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Developing Site-Specific Immobilization Strategies of Peptides in a Microarray

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Abstract—In peptide-based microarrays, most existing methods do not allow for site-specific immobilization of peptides on the glass surface. We have developed two new approaches for site-specific immobilization of kinase substrates onto glass slides: (1) slides were functionalized with avidin for attachment of biotinylated peptides; and (2) slides were functionalized with thioester for attachment of N-terminally cysteine-containing peptides via a native chemical ligation reaction.

Introduction

Peptide-based microarray is one of the most promising technologies which allow for potential miniaturized, high-throughput screenings of enzymatic activities, antigen–antibody or ligand–receptor interaction and so on.[1,2] At present, however, this technology is still in its infancy with a number of issues remaining unsolved. For example, immobilization of peptides, as well as proteins, on glass surface while retaining their biological activities, is still not well established. Although unlike their counterparts, proteins, peptides do not typically possess well-defined three-dimensional structures, immobilization of peptides on glass surfaces with a correct orientation is still imperative in order for the peptides to interact effectively with their targeting proteins. Most peptide- and protein-based microarrays reported to date use non-specific, covalent immobilization of molecules to the slide,[1,3] thereby failing to address this issue. Falsey et al. recently reported chemoselective N-terminal attachment of peptides onto a slide.[4] Using glass slides functionalized with glyoxylic acid, they were able to immobilize N-terminally cysteine-containing peptides via oxime-bond or thiazolidine-ring ligation reaction. This strategy, while elegantly designed to site-specifically immobilize peptides, may be of limited applications as the oxime bond is relatively unstable, and the five-membered ring thiazolidine may present immobilized peptides in an unfavorably restricted orientation to interact with their targeting proteins. While our manuscript was under review, another group has recently reported the use of Diels–Alder reaction for site-specific immobilization of peptides onto glass slides.[5] However, this method requires the conjugation of peptides with an unnatural cyclopentadiene moiety, making it synthetically challenging and not easily accessible.

In this report, we have developed two new approaches for site-specific immobilization of peptides in a microarray. By exploring the chemoselective chemistry used in native chemical ligation,[6] and the chemistry of biotin-avidin interaction,[7] peptide kinase substrates were readily modified, immobilized onto a 3 inch × 1 inch glass slide in both site-specific and regiospecific fashions, and were shown to retain their biological activity.

Results and Discussion

The p60, PKA and JAK substrates (YIYGSFK, ALRRASLG and KGTGYIKTG respectively) containing appropriate N-terminal residues, were synthesized as previously described.[8] The phosphorylated substrates were synthesized with Fmoc-Tyr(PO(Obzl)OH)-OH and Fmoc-Ser(PO(OBzl)OH)–OH. An extended cycle (4 h

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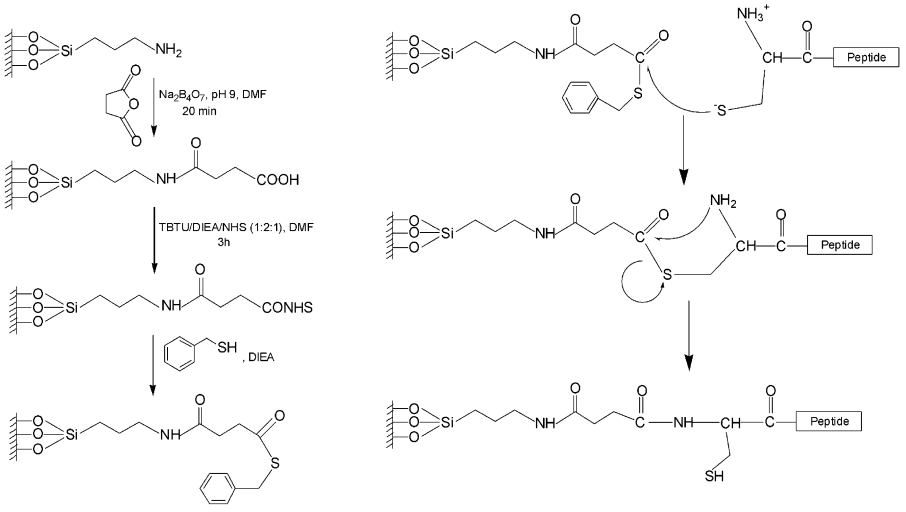
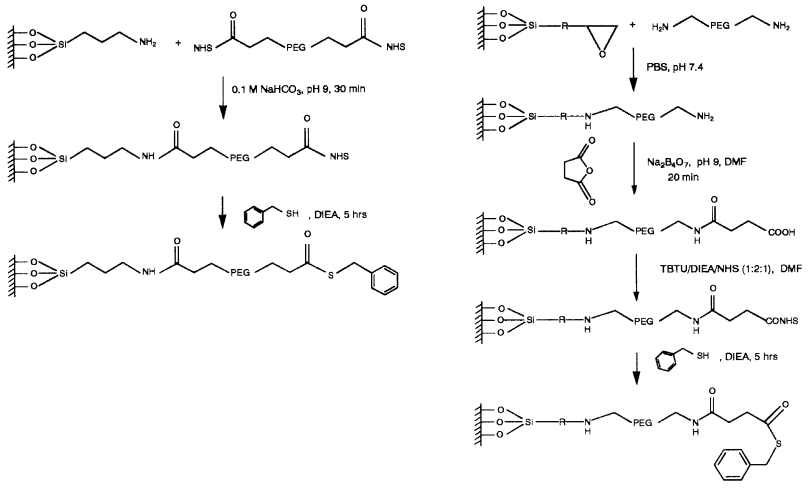
coupling) was used to couple the phosphorylated serine and tyrosine, as well as the N-terminal biotin, to the peptides.

