Review

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Immobilization strategies for small molecule, peptide and protein microarrays‡

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Protein, peptide and small molecule microarrays are valuable tools in biological research. In the last decade, substantial progress has been achieved to make these powerful technologies more reliable and available for researchers. This review describes chemical preparation methods for these microarrays with focus on site-selective and bioorthogonal immobilization reactions, particularly the Staudinger ligation and the thiol-ene reaction. In addition, the application of peptide microarrays, which were prepared by Staudinger ligation, to substrate specificity mapping is illustrated. Copyright © 2009 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: small molecule microarrays; peptide microarrays; protein microarrays; biochips; immobilization methods; Staudinger ligation; thiol-ene reaction; chemoselective reactions; bioorthogonal reactions; high throughput screening

Background

Almost two decades after the first example of a peptide microarray [1] and one decade after printed small molecule- and protein microarrays were published [2,3], these technologies have matured to the point where they now are broadly applicable and more accessible to researchers [4–6]. They are referred to as either microarrays or biochips and are used, for example, for ligand discovery for proteins [4], analytical and functional protein studies [5] and substrate profiling [6]. The principle of using an array of spatially addressable compounds on a planar surface, and incubating this array with biomolecules of interest to investigate thousands of biomolecule interactions in parallel, had first been described by Edwin Southern for oligonucleotide probes [7] and has opened up a whole new technology field.

Biochips in general offer the advantages of high throughput screening while only requiring small amounts of analytes [4]. The feature that enables these advantages and distinguishes biochips from microtiter plate solution phase assays is the linkage of the compounds to the planar surface. This linkage can be covalent or noncovalent, and over the years, many researchers have contributed to its optimization and to expanding the immobilization method repertoire [4,8–10]. However, the immobilization of a compound can also be a disadvantage: If the compound moiety, which is attached to the surface and therefore masked, is relevant for binding to the biomolecule of interest, the compound might not be recognized by the biomolecule, which would lead to false negative results. Therefore, special attention has been paid to chemoselective and bioorthogonal reactions for the immobilization.

For small molecule microarrays several reactions are reported, such as thiazolidine ring formation via glyoxylyl group reaction with 1,2-amino thiols [11], 1,3-dipolar cycloaddition of terminal alkynes with azides [12,13] and Diels-Alder product formation of benzoquinone with cyclopentadiene [14]. Peptide microarrays are fabricated either through printing or on-chip synthesis, which can be accomplished because peptides are built in a linear fashion and can thus be site selectively immobilized via the first amino acid. The first examples were based on on-chip synthesis using either photolithography [1] or SPOT synthesis on membrane supports [15]. On-chip synthesis is very powerful in creating large libraries fast [16], but it has a disadvantage in that the quality control of the peptides is very difficult [9]. Alternatively, peptides have been printed using the same bioorthogonal approaches as for small molecules [9] and also native chemical ligation (NCL) [17]. The site-selective immobilization of proteins has an additional challenge to overcome: The residue, which enables a chemoselective reaction, has to be introduced to the protein already in a site-selective manner. This was achieved for example by fusing GST–His6 to the *N*-termini of yeast proteins and printing them onto Ni NTA slides [18]; however this immobilization is not covalent. For selective covalent binding, NCL can be applied with the thioester located either at the *C*-terminus of the protein [19], or on the surface [20]. Using the *C*-terminal thioester approach, other functional groups, such as biotin, can be introduced site selectively for other immobilization methods [8].

Immobilization by Staudinger Ligation

We approached the issue of site-selective immobilization for small molecule- and peptide array preparation by defining the following design criteria, which our new coupling strategy had to fulfill: Employment of uniquely reactive functional groups, which (i) tolerate a diverse array of other functionalities, (ii) react efficiently under mild reaction conditions in an atmosphere

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Biography

Maja Köhn was born in 1975 in Kiel, Germany. In 2001, she graduated in Chemistry at the Christian-Albrechts-University in Kiel. She obtained her Ph.D. at the Max-Planck-Institute for Molecular Physiology and the University of Dortmund, Germany. The thesis was focused on immobilization methods for the preparation of microarrays. She conducted her postdoctoral studies at Harvard University, Cambridge, Massachusetts, USA, where she was working on oligonucleotide inhibitors for the hepatitis C virus. Since 2007 she is a group leader at the European Molecular Biology Laboratory. Her research interests lie in the interface between chemistry and biology. Her group is developing tools and inhibitors to study protein phosphatases, mainly those that are linked to diseases. These tools are based on peptide as well as phosphoinositide chemistry, protein semisynthesis, and also molecular biology approaches.

containing oxygen and water, and (iii) are compatible to the strategies of solid-phase combinatorial chemistry.

