Microarray Immobilization

Fabrication of Chemical Microarrays by Efficient Immobilization of Hydrazide-Linked Substances on Epoxide-Coated Glass Surfaces**

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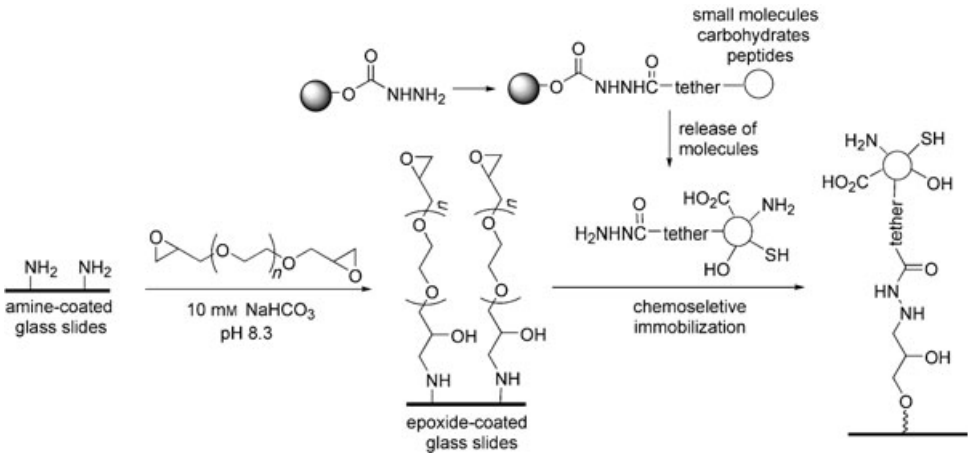
Small molecules that regulate biological processes serve as valuable tools in studies of the functions of biomolecules, especially proteins, as well as in the development of drugs.[1] An important component of efforts that target the discovery of bioactive molecules is high-throughput screening. Technologies that rely on the use of DNA, protein, and carbohydrate microarrays have been widely employed to accelerate the selection of lead compounds and as high-throughput analytical tools in genomic, transcriptomic, proteomic, and glycomic research.[2–4] Microarray platforms enable the simultaneous assessment of a large number of samples that are available in limited quantities.

Similarly, small-molecule microarrays have been used as high-throughput methods to identify substances that selectively bind to proteins.[5] Most of the small molecules of interest in these efforts possess a number of different functional groups, such as hydroxy (OH), amino (NH_2), carboxy (CO_2H), and sulfanyl groups (SH). The major requirement of techniques used to fabricate small-molecule microarrays is that immobilization of the diversely functionalized compounds to the modified surfaces must be highly selective. Strategies that employ efficient and chemoselective ligation processes would be generally applicable to the fabrication of microarrays that possess covalently linked, biologically interesting molecules. Herein we describe a novel chemoselective immobilization process in which hydrazide-containing compounds react with epoxides coated on glass slides. This new technique has been applied to the efficient construction of chemical microarrays, which have been used to evaluate protein binding to peptides and small molecules.

Several criteria must be met in designing a general method to prepare diverse chemical microarrays. Firstly, the diverse substances containing the specific functional groups used for selective reactions with the modified solid surfaces must be easily prepared by solid-phase synthesis. Also, functional groups that will selectively react with the small molecules must be readily incorporated onto the solid surfaces. Lastly, following their release from a solid support, the diversely structured and functionalized small molecules must undergo site-specific covalent attachment to the modified surfaces. Strategies employing highly chemoselective ligation reactions fit these criteria. We have investigated a novel technique for immobilization, which relies on the use of reactions between hydrazide-containing small molecules and epoxide-coated glass slides (Scheme 1). The hydrazide groups are incorporated into the small molecules while they are attached to a solid support and are used as a handle in their solid-phase synthesis.[6] The epoxide-derivatized glass slides are easily created by immersing amine-coated glass slides into a solution of poly(ethylene glycol) diglycidyl ether (3% solution in 10 mM $NaHCO_3$, pH 8.3).[7]



Scheme 1. Strategy for the fabrication of a chemical microarray based on the immobilization of hydrazide-containing compounds on epoxide-derivatized glass slides.

