold.[3] Polymer gels can be used as synthetic affinity materials[4] and have been suggested as possible molecular recognition mimics of proteins.[5] Like proteins, polymer gels present a three-dimensional surface but are typically built from a limited set of monomers when compared to the chemically diverse set of 20 amino acids used to build proteins. Unlike proteins, precise sequence control of monomers within a polymer gel is difficult.[6] Conversely, DNA and peptide microarrays[7–9] constructed on planar glass substrates present a precisely controlled and chemically diverse set of recognition elements, but are not representative of the three-dimensional protein surfaces used in nature. Combining the three-dimensional surface of polymer gels with the precisely controlled chemical diversity of microarrays is a potentially powerful approach for high-throughput screening of biomimetic affinity materials.

Here we describe the preparation of large arrays of three-dimensional peptide grafted polymer affinity materials, allowing the screening of 10000 material combinations in parallel for protein affinity. While there are several classes of molecules which can be used to design protein-specific recognition elements including oligonucleotides,[10,11] proteins,[7] and peptides,[12] peptides were selected because they can be synthesized in situ[12,13] and peptides grafted to three-dimensional polymer scaffolds have the potential to serve as a general platform for developing biomimetic affinity materials, that is affinity materials that topographically and chemically resemble the surface of a protein.

Since peptide combinatorial space is vast (20^n, n = length of peptide), it is critical to develop both methods capable of screening large numbers of peptide/polymer combinations in parallel and methods of efficiently searching this space. Commonly, combinatorial peptide affinity screening is done using pre-synthesized peptides printed on planar glass or hydrogel-coated[14] substrates. In addition, affinity screening can be done using peptides synthesized in situ on membrane supports using SPOT synthesis.[13] Printing arrays of thousands of pre-synthesized peptides involves costly synthesis and equipment. SPOT synthesis on the other hand has a much lower per peptide cost but has limited feature density (<20 features/cm^2)[15] making it difficult to synthesize and screen tens of thousands of material combinations in parallel using this method. Alternatively, bead-based libraries are an effective method for screening millions of peptides.[16] However unlike array-based libraries, bead libraries are not addressable and require a sequencing step or specific encoding scheme [17] to determine the identity of the active peptides. In order to maintain library addressability, avoid the time and cost of pre-synthesizing a large library of peptides and to achieve feature densities much higher than what is attainable using SPOT synthesis, light-directed synthetic methods were used to construct a library of 10^4 peptide/polymer material combinations with high feature densities ($>10^4$ features/cm^2). To demonstrate this approach to screening large libraries of affinity materials, a library of 10^4 mutants of a peptide (EGEWTEGKLSLRGSC) known to bind the regulatory protein[18] Gal80 was synthesized in situ on a three-dimensional porous polymer scaffold and screened for Gal80 affinity. To efficiently search the binding space, an initial library of peptide/polymer material combinations containing point mutations in the starting peptide was screened to identify the critical residues for Gal80 binding. This scan identified four positions most critical to Gal80 binding and this information was used to define eight constant and four variable regions in the peptide EGE(W)TEGK(L)S(L)(R)GSC (mutated positions in parentheses, the C-terminal GSC tripeptide was used as a linker for all peptides). Identifying the key positions and limiting the amino acids to a set of ten chemically heterogeneous amino acids (R, K, S, T, G, E, L, W, D, and I) reduced the combinatorial possibilities from 10^{15} to 10^4 allowing construction of three replicates for each peptide/polymer

[*] Prof. N. W. Woodbury, M. P. Greving
Department of Chemistry and Biochemistry
Biodesign Institute, Arizona State University
Tempe, AZ 85287 (USA)
E-mail: nwoodbury@asu.edu
Dr. T. R. Northen
Life Sciences Division
Lawrence Berkeley National Laboratory
1 Cyclotron Road, Berkeley, CA 94720 (USA)

[**] T.R.N. and M.P.G. contributed equally to this work, and were both supported by NSF IGERT Fellowships. This work was funded by The Biodesign Institute at Arizona State University, Department of Energy (grant DE-FC36-05GO15016 and DE-AC02-05CH11231), NSF (grant DGE-0114434), and NSF grant no. CHE-0131222 (mass spectrometer). We would also like to thank Paul Belcher for the SPR measurements, Rashaad Sidique, and the Center for Solid State Science at Arizona State University for SEM images. Supporting Information is available online from Wiley InterScience or from the authors.

ADVANCED MATERIALS

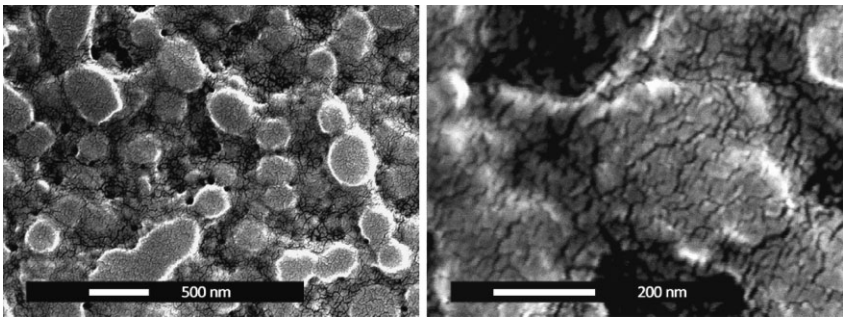

Figure 1. SEM images of three-dimensional porous polymer scaffold. Spin-coated polymer made with polymeric porogens is comprised of <100nm pores and is estimated to be 1 μm thick using confocal microscopy (data not shown).

material combination for a total of 30,000 features. These material combinations are found to have extremely high affinity with easily detectable binding of Gal80 at 25 pM, significantly less than the solution-based dissociation constant ($K_d = 4\ \mu M$)[19] suggesting that the three-dimensional polymeric scaffold/peptide combination does indeed increase affinity relative to the peptide alone.