As the immobilization reaction, the Staudinger ligation between azides and appropriately substituted phosphanes was chosen, in which a chemically stable amide bond is formed [21], because this reaction had been shown to be chemoselective and bioorthogonal in many different approaches [22]. The coupling strategy was the following: Azide-functionalized small molecules and peptides are generated by solid-phase organic synthesis (SPOS). The application of a Kenner-type linker allows the azide moiety to be introduced within the safety-catch cleavage step (Figure 1(a), A) [23], in order for the azide not to interfere with other reactions on the solid phase. In the directly following step, the activated library molecules B are selectively immobilized to glass substrates through Staudinger ligation with surface bound phosphane groups (Figure 1(a), C). The surface-ordered molecules (Figure 1(a), D) are then susceptible to ligand-binding reactions using, for example, fluorescently labeled proteins [24].

Several different compounds (such as **1**, **3**, **4** in Figure 1(b)) as well as the appropriate negative control (for example, **2** in Figure 1(b)) were immobilized. Only the azide-containing molecules could be detected. Compounds **3** and **4** were synthesized according to the outlined strategy. Spotting different concentrations of molecule **1** revealed concentration dependence of the fluorescence signal. Thus, it was demonstrated that this immobilization strategy fulfills all of the above-listed criteria and enables rapid access to the compounds to be immobilized [24].

With this methodology in hand, we intended to develop a microarray with which preferred peptide substrates of protein tyrosine phosphatases (PTPs) can be detected. PTPs and their biological substrates are integrated within signal transducing networks that are essential for processes such as cell growth, differentiation, cell–cell communication, and immune response [25]. Defective operation of these networks leads to aberrant tyrosine phosphorylation, which contributes to the development of many human diseases including cancers and diabetes [25]. Deciphering preferred peptide substrates for different PTPs provides information that can lead to identification of their biological substrates and therefore their roles in biological pathways, and enables discovery of drug candidates [26–29]. For many PTPs however, substrate specificities still have to be

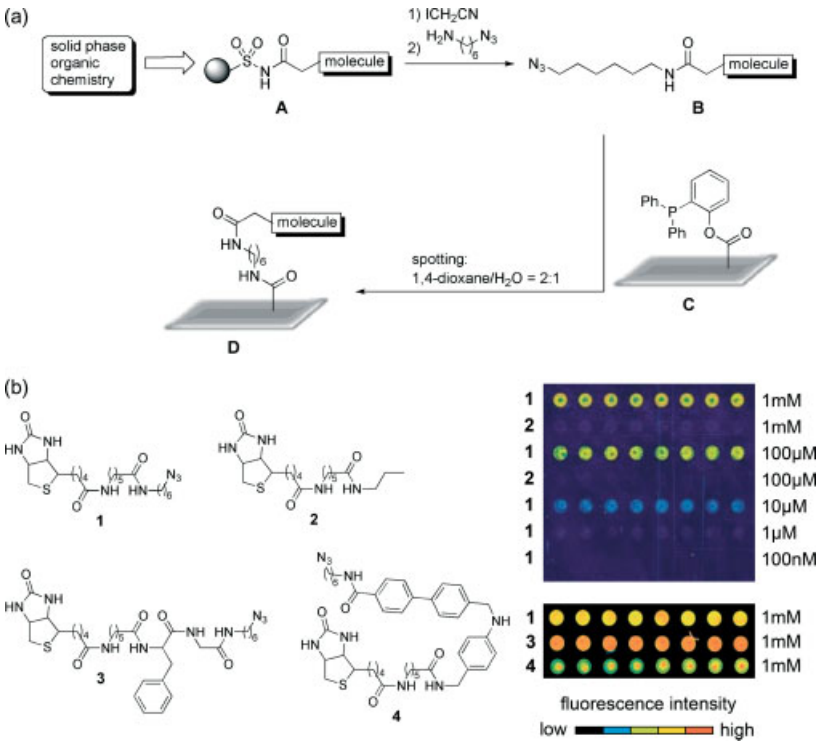

Figure 1. Site-selective immobilization by Staudinger Ligation. (a) Coupling strategy; (b) Detection of biotinylated compounds using Cy5-labeled anti-biotin antibody (100 nM). Numbers of spotted compounds are shown on the left and their concentrations on the right of the corresponding spots on the microarray.

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identified [25,26]. Our microarray strategy would accomplish this in a high throughput fashion.

The following approach was applied: A phosphotyrosine(pTyr)-peptide library was synthesized (Figure 2(a), E) and immobilized on a phosphane-modified glass slide (Figure 2(a), F, G) following the Staudinger Ligation coupling strategy. Since phosphane **5**, which leads to the 'nontraceless' ligation product, showed a better coupling efficiency than the phosphane used before (Figure 1(a), C), it was used for all following applications. The array was incubated with the phosphatase of interest, washed (Figure 2(a), H), treated with a fluorescently labeled anti-pTyr antibody and washed again (Figure 2(a), I). Preferred substrates of the PTP show signal decrease compared to a buffer incubated array, whereas no change in signal intensity indicates nonsubstrates (Figure 2(b)) [30].