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A hydrazide-linked fucose probe (**S**- or **L**-Fuc-$NHNH_2$, **S**: short, **L**: long; Scheme 2) was used to probe optimal conditions (pH, time, and concentration) for the immobilization process. Preparation of **S**- or **L**-Fuc-$NHNH_2$ was initiated by transforming alcohol groups on a Wang resin into *p*-nitrophenyl carbonates.[8] The resulting resin was then treated with hydrazine to yield the hydrazide-containing resin, which was coupled with the Fmoc-protected **L** or **S** tether in the presence of NEM, BOP, and HOBt. The amino groups

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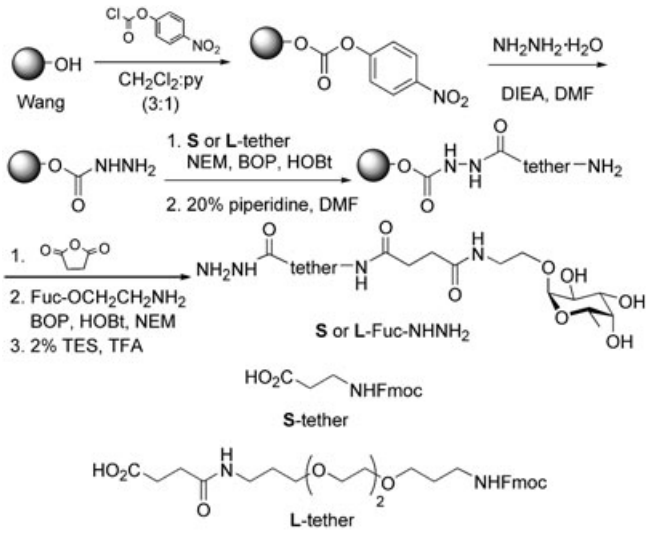
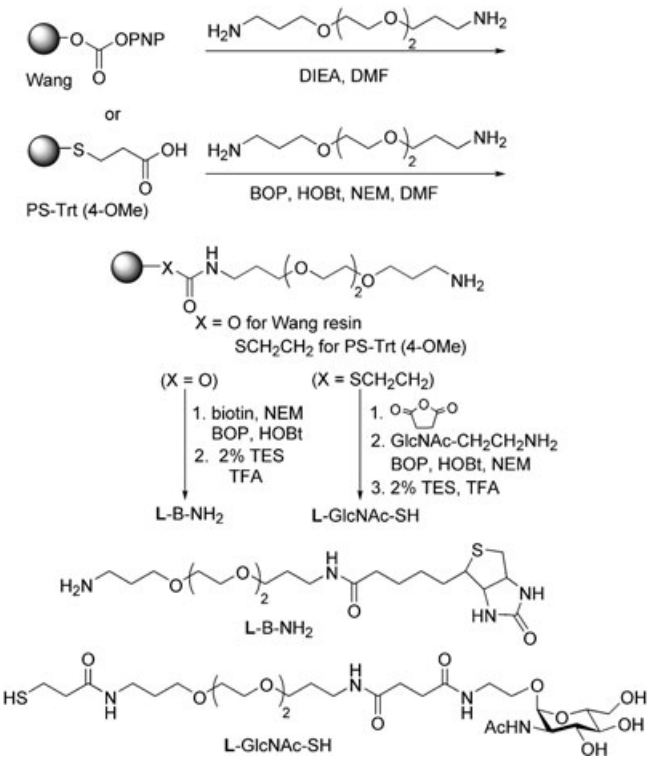


Scheme 2. Synthesis of the hydrazide-linked fucose probes **S**- and **L**-Fuc-$NHNH_2$. Py: pyridine, DIEA: ethyldiisopropylamine, DMF = *N*,*N*-dimethylformamide, NEM = *N*-ethylmorpholine, BOP = 1-benzotriazolyloxytris(dimethylamino)phosphonium hexafluorophosphate, HOBt = 1-hydroxybenzotriazole, TES = triethylsilane, TFA = trifluoroacetic acid, Fmoc = 9-fluorenylmethoxycarbonyl.



Scheme 3. Synthesis of amine-linked biotin (**L**-B-NH_2) and thiol-linked GlcNAc (**L**-GlcNAc-SH) probes. PNP = *p*-nitrophenyl.

produced by treatment of the resin with piperidine were treated with succinic anhydride to generate carboxylic acids, which underwent an amide-bond-forming reaction with aminoethyl α-fucopyranoside. Finally, the desired compounds were released from the resin by treatment with 2% TES in TFA.