For the three-dimensional porous polymer support, monomers 2-hydroxyethyl methacrylate (HEMA) and ethylene dimethacrylate (EDMA) were selected for their optical properties and the photoinitiator azobisisobutyronitrile (AIBN) was selected because the aliphatic photoproducts are nonfluorescent. The hydroxyl groups on HEMA serve as handles for synthesis through an ester linkage to the first amino acid. Porous structures are obtained through phase separation between the monomer solution and the growing polymer using polymeric porogens (Fig. 1). Photopolymerization is accomplished using a UV lamp or a micromirror array (MMA). The latter approach can be used to pattern the porous polymer structures in three-dimensions. However, superior performance is observed with spin-coated films polymerized using a UV lamp in the presence of nonvolatile polymeric porogens, which are subsequently removed by leaching in organic solvent. In general, the thin spin-coated polymer allows rapid diffusion of reagents, has low light scattering and is homogeneous. These properties are essential to high fidelity in situ synthesis, high contrast photopatterning, and fluorescence based affinity assays.

To provide a handle for subsequent peptide grafting, the polymer's pendant hydroxyl groups are esterified with glycine resulting in a high density of amino groups (90 nmole cm^{-2}) as determined by benzofulvene-piperidine absorbance. This is roughly 1000-fold higher than the density attainable on silanized planar glass substrates (<50 pmole cm^{-2}).[20] In fact, solid-phase synthesis colorimetric tests (TNBS, ninhydrin, and bromophenol blue) are easily visible to the naked eye; however more quantitative fluorescent assays are used for stepwise yield determination. This increased peptide density facilitates characterization of synthesis products using mass spectrometry and we have recently shown that combining photolithographic methods with high site-density polymeric supports allows both high resolution peptide grafting and MALDI-MS characterization.[21]

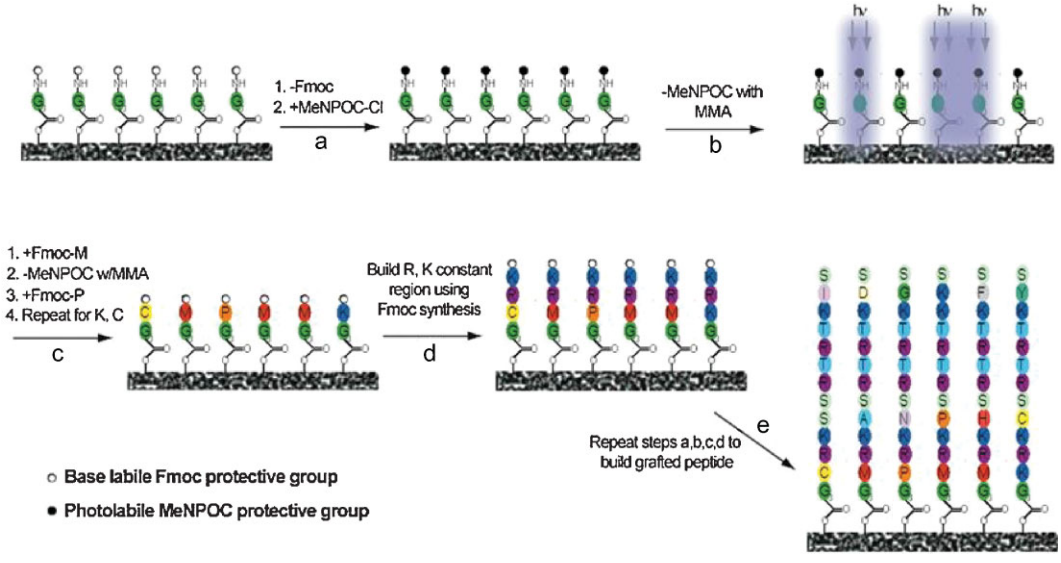

Figure 2. Illustration of the method used to construct peptide grafted polymer libraries using mixed Fmoc/MeNPOC N-terminal protecting groups. Peptides are designed with constant and variable regions compatible with chemically directed Fmoc synthesis and light-directed MeNPOC deprotections respectively. a) A polymer gel surface modified with Fmoc-glycine is deprotected and the Fmoc group replaced with the photolabile protecting group MeNPOC. b) The MeNPOC group is selectively removed with masked light projected by a micromirror array (MMA). c) The desired Fmoc amino acid is coupled and additional masks are projected for different amino acids in that layer. d) Constant regions within the peptides are built using Fmoc chemistry. e) This process is repeated for additonal layers and amino acids to construct the desired peptide grafted polymer library. Note that the photolabile group is coupled *in situ* allowing the use of commercially available Fmoc amino acids.

The synthetic approach is summarized schematically in Figure 2 and comprises (i) preparing the three-dimensional porous polymer support, (ii) use of alternating protecting groups where the photolabile group is introduced in situ, and (iii) photolysis using a micromirror array and thiol-based scavenging system.