This array strategy was validated using the phosphatase PTP1B, a well-studied phosphatase with respect to its substrate specificity [26,27,31]. Then, the catalytic domain of the receptor-like phosphatase RPTPμ was screened on a chip containing 48 different pTyr-peptide substrates to map its peptide substrate specificity, since little is known about its substrate specificities, intracellular substrates and signaling properties [32]. The on-chip results were validated through biochemical dephosphorylation assays, yielding good agreement and reproducibility, and the reaction kinetics for the best substrate of RPTPμ was determined. In addition, by replacement of the phosphotyrosine of the best substrate with phosphonomethyl-phenylalanine, an inhibitor active in the micromolar range was created. Taken together, we demonstrated that our microarray strategy enables screening for PTP substrate specificities, and that substrates, which were identified using our microarray, can be used for kinetic studies with, and inhibitor design for PTPs [30].

Since the Staudinger ligation proved to be efficient, bioorthogonal and chemoselective, it was also applied to the site-selective immobilization of proteins for protein-microarray preparation. Azides are not naturally occurring in proteins and therefore a building-block strategy was designed, which enables the site-selective introduction of functional groups into proteins via expressed protein ligation. This extra step to bring in the azide site selectively into a protein might be considered a disadvantage. However, our strategy offers the possibility of the addition of two different groups to the *C*-terminus of a protein at the same time.

Azide-functionalized N-Ras proteins as well as unmodified N-Ras proteins as negative control were immobilized onto phosphane-modified glass surfaces. The read-out was performed using fluorescently labeled anti-Ras antibody, which only detected N-Ras proteins containing the azide, demonstrating that the immobilization occurred site selectively. The anti-Ras antibody recognizes a helix belonging to the active site of Ras, and thus the positive read-out demonstrated indirectly the perpetuation of protein activity [33,34].

Using a mercaptomethyl-substituted phosphane, Raines and coworkers also applied the Staudinger ligation to the preparation of peptide and protein microarrays. This, together with our results, demonstrates the excellent applicability of this reaction for this purpose [35,36].

Photoactivatable Immobilization

For many biotechnological applications, such as directed cell growth for the preparation of cell microarrays, it is important to control size and dimension of the patterns of the immobilized proteins, peptides or small molecules [8,37,38]. Patterns can be generated for example through photolithography using photoactivatable reactions, such as the conversion of arylazides to reactive nitrenes and subsequent insertion into C–H bonds [4,8,37]. Whereas photoactivatable immobilization offers the possibility to structure surfaces by light exposure through a mask, commonly used reactions do not lead to a site-selective attachment. Thus, we intended to develop a mild, chemo- and regioselective immobilization reaction to be able to create microstructured surfaces. Since the thiol-ene reaction, which is the photo-induced addition of thiols to terminal alkenes yielding a thioether linkage, seemed to be promising to fulfill these criteria [39–41], it was chosen for this application.

The allyl amides of several compounds, such as biotin and a pTyr-peptide, were synthesized and immobilized on thiol-modified silicon wafers (Figure 3(a)). Immobilization was accomplished via covering the surface with a thin film of the compound and subsequent light exposure through a mask. In addition, streptavidin, which was modified with 5-pentenoic acid, was immobilized as a model protein. The immobilized molecules were detected using corresponding fluorescently labeled proteins, i.e. streptavidin and anti-pTyr antibody, and patterned streptavidin was visualized with Cy5-labeled biotin. Signals were obtained only in the regions where the light could reach the surface through the mask, and all the compounds including the protein streptavidin retained their ability to bind their corresponding binding partners after irradiation (Figure 3(b)) [42]. These results demonstrated the remarkable bioorthogonality, chemoselectivity and mildness of the radical thiol-ene reaction.

The versatility of this method was then further demonstrated by immobilizing biotinylated alkaline phosphatase as well as complexes of Ras-GDP and Ras-GppNHp, a constitutively active form of Ras, on streptavidin-patterned slides, which were obtained through biotin-allyl amide patterning of the silicon wafers. These microstructured surfaces were used to study several protein–protein interactions [43]. Surface micropatterning was additionally achieved by using a laser for the immobilization instead of a mask, opening up possibilities to create a wide variety of patterns [42,43].