In order to find the proper immobilization conditions, a solution of **L**-Fuc-$NHNH_2$ was printed onto the epoxide-derivatized glass slides and then the resulting slides were probed with Cy5-labeled *Aleuria aurantia* lectin (Cy5-AA) for 1 h (Cy5: indodicarbocyanine).[9] It was found that substrate concentrations of 0.5–1 mM and times of 3–4 h at pH 3–5 were appropriate for efficient immobilization reactions.[8]

An important feature of the method we have developed for constructing small-molecule microarrays is chemoselectivity. This was demonstrated in studies of selective attachment of hydrazides onto the epoxide-coated surfaces in the presence of other potent nucleophilic groups, such as amines and thiols. For these studies, amine-linked biotin (**L**-B-NH_2) and thiol-linked *N*-acetylglucosamine (**L**-GlcNAc-SH) probes were synthesized on solid supports by using *p*-nitrophenol-activated Wang and 2-carboxyethanethiol 4-methoxytrityl polystyrene (PS-Trt-(4-OMe)) resins, respectively (Scheme 3).[8]

A 1:1 mixture of **L**-Fuc-$NHNH_2$ and **L**-GlcNAc-SH (1 mM in 30% glycerol in 100 mM sodium phosphate, pH 3–10) was applied to the epoxide-functionalized slides, which were then treated with Cy5-AA and Cy3-labeled *Triticum vulgaris* lectin (wheat germ agglutinin; Cy3-TV; Cy3: indocarbocyanine).[9] Fluorescence analysis showed that the hydrazide substrate (**L**-Fuc-$NHNH_2$) was selectively immobilized in reactions conducted at low pH values, while the thiol substrate (**L**-GlcNAc-SH) underwent a more efficient reaction with the epoxide residues at a higher pH value, when the thiol moiety is deprotonated (Figure 1a).

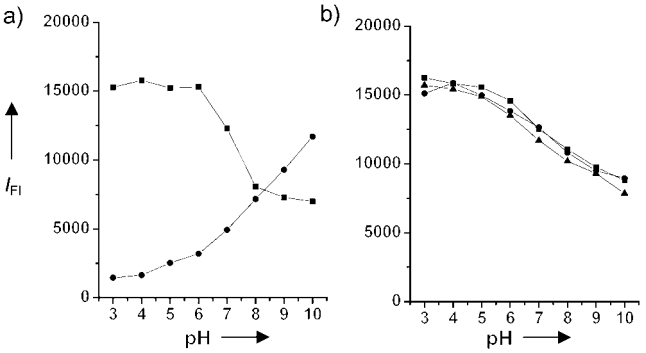
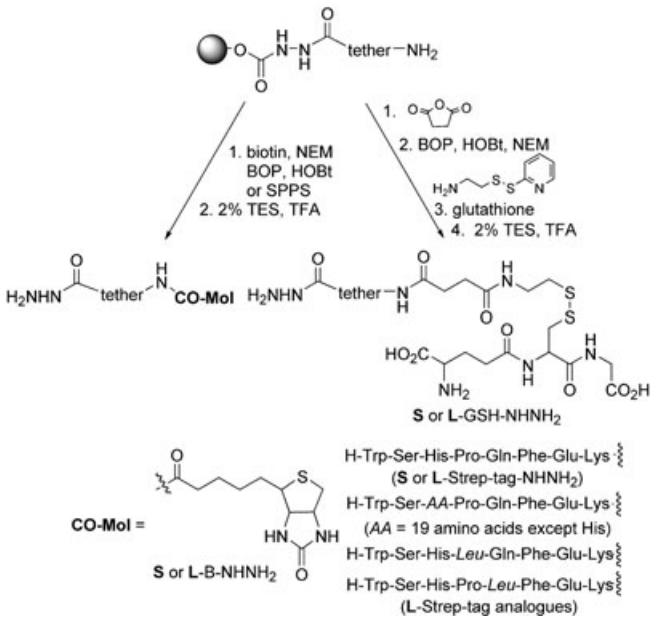


Figure 1. a) Immobilization of **L**-Fuc-$NHNH_2$ in the presence of one equivalent of **L**-GlcNAc-SH (■ probed with Cy5-AA, ● probed with Cy3-TV). b) Immobilization of **L**-Fuc-$NHNH_2$ in the presence of **L**-B-NH_2 (■ **L**-Fuc-$NHNH_2$ only, ● **L**-Fuc-$NHNH_2$:**L**-B-NH_2 (1:1), ▲ **L**-Fuc-$NHNH_2$:**L**-B-NH_2 (1:4); all probed with Cy5-AA).