Syntheses were performed on a custom-assembled automated system using amino acids protected by the photolabile protecting group, 5′-(α-methyl-2-nitropiperonyl)oxycarbonyl (MeNPOC). However, unlike conventional approaches requiring synthetic preparation and purification of the photolabile protected amino acids,[12,22] in this approach they are prepared in situ by removal of the terminal fluorenylmethyloxycarbonyl (Fmoc) group and subsequent coupling of MeNPOC before photopatterning. This strategy of switching between the base labile Fmoc group and the photolabile MeNPOC group addresses several problems: First, one very practical benefit is that Fmoc amino acids are commercially available whereas MeNPOC protected amino acids are not. More importantly, Fmoc deprotection is essentially quantitative (Fig. 3), extending the lengths of peptides that can be probed with non-quantitative photolysis yields (for invariable positions only Fmoc chemistry is required). Finally, after removal of MeNPOC with light, introduction of the Fmoc protected amino acid makes the growing peptide chain insensitive to scattered light from subsequent photopatterning in a neighboring region. This is important, because low levels of scattered light will integrate over all light-directed steps in a layer, creating cross additions between patterned features. The additional cost in steps of this approach is relatively small since Fmoc deprotection and MeNPOC coupling are rapid and the exchange only occurs once per photopatterned layer.

Micromirror arrays are commonly used in photolithographic synthesis[23] because they are flexible (virtual masks), can achieve resolutions better than 4 μm^2[24] and are efficient in that all features are illuminated in parallel. One of the great difficulties in utilizing photolabile protecting groups is that intense UV irradiation can result in highly reactive photoproducts. Upon photolysis, the local concentration of photoproducts is extremely high and this effect is compounded in porous materials due to restrictions on diffusion versus planar materials. Without effective scavenging systems these accumulate and reduce the synthesis yield. Conventional scavenging systems[25] were found to be insufficient for this application and resulted in low stepwise yields (ca. 60%). However, it was found that use of 30% β-mercaptoethanol and 7% di-isopropylethylamine in DMF could increase photolysis yields to about 93% yield (Fig. 3). This result is consistent with reports describing the ability of thiols to quench radicals,[26] and successful application in caged ATP studies.[27] In addition, to avoid accumulation of point mutated peptides, capping steps are performed after every coupling step using acetic anhydride. This results in some truncated peptides at each step, but reduces the number of point mutations that are more likely to complicate the functional analysis.

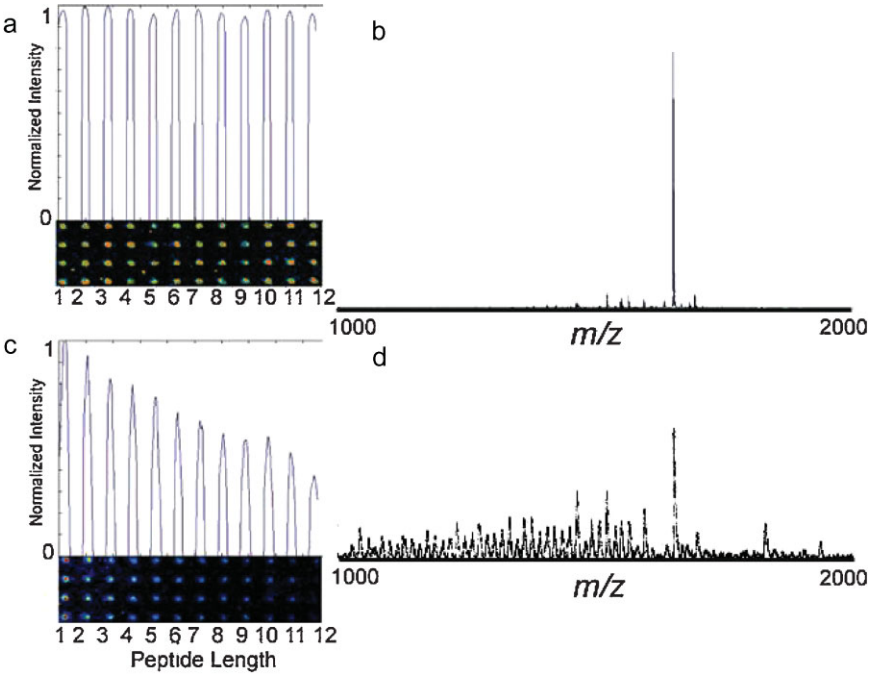

Figure 3. Characterization of stepwise yields and products. a) and c) Fluorescence from N-terminal fluorescent dye (TAMARA-SE) attached to series of poly-glycine peptides with 1–12 repeats plotted versus number of (a) Fmoc and (c) photodeprotection steps revealing 98% and 93% stepwise yields for Fmoc and photodeprotection steps, respectively. b) The fidelity of synthesis has been confirmed for Fmoc synthesis where the peptide EGEWTEGKLSLRGSC is the major product as it is when four light-direct steps are used d) albeit with a lower yield.

Fmoc and light-directed synthesis steps were performed on three-dimensional porous polymer supports modified with a glycine linker. Following the scheme shown in Figure 2, poly-glycine synthesis was performed with a capping step following each glycine coupling to terminate any remaining free amines. At the end of the synthesis, the N-terminal amine was deprotected and derivatized with the fluorescent dye TAMARA-succinimidyl ester. Comparison of the fluorescence intensity from peptides of different lengths allows determination of the stepwise synthesis yield. This approach was used to construct a series of peptides with chain lengths 1–12 using both Fmoc and light-directed deprotection (Fig. 3a and c). This reveals a near quantitative yield for Fmoc synthesis (98%) and a lower (93%) photodeprotection yield. These yields reflect the upper-limit on the synthetic yield since glycine is less hindered than other amino acids, however in general the coupling yields for HATU mediated peptide synthesis are near quantitative. Overall, this confirms that the highest overall peptide grafting yields will be obtained with libraries that utilize Fmoc

ADVANCED MATERIALS

chemistry for invariable regions and limit light-directed chemistry to variable regions.

