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Facile Construction of Fluorescent Peptide Microarrays: One-Step Fluorescent Derivatization of Sub-microscale Peptide Aldehydes for Selective Terminal Immobilization

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Abstract

In this note, we demonstrate the utility of bifunctional fluorescent linkers to facilitate the construction of peptide microarrays with either an N or C-terminal alkylamine for directionally preferred peptide immobilization. Significantly, these small tags facilitate HPLC profiling, while limiting interference with antigen-antibody interactions after peptide immobilization. In a model peptide-antibody binding assay, a sequence-dependent orientation effect of antibody binding to a series of peptide ligands was demonstrated. This approach provides a strategy that can be applied to a variety of peptide-microarray based detection systems.

Keywords

peptide microarray; fluorescent derivatization; HPLC; immobilization

Peptide and protein microarrays [1] represent an emerging platform technology for analyzing enzyme activity [2], ligand/receptor [3], as well as peptide-cell interactions [4]. Peptides have several advantages over proteins as on-chip probes. Firstly, advances in solid phase peptide synthesis provide opportunities to conduct combinatorial peptide synthesis in a semi or fully automated instrument. In particular, high performance liquid chromatography (HPLC) equipped with online UV/fluorescence/MS detectors allows facile characterization and subsequent purification. Secondly, unlike proteins, peptides can withstand relatively harsh experimental conditions with minimal structural effects. Most importantly, peptides can be prepared to incorporate chemical functionalities for site-selective immobilization using

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Supplementary data

Supplementary data associated with the article can be found in the online version.

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orthogonal chemical reactions. The functional groups on the amino acid side chains can be readily changed and not limited to those of natural amino acids. Non-canonical amino acids, such as D-amino acids, cyclic and N-methylated amino acids and many other variants can be readily included at any position along the primary peptide sequence.

Despite the varied approaches to construct peptide microarrays, most have been fabricated through N-terminal immobilization of a peptide onto a substrate. Recently, Bier described a peptide microarray whereby immobilization was controlled by positioning a biotin group at the desired end of the peptide sequence for coupling to a NeutrAvidin or streptavidin activated glass surface [5]. In this report, we investigated the effect of peptide orientation on antibody binding through construction of a microarray derived from a one-step reaction of peptide aldehydes with one of two bifunctional fluorescent tags. Ideally, the fluorescent tag should facilitate peptide separation by HPLC and subsequent immobilization through the remaining functional group. In this regard, we have recently developed a bifunctional fluorescent linker, 2-amino-N-(2-aminoethyl)-benzamide (AEAB) [6], which can selectively react with the C-terminal aldehyde group of a peptide by reductive amination through the arylamine, while the remaining primary alkylamine can be used for solid-phase immobilization. In contrast, 2-aminobenzamide (2-AB) reacts with the C-terminal aldehyde group to generate an inert amide group, while providing a free amine at the N-terminus for surface immobilization (Scheme 1). As a model study, a peptide derived from human Protein C (HPC4) [7] and related analogs were synthesized for construction of a microarray presenting peptide ligands in defined orientation. Both printing and detection of these peptides by binding to a Ca^{2+} -dependent anti-HPC4 antibody was demonstrated with antibody binding affinities strongly dependent on peptide orientation.

Model peptides were derived from the known binding sequence (EDQVDPRLIDSK) of the mouse anti-human protein C monoclonal antibody (clone HPC4). This parent sequence was referred to as the HPC4 peptide (Supplemental Data Table S1). To evaluate the relative importance of particular amino acids within the HPC4 native peptide, deletions of one or two amino acids at the N or C terminus were implemented. Alanine residues were incorporated at the opposite end of the deletion point to maintain the length of the primary sequence.

All peptides were synthesized on a preloaded leucine solid phase support using an oxazolidine linker, which upon cleavage generates a C-terminal aldehyde [9]. Solid phase synthesis was carried out in a 96-well filter plate on a 0.25 μmol scale. Each peptide was prepared in duplicate and combined after the two-step cleavage reaction. The cleaved and deprotected peptide aldehydes were conjugated to one of two fluorescent tags by reductive amination in DMSO/AcOH/ NaBH_3CN . Specifically, peptides **1-5** were reacted with 2-aminobenzamide (2-AB) generating an inert amide group at the C-terminus, while providing a free amine at the N-terminus for surface immobilization. Notably, neither the N-terminal amine nor the ϵ -amino group on lysine, present within the sequence, reacted with the aldehyde, as confirmed by the absence of peptide oligomers on HPLC and MALDI (Supplemental Data Fig. S1). Avoiding undesired side reactions can be ascribed both to the large excess of the fluorescent dye and the lower pK_a of the arylamine as compared to the alkylamine. Proper selection of reaction medium facilitates control of the protonation state of various amine functionalities. In this instance, high selectivity for reductive amination between the arylamine and aldehyde could be achieved through fine tuning solution acidity. Peptides **6-10** with sequences identical to peptides **1-5** were treated with AEAB to generate an active C-terminal amine for surface immobilization. The incorporation of a fluorescent tag simplifies HPLC characterization, preparation, and quantification due to the sensitivity of fluorescence detection, especially for small-scale synthesis. Moreover, the small size of the fluorescent tags used in this study may limit unintended problems in HPLC separation or peptide antigen/antibody interactions.