Perspectives

Microarray technologies have become valuable tools for biological research, with DNA microarrays being the most commonly used and basically essential tool for certain applications. With the progress achieved in the fabrication methods of protein, peptide and small molecule microarrays, these techniques are now more available and reliable for researchers. They represent powerful and advantageous alternatives to existing high throughput techniques such as microtiter plate solution assays. Protein, peptide and small molecule microarrays, in particular ones prepared by site-selective immobilization procedures, are however not as widely used as DNA microarrays yet, in part due to the surfaces required for chemoselective immobilization not being commercially available and because introduction of the bioorthogonal functional group into the molecule of interest is a necessary additional step. Interdisciplinary setup or collaborations are still needed to fully exploit the scope of these microarrays at this point. Nevertheless, with the interdisciplinary field of chemical biology growing quickly in the last decade, and further advances being made in the

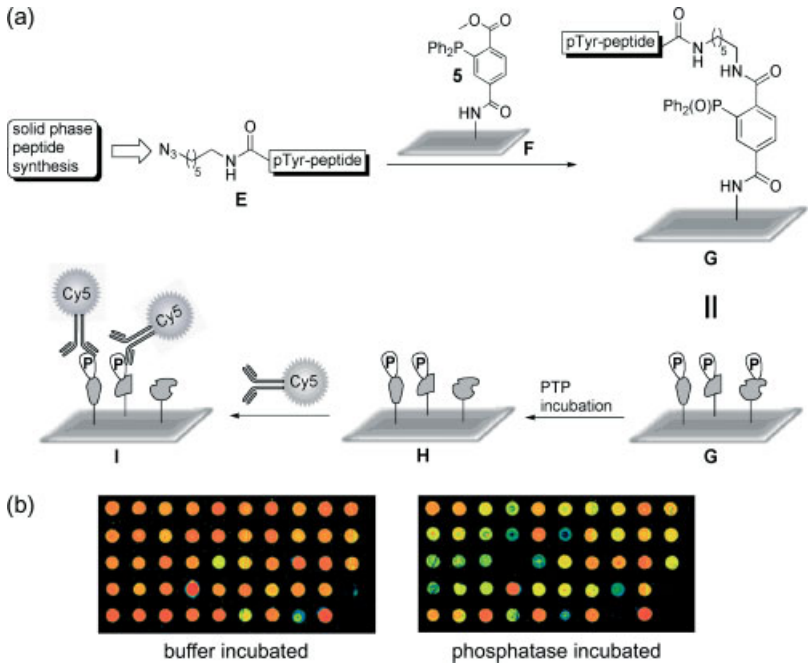

Figure 2. A microarray for mapping of phosphatase substrate specificities. (a) Array strategy; (b) Fluorescence read-out of a microarray incubated with buffer *versus* a microarray incubated with a phosphatase (PTPμ, 5 µg/ml). Read-out was achieved using Cy5-labeled anti-pTyr antibody (50 nM). For a color intensity scale see Figure 1(b).

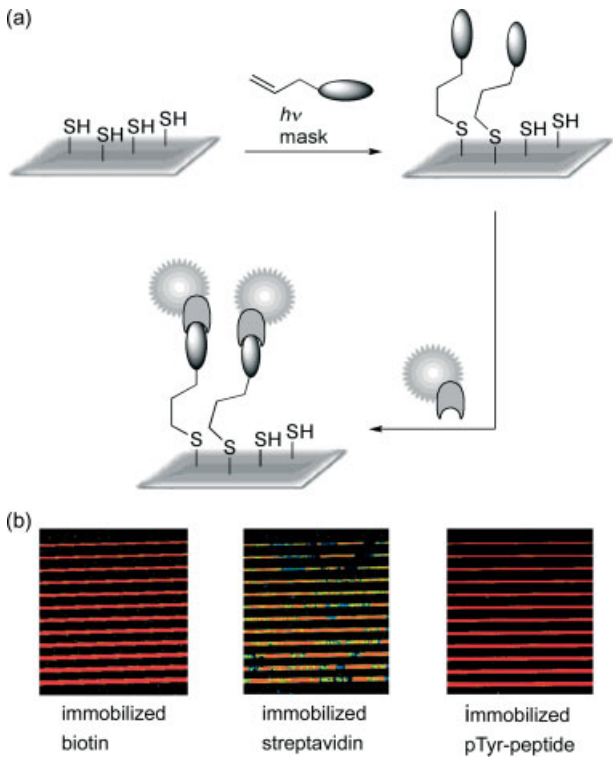

Figure 3. Microarrays fabricated using the thiol-ene reaction. (a) Immobilization strategy; (b) Read-out of surfaces patterned with ene-modified biotin (10 mM), streptavidin (200 µM) and pTyr-peptide (500 µM) by incubation with Cy5-labeled streptavidin (100 nm), biotin (10 nM) and anti-pTyr antibody (50 nM), respectively. For a color intensity scale see Figure 1(b).

microarray field, these technologies are likely to play a very important role in biological research in the future.

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