MALDI-MS characterization was performed to confirm the in situ synthesis of the peptide on the three-dimensional polymeric support. The Gal80 binding peptide EGEWTEGKLSLRGSC was synthesized on a polymeric support using Fmoc chemistry, a second synthesis of the same peptide using four light-directed steps EGE(W)TEGK(L)S(L)(R)GSC was performed to allow direct comparison of Fmoc and light-directed chemistry. MALDI-MS analysis (Fig. 3b and d) reveals that the primary product in both syntheses is the desired peptide. However, consistent with stepwise yield data, inclusion of the light-directed steps results in an apparent decrease in the signal-to-noise.

To test this approach to screening large libraries of peptide grafted polymer affinity materials, we selected the protein Gal80 as a target since our previous work has revealed that the peptide EGEWTEGKLSLRGSC has a K_d of approximately 4 μM for Gal80,[19] thereby providing a template peptide for the library. To narrow the chemical search space, critical residues for Gal80 binding were identified by constructing a small library of point mutation peptides on the three-dimensional polymeric support where the amino acid in every position was replaced with (1) alanine, (2) proline, (3) deleted, or (4) substituted with a heterolog (polar/nonpolar amino acid substitutions, polar residues substituted with Gly and nonpolar residues substituted with Ser). After synthesis, the surface was blocked with BSA to reduce non-specific binding. Immediately after blocking, Alexa-647 labeled Gal80 (250 pM) was incubated in a 1 mg mL^{-1} *E.coli* extract (to ensure Gal80 specificity), surface washed then imaged. Comparison of fluorescence intensity reveals the critical residues for Gal80 binding as shown in Figure 4. Overall, the different types of point mutants agreed well at each position in terms of the effect on Gal80 affinity. Several point mutations within the peptide appear to be deleterious. Positions 3, 6, and 8 appear to produce the greatest decrease in intensity, with position 8 being a particularly critical residue for binding.

In order to search the chemical space for higher affinity peptide/polymer material combinations, it was decided to mutate amino acid positions that had relatively small effects on binding in the positional scan but were near the critical residues that played the greatest role in binding. The rationale for this approach is that direct mutation of the critical residues would likely reduce binding whereas mutations near these residues may augment the effect of these favorable interactions.

Using these design considerations, a library was designed where 10 amino acids (R, K, S, T, G, E, L, W, D, and I) were substituted into 4 positions EGE(W)TEGK(L)S(L)(R)GSC with 3 replicates for each peptide/polymer material combination resulting in an array with 30,000 features as shown in Figure 5. Following a preblock with BSA and incubation with 25 pM Gal80 for 1 h in the presence of ~1 mg mL^{-1} *E.coli* lysate competitor, washing, and imaging, the binding data was analyzed using GenePix (Table 1, Supporting Information). The tabulated

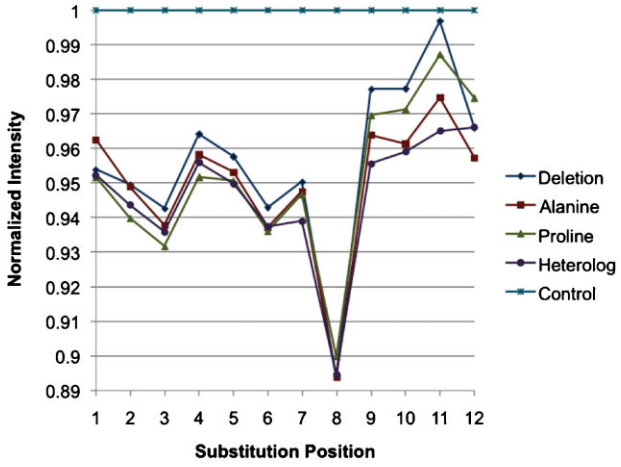

Figure 4. Identification of the most important residues for Gal80 binding. Construction of a small library of peptide grafted polymers with point deletions and alanine, proline, and heterolog substitutions within the starting peptide EGEWTEGKLSLRGSC (C-terminal GSC used as a linker). Binding of fluorescently labeled Gal80 (250 pM) in 1 mg mL^{-1} *E.coli* lysate and subsequent imaging reveals those residues with the biggest effects on Gal80 binding. (The control line is the starting peptide without substitution. Standard errors at each point are 0.005 or less.) Because the concentration of target protein was not optimized for maximum dynamic range in this initial scanning study, the changes observed in fluorescence intensity are not linearly related to binding constants. In the linear range of target binding, there presumably would have been much larger changes in fluorescence intensity with position. However, this was found to be adequate for determining which residues were most important for binding.

data contains the fold enhancement over the starting peptide/polymer material combination, the grafted peptide sequence and the standard error between the three replicates.