The quantity of each peptide was measured by HPLC according to its fluorescence and a stock solution for each peptide was prepared by dissolving in water to a final concentration of 1 mM. After serial dilution, all peptides were printed on a NHS-activated glass surface at 100 μ M, 20 μ M, 4 μ M and 0.8 μ M using a piezoelectronic non-contact printer. The average spot size was $\sim$ 100 μ m. Each concentration was printed as six duplicate spots by depositing 0.33 nL of peptide solution. Slides were incubated at 50°C for 1 hour in a moisture controlled chamber and then blocked with 50 mM aqueous ethanolamine to prevent non-specific absorption of the primary or secondary antibodies.

Peptide arrays were first incubated with the anti-HPC4 antibody at concentrations ranging from 0.01 to 100 μ g/mL for 1 hour at room temperature. Detection of immobilized antibody was accomplished by incubation with Alexa488 goat anti-mouse IgG at 5 μ g/mL. Slides were assessed using a microarray scanner with fluorescence detection at 495 nm/519 nm (Ex/Em) (Supplemental Data Fig. S2). Both fluorescent tags, 2-AB and 2-AEAB, display an excitation wavelength of $\sim$ 320 nm, which should not interfere with the ability to quantify binding of the Alexa488 labeled antibody. Characteristically, the array was divided into 42 subdomains with each domain contains five duplicates comprising spotted samples of ten peptides at four different concentrations, along with PBS and biotin reference domains. The images were then translated into fluorescence plots for semi-quantitative analysis.

We confirmed that antibody binding was Ca^{2+} -dependent and fluorescence intensity increased with increasing concentrations of the anti-HPC4 antibody (Supplemental Data Fig. S3). However, antibody concentrations above 10 μ g/mL increased non-specific binding for several peptide sequences, in which significant fluorescence intensity was observed in the absence of Ca^{2+} (Supplemental Data Fig. S3 H, J). Thus, caution is dictated under conditions of high antibody concentration that may increase the potential of a false positive result.

To determine the influence of bound peptide orientation on antibody binding, peptides were incorporated onto the microarray through either an N- or C- terminal amine. We observed that binding events were favored by arrays composed of C-terminal immobilized peptides, particularly at low anti-HPC4 antibody concentrations (Fig. 1). Notably, the effect of bound peptide orientation on antibody binding was most pronounced when peptides were printed at low concentration (20 μ M). For example, with the exception of native HPC4 peptide sequences (**3**, **8**), C-terminal immobilized peptides generated consistently higher signals, even in the presence of deletions of even one or two amino acids that otherwise completely disrupted antibody binding when peptides were immobilized through the N-terminus (Supplemental Data Fig. S4).

Antibody binding affinity to all peptides was determined for C-terminal immobilized peptides at a printing concentration of 20 μ M and antibody concentrations of 0.1, 1, 10, 100 μ g/mL yielding a saturation binding curve for each peptide antigen-antibody pair. Under equilibrium conditions, K_d can be calculated for each peptide-antibody pair using $F = F_{\text{max}}[Ab]_o / (K_d + [Ab]_o)$, where F is the fluorescent intensity of individual spots, F_{max} is the maximum fluorescence intensity, $[Ab]_o$ is the incubation concentration of the primary antibody, and K_d is the dissociation constant [3a,11]. K_d values (fitting curve for peptides **6** and **8** in Supplemental Data Fig. S5) were 182, 62, 32, 50, and 63 nM for peptides **6** through **10** with the highest binding affinity determined for the native sequence (peptide **8**). Admittedly, lysines in the peptide backbone are reactive towards NHS-activated surfaces. While the extent to which peptides are bound through lysine immobilization is difficult to measure, its effect can be statistically excluded and should not prevent a comparison of binding affinity displayed by N- and C-terminal oriented peptides. Given the N- and C-terminal specific immobilization schemes described above, enrichment of oriented peptides is clearly achievable. In rare

instances when absolute control is needed, protecting groups can be used to avoid side-chain reactions followed by deprotection of bound peptides on the array.

In conclusion, we have demonstrated a simple strategy for one-step fluorescent derivatization of peptides that generates either an N or C-terminal alkylamine for directionally preferred peptide immobilization. Significantly, peptides can be synthesized in parallel at a sub-micromole scale in 96-well filter plates with fluorescent tags, 2AB and AEAB. These small tags facilitate HPLC profiling, while limiting interference with antigen-antibody interactions after peptide immobilization. Significantly, peptide orientation had a dramatic effect on antibody binding in a sequence-dependent manner. We believe that this strategy provides a useful approach to screen potential inhibitors of protein-protein, protein-carbohydrate, or protein-cell binding events.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Effect of terminal immobilization on antibody binding

Peptides were printed on NHS activated slides at 100 µM (**A and B**). The RFU value is the mean fluorescent intensity of 6 individual spots. (**A**) [HPC4 antibody] = 0.01 µg/mL. (**B**) [HPC4 antibody] = 1 µg/mL.

Scheme 1.

Preparation of fluorescently labeled peptides for selective N- or C-terminal immobilization.