Next, selective attachment of hydrazides to the epoxide-coated slides in the presence of amines was examined. Mixtures of **L**-Fuc-$NHNH_2$ and **L**-B-NH_2 (1:1 and 1:4 in 30% glycerol in 100 mM sodium phosphate, pH 3–10) were applied to the epoxide-derivatized slides and then probed with Cy5-AA. Since in some biologically interesting substrates (especially peptides) several amino groups are present, these immobilization reactions were run with mixtures

containing an excess of **L**-B-NH_2 of up to four molar equivalents. As shown in Figure 1 b, the fluorescence intensity of spots containing **L**-Fuc-$NHNH_2$ was barely changed when **L**-B-NH_2 was present, a result indicating that hydrazide-linked fucose (**L**-Fuc-$NHNH_2$) was selectively attached to the surface over the range of pH 3–10 even when a large excess of amine-containing biotin was used. The results of these competition experiments demonstrate that hydrazides are immobilized on the epoxide-coated surface more rapidly than substrates with both amine and thiol functional groups.

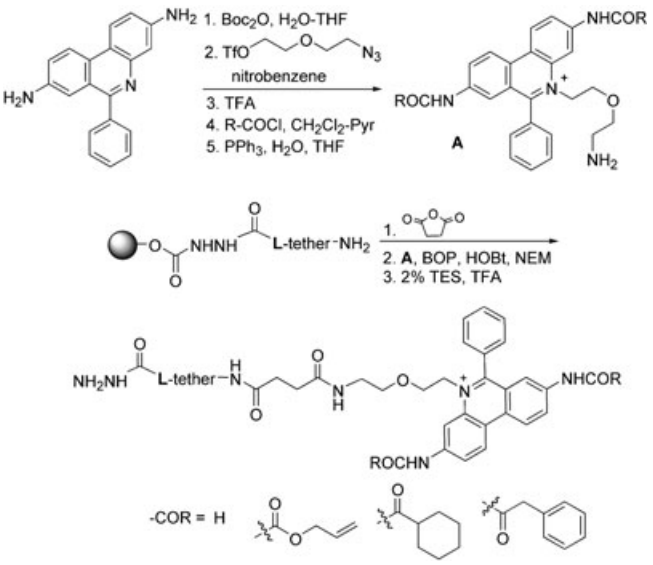
As part of a practical application of this technique to the fabrication of chemical microarrays, we synthesized hydrazide-linked substrates containing biotin (**S**- or **L**-B-$NHNH_2$), peptides (**S**- or **L**-GSH-$NHNH_2$, **S**- or **L**-strep-tag-$NHNH_2$ and its analogues), and phenanthridinium derivatives (Schemes 4 and 5).[8] The strep-tag peptide sequence and its



Scheme 4. Synthesis of hydrazide-linked probes containing biotin, glutathione (GSH), or the streptavidin tag (strep tag) and its analogues.

analogues were directly assembled on solid supports by using the conventional Fmoc strategy. Strep-tag analogues were prepared by replacing the histidine residue with 1 of the other 19 amino acids or by replacing the proline and glutamine residues with leucine. GSH was coupled to the resin by using a disulfide exchange reaction. Four phenanthridinium derivatives for acetylcholinesterase (AChE) inhibitors were prepared by solution-phase synthesis and subsequently coupled to the carboxylic acid derivatized resin.

Eight **S**/**L**-tethered fucose-, glutathione-, biotin-, and strep-tag-containing hydrazides (1 mM in 30% glycerol in 100 mM sodium phosphate, pH 5) were microspotted onto the epoxide-derivatized glass slides and then incubated with Cy5-AA, Cy3-streptavidin, Cy3-avidin, or glutathione S transferase (GST). GST incubation was followed by treatment with fluorescein isothiocyanate (FITC) labeled anti-GST antibody. As shown in Figure 2 a–d, fucose, glutathione, and the strep



Scheme 5. Synthesis of probes containing phenanthridinium derivatives. Boc: *tert*-butoxycarbonyl, Tf: trifluoromethanesulfonyl.