The highest affinity material combination contained the grafted peptide EGE(I)TEGK(K)S(K)(I)GSC and was found to have >10× the affinity of the starting peptide/polymer combination. Analysis of all of the highest affinity material combinations showed that specific substitutions at three of the four mutated positions were particularly important: lysine in positions 9 and 11 and to a lesser extent isoleucine in position 4. The standard replicate errors range from 2–9%, suggesting reasonable reproducibility.

Perhaps the most surprising result of this work is the high target affinity and specificity exhibited by Gal80 binding peptides grafted on the three-dimensional porous polymer support. The high levels of binding observed at 25 pM target concentration in the presence of ~10^6 fold higher concentration of competitor is behavior one might expect from a very good antibody. In addition, 25 pM target concentration is 5 orders of magnitude below the K_d of the starting peptide measured using surface plasmon resonance (SPR) assays.[19] While it is not certain which properties of the peptide/polymer material combination confer the observed high levels of binding, presumably the avidity that arises from the close proximity of multiple recognition elements within the porous polymer plays a significant role, much like the multivalency of antibodies.[28] It may also be the case that the three-dimensional topology of the peptide recognition elements is

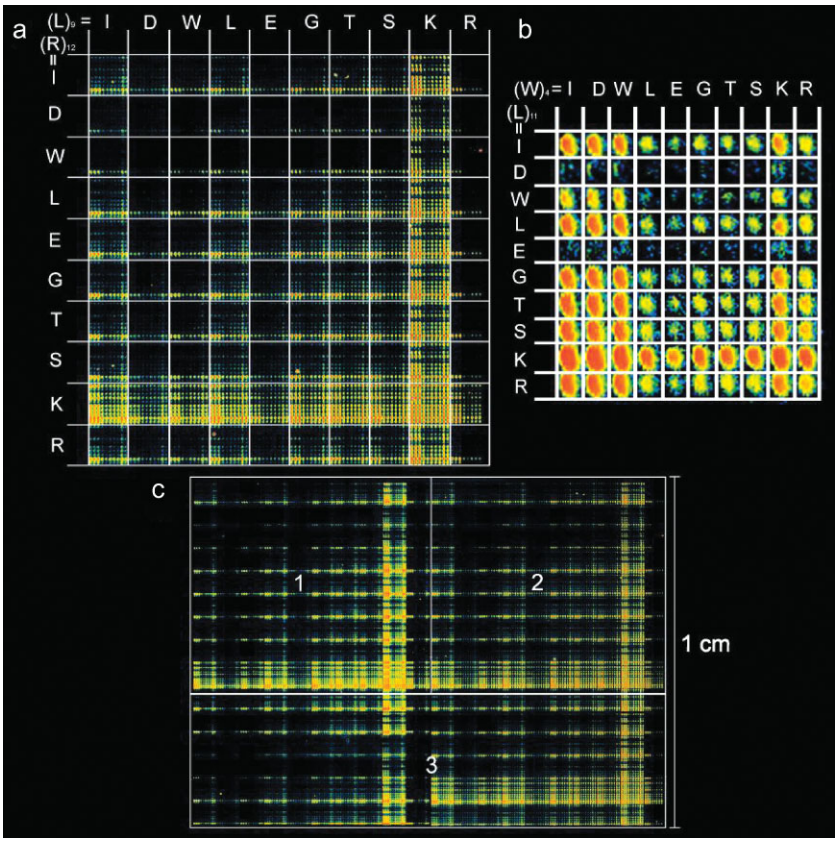

Figure 5. Fluorescence image of the peptide grafted polymer library following binding of Gal80 at 25 pM in 1 mg mL^{-1} *E.coli* lysate for 1 h. a) Image of one replicate block where the rows and columns describe the layout of the library and provide a substitution index for mutated positions 9 and 12. b) Expanded view of EGE(*)$_4$TEGK(K)$_9$S(*)$_{11}$(I)$_{12}$GSC which describes the substitution index for mutated positions 4 and 11 for each white square shown in panel (a). c) Image of the entire peptide grafted polymer library containing three replicate blocks (outlined in white and numbered 1, 2, 3). The total polymer surface area is ~1 cm^2 with each library feature measuring 45 μm diameter and the template peptide was EGE(W)$_4$TEGK(L)$_9$S(L)$_{11}$(R)$_{12}$GSC.

key to high affinity interactions. In this presentation, the peptide grafted polymeric surface may be more similar to a three-dimensional protein surface than peptides grafted to planar surfaces.

In conclusion, we have presented general methods for efficiently constructing and screening libraries of peptides grafted to three-dimensional polymer gels. These have been screened for targeted protein binding resulting in the identification of materials that bind Gal80 with high affinity. In general, this approach may prove particularly useful for the development and optimization of biomimetic affinity materials. Applications for such materials include both biomaterials as well as multiplexed, high-sensitivity protein affinity materials for biomedical diagnostics.