Avidin-derivatized slides were prepared from plain glass slides (Fisher Scientific, USA). First, glass slides were cleaned in a piranha solution and derivatized with a 1% solution of 3-glyicidoxypropyltrimethoxisilane (95% ethanol, 16 mM acetic acid) for 1 h and cured at 150 °C for 2 h. The resulting epoxy slides were reacted with a solution of 1 mg/mL avidin in 10 mM $NaHCO_3$ for 30 min, washed with water, air dried and the remaining epoxide was quenched with a solution of 2 mM aspartic acid in a 0.5 M $NaHCO_3$ buffer (pH 9). Thioester-containing slides were prepared from amine slides as shown in Scheme 1. First, amine slides were placed in a solution of 180 mM succinic anhydride in DMF, pH 9 ($Na_2B_4O_7$) for 30 min and subsequently in boiling water for 2 min. The slides were washed with ethanol, dried and the carboxylic acid was then activated with a solution of *O*-benzothiazol-1-yl-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate (TBTU), *N*,*N*-diisopropylethylamine (DIEA) and *N*-hydroxysuccinimide (NHS) (1:2:1) in DMF for 3 h and reacted overnight with a solution of 120 mM DIEA and 100 mM benzylmercaptan in DMF. PEGylation of slides was done as described in Scheme 2: (a) amine slides were reacted with PEG-succinimidyl propionate (SPA-PEG; Shearwater, USA) for 30 min in 0.1 M $NaHCO_3$, pH 9 and overnight with a solution of 120 mM DIEA and 100 mM benzylmercaptan in DMF; (b) alternatively, slides were prepared by first functionalization with epoxy groups, and then with a 100 mM solution of diamine-PEG (Shearwater, USA). The slides were subsequently placed in a solution of 180 mM succinic anhydride in DMF, pH 9 ($Na_2B_4O_7$) for 30 min and subsequently in boiling water for 2 min. The carboxylic acid was then activated with a solution of TBTU/DIEA/NHS (1:2:1) in DMF for 3 h and reacted overnight with a solution of 120 mM DIEA and 100 mM benzylmercaptan in DMF. The derivatization of antibodies with Cy3-NHS (Amersham, UK), Cy5-NHS or Fluorescein-NHS, their on-chip detection and kinase assay were as described.[8]

Falser et al. recently reported chemoselective N-terminal attachment of peptides onto a slide using oxime and thiazolidine chemistry.[4] Zhu et al. investigated immobilization of terminally His-tagged proteins onto a glass



Scheme 1. Thioester-containing slides and native chemical ligation.



Scheme 2. PEGylation of thioester slides.

slide functionalized with Ni–NTA.[2] We reason that, in order for kinase assay to be performed efficiently and accurately in a microarray format, the substrates need to be attached at their terminal residues via a site-specific immobilization method. We took advantages of the interaction between avidin and biotin, one of the strongest non-covalent interactions known ($K_d = 10^{-15}$ M) to immobilize N-terminally biotinylated peptides onto a glass slide functionalized with avidin. Avidin is also a highly stable protein that maintains its functions even under extremely harsh conditions,[9] and therefore is an ideal candidate for slide derivatization and immobilization. Although the biotin-avidin interaction is well studied, this is the first report of its use for site-specific immobilization of peptides in a peptide array. An N-terminally biotinylated Janus Tyrosine Kinase (JAK) substrate with an additional GG spacer (Biotin-GG-KGTGYIKTG) was dissolved in PBS, pH 7.4, and arrayed on the avidin slide. After a few minutes of incubation, the slide was washed with PBS, water and then dried. Successful detection of the JAK peptide was accomplished by incubation for 1 h with a Cy3-labeled antibody raised against JAK (Fig. 1a). The extremely high biotin-binding affinity towards avidin allows for tight and efficient immobilization of the biotinylated peptide on the glass slide. In addition, since the avidin-biotin binding is instantaneous, no incubation time was needed for the immobilization of the peptide onto the slide, in contrast with other existing methods which require long incubation time for immobilization to occur.[1,3,4] The use of avidin-functionalized glass slides also provides a molecular layer between the glass surface and proteins interacting with the immobilized peptides, thereby eliminating the BSA blocking and minimizing nonspecific binding on the glass surface. Kinase assay was also performed on avidin-functionalized slides with a biotinylated, non-phosphorylated p60 substrate containing an additional GG spacer (Biotin-GG-YIYGSFK). Upon incubation with p60 tyrosine kinase for an h, the slide was probed with FITC-labeled anti-phosphotyrosine (Fig. 1b). Successful phosphorylation and detection of the p60 substrate further demonstrate the utility of this new method for site-specific immobilization of peptides on glass slides.