Experimental

Materials: Calmix2 and *N*,*N*-Dimethylformamide (DMF) was from Applied Biosystems Inc. (Foster City, CA). 3-(trimethoxysilyl)propyl methacrylate, Cyclohexanol, azo-bis-isobutyronitrile (AIBN), β-mercaptoethanol, piperidine, dicloromethane (DCM), α-cyano-4-hydroxycinnamic acid, triisopropyl silane (TIS), diisopropylethylamine (DIPEA), polyvinylacetate (PvAc), diethylene glycol dimethyl ether (diglyme), Trifluoroacetic acid (TFA), Acetonitrile, Bovine Serum Albumin (BSA) were from Sigma–Aldrich Chemical Co. (Milwaukee, WI). Glass coverslips were from Bioptechs (Butler, PA). ((α-methyl-2-nitropiperonyl) oxy) carbonyl chloride (MeNPOC-Cl) was purchased from Cambridge Major Laboratories Inc. (Germantown WI). Bromophenol blue was from Alfa Aesar (Ward Hill, MA). Methanol, sulfuric acid, acetic anhydride, hydrochloric acid were purchased from Mallinckrodt Inc. (Paris, KY). Fmoc amino acids, *O*-(7-Azabenzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluroniumhexafluorophosphate (HATU) and tetramethylrhodamine-X succinimidyl ester (TAMARA-X SE) were purchased from Anaspec Inc. (San Jose, CA). Fmoc-Rink amide linker was from NovaBiochem, a division of EMD Biosciences, Inc. (San Diego, CA). Alexa-647 fluorophore was obtained from Invitrogen (Carlsbad, CA). Tween-20 was obtained from USB (Cleveland, OH). Alexa-647 labeled recombinant Gal80, purified to ~95% from *E.coli*, was a kind gift from Prof. Stephen A. Johnston.

Glass Surface Functionalization: Glass coverslips were soaked for 30 min. at room temperature in hydrochloric acid:methanol (1:1, v:v), then in concentrated sulfuric acid at room temperature for 30 min. and finally in boiling water for 20 min. Between the aforementioned steps, the slides were immersed in nanopure water (ELGA PURELAB Ultra, High Wycombe UK.) at room temperature for 2 min. A solution of 3-(trimethoxysilyl)propyl methacrylate in methanol:water (5% in 95:5 methanol:water, by vol.) was prepared and stirred for 1 min, the slides were immersed in this solution at room temperature and allowed to react for 1 h with gentile agitation. Slides were then immersed in methanol for 3 min and placed in a nitrogen purged 100 °C oven for 12 h.

Spin-Coating Three-Dimensional Porous Polymer Surfaces for High Spatial Density Library: An argon sparged monomer solution (15% 3:1 HEMA:EDMA, 1% AIBN and 84% porogenic solvent: 6% 113 kDa PvAc (nonvolatile porogen) in diglyme (nonvolatile solvent)) was spin-coated for 30 s at 2000 rpm on a 40 mm diameter glass cover slide functionalized with a methacrylate monolayer using a Laurell WS-400B-6NPP-LITE spin processor (North Wales, PA). UV Curing of spin-coated polymer was performed with a Dymax 5000 (Torrington, CT) light source for 30 s in a KEMS (Baltimore, MD) controlled atmosphere glove box purged with argon. After curing, the surface was soaked in methanol for 12 h to remove excess monomer and porogen.

SEM Imaging of Three-Dimensional Porous Polymer: Scanning electron microscopy (SEM) of porous polymer coated glass slides was performed using a XL30ESEM environmental SEM, FEI Co. (Hillsboro, OR). The porous polymer was coated with 3.5 nm palladium/gold or 8 nm gold with accelerating voltages of 3–20 kV.

Amine Functionalization of Three-Dimensional Porous Polymer Scaffold: Porous polymer amine functionalization was performed by esterification of Fmoc-Glycine with the available hydroxyl groups on the polymer. The coupling solution consisted of 0.1 M Fmoc-Glycine, 0.1 M HATU, 0.4 M DIPEA in DMF. Coupling reactions were performed for 2 h in an FCS2 flow chamber (Bioptechs Inc., Butler, PA) at 50 °C. Amine density was measured by UV-absorbance of the

ADVANCED MATERIALS

benzofulvene-piperidine Fmoc deprotection product ($\varepsilon_{301} = 7800\ \text{M}^{-1}\ \text{cm}^{-1}$) resulting from Fmoc deprotection (as described in a following paragraph) on a well-defined area of porous polymer confined by a gasket and sealed within an FCS2 flow chamber (Bioptechs Inc., Butler, PA). After Fmoc deprotection the solution was collected and UV-absorbance measured on a Cary 50 UV-Vis spectrophotometer (Varian, Palo Alto, CA).

Automated Synthesis of Peptide Grafted Polymer Library: Synthesis of peptide grafted polymer libraries was fully automated using a custom assembled system comprised of a Milligen 9050 peptide synthesizer complete with an amino acid handler (Millipore, Billerica, MA), an FCS2 flow through optical chamber (Bioptechs Inc., Butler, PA), an Intelligent Micropatterning SF-100 DMD maskless exposure system (16 μm^2 pixels) (St. Petersburg, Fl), a computer controlling the peptide synthesizer with custom protocols for constructing the peptide grafted polymer libraries and a master computer with custom software for creating digital masks, projecting the masks for a programmed duration and communicating with the peptide synthesizer to synchronize digital mask exposure by the SF-100 with reagent delivery from the peptide synthesizer.

Invariable amino acid positions in the peptides were coupled using standard Fmoc synthesis protocols. Briefly, the Fmoc protecting group at the N-terminus of the growing peptide chain was removed with 20% piperidine in DMF for 20 min. After deprotection, the next Fmoc protected amino acid was coupled to the N-terminus of the peptide chain (0.1 M Fmoc amino acid, 0.1 M HATU, 0.4 M DIPEA in DMF). Amino acid coupling times varied depending on the size of the amino acid, smaller amino acids were coupled for 30 min and larger amino acids were coupled for 60 min. Three amino acids (GSC) were sequentially coupled to the polymer using Fmoc synthesis to act as a linker between the peptide and the three-dimensional polymer scaffold. At variable sequence positions, the photolabile protecting group MeNPOC was coupled and deprotected as described in a following paragraph. Finally, unreacted N-terminal amines were acetylated between coupling cycles using 5% acetic anhydride, 5% pyridine in DMF for 10 min to terminate unreacted peptide chains and minimize the formation of unwanted full-length peptide sequences.

Variable amino acid positions were coupled using a parallel light-directed synthesis protocol. First, Fmoc protecting groups were removed from all peptides on the polymer using 20% piperidine in DMF for 20 min. Next, MeNPOC-Cl (the acid chloride activated form of MeNPOC) photolabile protecting group was added and allowed to couple to the N-terminus for 30 min in DMF. After *in situ* substitution of MeNPOC, the polymer was soaked in photolysis solution consisting of 30% β-mercaptoethanol and 7% DIPEA in acetonitrile. With photolysis solution present in the flow chamber, a digital mask was projected through a 380/50 bandpass filter (Chroma Technologies, Rockingham, VT) onto the polymer surface using the SF-100 DMD (8 mW cm^{-2} intensity, 5 min exposure), thereby selectively removing the photolabile group. The photolysis solution was exchanged with DMF and the next Fmoc amino acid was added and allowed to couple to the selectively deprotected peptide chains. Unreacted N-terminal amines were acetylated and the light-directed cycle was repeated until all amino acid substitutions for that layer were complete.

Average synthesis cycle times are 1.5 h, therefore a 15-mer polymer grafted peptide library with 4 variable positions substituted with 10 different amino acids (~55 total cycles, including photolabile group substitutions) takes about 3.5 d to complete.

Post Library Synthesis Modification: A final illumination of the entire polymer surface in photolysis solution removed the photolabile protecting groups from the interstitial areas. The amines in the interstitial areas were then acetylated in a solution of (v/v) 5% acetic anhydride, 5% pyridine in DMF for 15 min to reduce non-specific binding to the interstitial areas. Finally, acid labile side-chain protecting groups were removed with a 1 h soak in a solution of 95% trifluoroacetic acid, 2.5% water, and 2.5% triisopropyl silane. Side-chain deprotection in 95% trifluoroacetic acid lasting longer than 1 h resulted in loss of peptide from the polymer surface, presumably due to hydrolysis of the ester bond linking the peptide to the polymer. Subsequent MALDI-MS characterization indicated that a 1 h exposure to 95% TFA was sufficient to remove the amino acid side-chain protecting groups. After post synthesis workup, the polymer was soaked overnight in 1:1:1 Water:Methanol:Acetonitrile to remove synthesis reagents from the porous polymer.

MALDI-MS Characterization of Grafted Peptides: Light-directed and Fmoc synthesis products were characterized using a Voyager-DE STR MALDI-TOF (Applied Biosystems, Foster City, CA) and a Microflex MALDI-TOF, (Bruker, Billerica, MA). Light-directed and Fmoc synthesis of characterized peptides was done as described in preceding paragraphs with the addition of a Rink linker between the polymer scaffold and the C-terminus of the grafted peptide. After synthesis, side-chain protecting groups and peptide cleavage was performed for 1 h in a solution of 95% trifluoroacetic acid (TFA), 2.5% water, and 2.5% triisopropyl silane, with the polymer-coated glass substrate contained in a thin glass cuvette. The glass slide was then removed from the cuvette and the cleavage solution evaporated leaving a very small amount of white solid at the bottom of the cuvette. The white solid was redissolved in 0.1% TFA/water and the sample washed with a C-18 ZipTip (Millipore, Billerica, MA). Peptides were eluted directly from the ZipTip onto the MALDI-plate using MALDI matrix (saturated alpha-cyano-4-hydroxycinnamic acid in 0.1% TFA, 50:50 acetonitrile:water).

Protein Binding to Three-Dimensional Peptide Grafted Polymer Library: To prepare the peptide grafted polymer library for Gal80 binding, the surface was dried then soaked in an aqueous equilibration buffer solution (1X PBS pH 7.4) for 2 h at 4 °C. The equilibration buffer was poured off and a blocking solution (1X PBS pH 7.4, 2% bovine-serum albumin) was added and soaked for 2 h at 4 °C. After 2 h of pre-block, the polymer was briefly rinsed and a binding/competition buffer (1X PBS pH 7.4, 25 pM Gal80-Alexa647, 1 mg ml^{-1} *E. coli* lysate) was incubated with the array for 1 h on a rocking table at 4 °C. The choice of 25 pM Gal80 concentration was based on several preliminary studies as a function of concentration. It was determined that target protein concentrations in the low nanomolar range (1–10 nM) produce fluorescence quenching at the highest affinity features. Concentrations in the mid-picomolar range (250–500 pM) did not exhibit quenching but many features had a saturated signal intensity which resulted in an overall low dynamic range. In contrast, concentrations in the low pM range (1–25 pM) produce good dynamic range of signal across all features in the library. After binding, the polymer was washed several times with 1X PBS buffer then soaked for 4 h in PBS gradually reducing the salt concentration (to improve fluorescence image quality) over several buffer exchanges during that period.