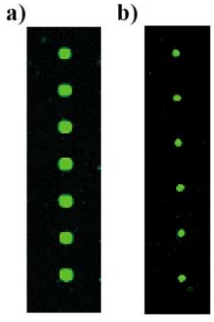


Figure 1. Immobilization of biotinylated peptides onto a glass slide functionalized with avidin. (a) JAK peptide on the slide was probed with a Cy3-labeled antibody raised against this peptide. (b) p60 substrate was spotted onto the slide, phosphorylated with p60 kinase and then probed with FITC-labeled anti-phosphotyrosine. Note no BSA blocking was used in this experiment.

We next explored the chemistry of native chemical ligation[6] for site-specific, covalent immobilization of N-terminally cysteine-containing peptide substrates onto glass slides functionalized with Thioester. Immobilization of peptides using native chemical ligation is highly specific since only the terminal cysteine would react with the thioester on the glass surface to form a stable native peptide bond (Scheme 1b). Presence of other reactive amino acid side chains, including internal cysteines, is tolerated. This immobilization method differs from those developed by McBeath et al. where either any sulfhydryl group may react with maleimide-functionalized slides,[10] or any nucleophilic group ($-NH_2$, –SH, –OH) may react with NHS- or epoxy-functionalized slides.[1,3] Formation of a stable peptide bond between the immobilized peptides and the glass surface using our method is also in contrast with that developed by Falser et al. where an unstable oxime bond or a rigid thiazolidine ring was formed.[4] A JAK peptide with an additional three amino acids CGG at the N terminus (CGG-KGTGYIKTG), together with the phosphorylated p60 peptide (CGG-YIYGSFK), were dissolved in PBS, pH 7.4, and spotted onto the thioester slide. Following incubation for 5 h, the peptides were successfully detected with their corresponding fluorescently-labeled antibodies (Fig. 2a and b). The two non-phosphorylated CGG-containing substrates of p60 and PKA (CGG-YIYGSFK and CGG-ALRRASLG, respectively) were also spotted onto the thioester-functionalized slide, incubated with their corresponding kinases, and detected successfully with FITC-labeled anti-phosphotyrosine and anti-phosphoserine (Fig. 2c and d), further demonstrating the compatibility of this immobilization method with microarray-based kinase assay.

Additional studies were conducted to further evaluate this novel thioester-containing glass surface. Cysteine-containing fluorescein (Fig. 3a) was dissolved in PBS, pH 7.4, serially diluted and arrayed on the thioester slide. Following overnight incubation, the slides were washed with PBS, water, dried and scanned. It was observed that the fluorescence intensity reaches saturation with 3 mM cysteine fluorescein, indicating the equivalent loading capacity of Thioester on the glass

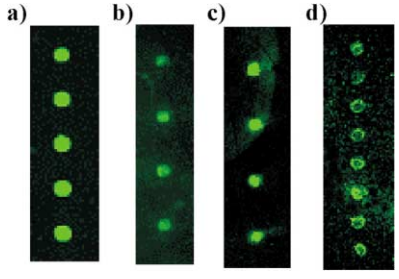


Figure 2. Native chemical ligation for chemoselective, covalent immobilization of N-terminal cysteine peptides onto a glass slide functionalized with thioester, as shown in Scheme 1. (a) Immobilized JAK peptide probed with Cy3-labeled anti-JAK. (b) Immobilized phosphorylated p60 peptide probed with FITC-labeled anti-phosphotyrosine. (c) Non-phosphorylated p60 peptide was immobilized, treated with p60 kinase and probed with FITC-labeled anti-phosphotyrosine. (d) Non-phosphorylated PKA peptide was immobilized, treated with PKA kinase and probed with FITC-labeled anti-phosphoserine.

surface (Fig. 3b). In order to evaluate the time needed for efficient immobilization of peptides, different concentrations of the cysteine-containing fluorescein in PBS (pH 7.4) were spotted and incubated for increasing periods of time (Fig. 3c). It was observed that the reaction took place within the first 30 min of incubation. After 3 h of incubation, spots corresponding to lower concentrations were eventually observed and the intensity reached saturation, indicating the completion of the native chemical ligation reaction, hence the complete peptide immobilization.