Fluorescence Imaging and Analysis of the Three-Dimensional Peptide Grafted Polymer Library: To prepare the peptide grafted polymer library for imaging, the surface was washed with nanopure water to remove salt on the surface that may affect imaging then dried with a low nitrogen stream. The polymer surface was imaged using a ScanArray ExpressHT (PerkinElmer, Wellesley, MA) using standard excitation laser settings for Alexa-647 dye, 5 micrometer resolution and 43% PMT. Peptide grafted polymer feature signal analysis was performed with GenePix (Molecular Devices, Sunnyvale, CA) microarray analysis software. All signals were background subtracted, using the unmodified regions of the polymer outside of the area containing grafted peptides along with unmodified interstitial regions designed within the patterned area as non-specific binding controls for background subtraction.

Characterization of Starting Peptide Solution-Phase Affinity: The starting peptide EGEWTEGKLSLRGSC dissociation constant (K_d) for Gal80 binding was determined using surface plasmon resonance (SPR) (Biacore T100, Uppsala, Sweden). Gal80 was immobilized on a CM-5 SPR chip (Biacore T100, Uppsala, Sweden) using immobilization protocols and reagents defined by the manufacturer. Reference-subtracted response units were measured across a series of starting

peptide concentrations flowed across the immobilized Gal80 in PBST buffer. The K_d was determined from a plot of concentration versus response.

Received: February 27, 2008
Revised: May 14, 2008
Published online: October 15, 2008

[1] B. B. Haab, M. J. Dunham, P. O. Brown, *Genome Biol.* **2001**, *2*, Research0004.
[2] R. Langer, D. A. Tirrell, *Nature* **2004**, *428*, 487.
[3] R. M. MacCallum, A. C. R. Martin, J. M. Thornton, *J. Mol. Biol.* **1996**, *262*, 732.
[4] T. Oya, T. Enoki, A. Y. Grosberg, S. Masamune, T. Sakiyama, Y. Takeoka, K. Tanaka, G. Q. Wang, Y. Yilmaz, M. S. Feld, R. Dasari, T. Tanaka, *Science* **1999**, *286*, 1543.
[5] N. A. Peppas, Y. Huang, *Pharm. Res.* **2002**, *19*, 578.
[6] N. A. Peppas, J. Z. Hilt, A. Khademhosseini, R. Langer, *Adv. Mater.* **2006**, *18*, 1345.
[7] G. MacBeath, S. L. Schreiber, *Science* **2000**, *289*, 1760.
[8] G. H. McGall, A. D. Barone, M. Diggelmann, S. P. A. Fodor, E. Gentalen, N. Ngo, *J. Am. Chem. Soc.* **1997**, *119*, 5081.
[9] M. A. Stiffler, J. Chen, V. Grantcharova, Y. Lei, *Science* **2007**, *317*, 364.
[10] M. B. Aberem, A. Najari, A. Ho, J. F. Gravel, P. Norbert, D. Boudreau, M. Leclerc, *Adv. Mater.* **2006**, *18*, 2703.
[11] A. D. Ellington, J. W. Szostak, *Nature* **1990**, *346*, 818.
[12] S. P. A. Fodor, J. L. Read, M. C. Pirrung, L. Stryer, A. T. Lu, D. Solas, *Science* **1991**, *251*, 767.
[13] R. Frank, *J. Immunol. Methods* **2002**, *267*, 13.
[14] P. Arenkov, A. Kukhtin, A. Gemmell, S. Voloshchuk, V. Chupeeva, A. Mirzabekov, *Anal. Biochem.* **2000**, *278*, 123.
[15] K. Hilpert, D. F. H. Winkler, R. E. W. Hancock, *Nat. Protocols* **2007**, *2*, 1333.
[16] K. S. Lam, S. E. Salmon, E. M. Hersh, V. J. Hruby, W. M. Kazmierski, R. J. Knapp, *Nature* **1991**, *354*, 82.
[17] M. Needels, D. Jones, E. Tate, G. Heinkel, L. Kochersperger, W. Dower, R. Barrett, M. Gallop, *Proc. Natl. Acad. Sci. USA* **1993**, *90*, 10700.
[18] K. K. Leuther, S. A. Johnston, *Science* **1992**, *256*, 1333.
[19] B. Williams, P. Belcher, C. Diehnelt, unpublished.
[20] M. C. Pirrung, *Angew. Chem. Int. Ed.* **2002**, *41*, 1277.
[21] T. R. Northen, D. C. Brune, N. W. Woodbury, *Biomacromolecules* **2006**, *7*, 750.
[22] K. R. Bhushan, *Org. Biomol. Chem.* **2006**, *4*, 1857.
[23] S. Singh-Gasson, R. D. Green, Y. J. Yue, C. Nelson, F. Blattner, M. R. Sussman, F. Cerrina, *Nat. Biotechnol.* **1999**, *17*, 974.
[24] T. Naiser, T. Mai, W. Michel, A. Ott, *Rev. Sci. Instrum.* **2006**, *77*, 063711.
[25] A. Patchornik, B. Amit, R. B. Woodward, *J. Am. Chem. Soc.* **1970**, *92*, 6333.
[26] H. G. Heine, W. Hartmann, D. R. Kory, J. G. Magyer, C. E. Hoyle, J. K. Mcvey, F. D. Lewis, *J. Org. Chem.* **1974**, *39*, 691.
[27] A. Barth, J. E. T. Corrie, M. J. Gradwell, Y. Maeda, W. Mantele, T. Meier, D. R. Trentham, *J. Am. Chem. Soc.* **1997**, *119*, 4149.
[28] A. Pluckthun, P. Pack, *Immunotechnology* **1997**, *3*, 83.