BSA is commonly used as a blocking agent to remove non-specific binding of proteins to the glass surface. However, it cannot be used directly in peptide-based arrays, presumably because it obscures the molecules of interest.[1] In order to solve this problem, slides functionalized with BSA containing reactive NHS were used to immobilize peptides.[1] However, peptides randomly reacted with NHS on the slide, resulting in non-specific immobilization on the surface. We derivatized glass slides with two types of PEG followed by treatments with other chemicals to give thioester surfaces containing a PEG layer in order to remove non-specific binding (Scheme 2). The phosphorylated CGG-containing p60 substrate was spotted onto the two PEG-COSR slides, then probed with the FITC-labeled anti-phosphotyrosine without any BSA blocking. Very little non-specific binding was observed on both surfaces and the background noise was very low (Fig. 4a), presumably due to the PEG layer between immobilized peptides and the glass surface. In addition to minimizing non-specific binding, PEG also acts as a spacer between the slide and the immobilized peptides which should facilitate phosphorylation by kinase. In order to confirm this, the non-phosphorylated CGG-containing p60 substrate was spotted onto the PEGylated thioester slide, incubated with p60 kinase and probed with FITC-labeled anti-phosphotyrosine (Fig. 4b). A positive signal with little background noise was observed, confirming the additional advantage of the PEG slide.

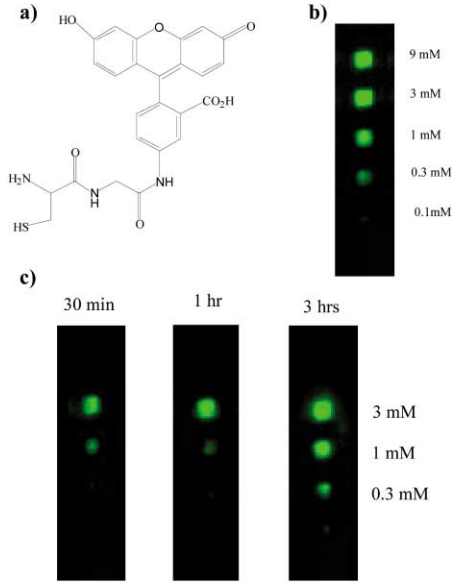


Figure 3. (a) Structure of cysteine-containing fluorescein used; (b) saturation studies of thioester slides using different spotting concentrations of cysteine-containing fluorescein; (c) determining the rate of chemical ligation reaction on-chip with differing amounts of cysteine-containing fluorescein following different incubation time.

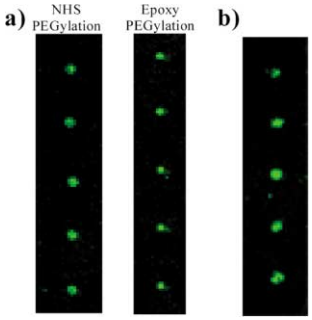


Figure 4. PEGylation of glass slides. (a) Phosphorylated p60 peptide was spotted onto glass slides functionalized with NHS PEGylation (left) and Epoxy PEGylation (right), and probed with FITC-labeled anti-phosphotyrosine. (b) Kinase assay on PEGylated slides. Non-phosphorylated p60 peptide was spotted onto a NHS PEGylated slide, treated with p60 kinase and probed with FITC-labeled anti-phosphotyrosine. Note no BSA blocking was used in this experiment.

Conclusion

The success of microarray-based technologies hinges largely on effective immobilization of molecules onto the glass surface in a correctly-oriented fashion. Few reports to date have focused on developing site-specific immobilization of molecules in a microarray. We reported here two new methods for site-specific attachment of N-terminally modified peptides in a microarray. Biotinylated peptides were spotted onto glass slides functionalized with avidin to achieve instantaneous immobilization. Avidin serves as both an immobilizing agent and an agent to minimize non-specific absorption of proteins on glass surface. Alternatively, peptides containing an N-terminal cysteine were chemoselectively immobilized onto thioester-funtionalized slides. Both methods show versatility in generating peptide-based microarrays suitable for high-throughput screenings of kinase activities, and potentially other enzymatic activities. The intercalation of a PEG layer between the slide surface and the immobilized peptides minimized non-specific binding on the glass surface, rendering it possible to eliminate the BSA blocking step.

Acknowledgements

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