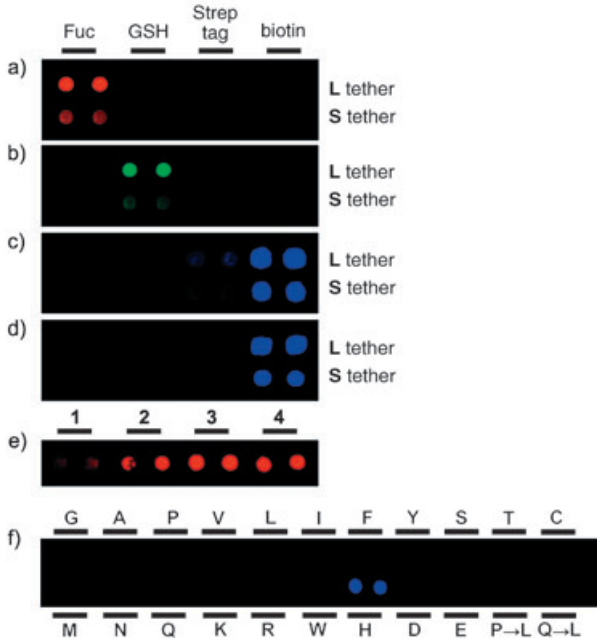


Figure 2. Fluorescence images of chemical microarrays containing fucose, GSH, the strep tag, and biotin, when probed with a) Cy5-AA, b) GST followed by FITC-labeled anti-GST antibody, c) Cy3-strepavidin, and d) Cy3-avidin. Fluorescence images of chemical microarrays containing e) phenanthridinium derivatives probed with Cy5-AChE (**1** COR = H, **2** COR = cyclohexylcarbonyl, **3** COR = allyloxycarbonyl, **4** COR = phenylacetyl) and f) strep tag and its analogues probed with Cy3-strepavidin (single letter amino acid codes given for the residue that replaced histidine in the strep tag, P→L: replacement of proline with leucine in the strep tag, Q→L: replacement of glutamine with leucine in the strep tag). Spot size: ≈100 μm, distance between centers of spots: ≈250 μm.

tag were selectively recognized by AA, GST, and streptavidin, respectively. The strep tag is known to bind to streptavidin rather than avidin and, as expected, microspots containing

this substance were recognized only by streptavidin.[10] Microspots containing biotin display much stronger fluorescence than strep-tag microspots because the binding affinity of streptavidin to biotin is much greater than that of streptavidin to the strep tag.[11] Interestingly, microspots produced by reaction of **L**-tethered substrates bound to the corresponding proteins more tightly than those with **S**-tethered substrates. The fluorescence intensities of microspots containing **L**-tethered compounds are more than twice as strong as those of microspots containing **S**-tethered ones.

Small-molecule microarrays with four phenanthridinium derivatives were also fabricated by following the method described above. Fluorescence analysis of slides treated with Cy5-AChE showed that substituted phenanthridinium derivatives bound to the proteins more strongly than their unsubstituted derivatives (Figure 2e).[12] Finally, we constructed peptide microarrays with the **L**-tethered strep tag and 21 of its analogues. The peptide microarrays, after probing with Cy3-streptavidin, showed that streptavidin only bound to the strep tag (Figure 2f). This is consistent with previous results showing that the amino acid sequence His-Pro-Gln is critical for streptavidin binding.[13]

In conclusion, a new, efficient, and simple method for fabricating chemical microarrays has been developed. The technique employs selective immobilization reactions of hydrazide-linked small molecules with epoxides on the solid surfaces. The length of the tether between the hydrazide groups and the active ligands governs protein binding; slides containing longer tethers exhibit stronger binding of proteins than those with shorter tethers. The immobilization technique is suitable for covalently attaching diverse compounds, including small molecules, carbohydrates, and peptides, to glass surfaces. The utility of this method is shown by its application to the fabrication of peptide and small-molecule microarrays, which have been used to screen for selective protein binding. We believe that the chemoselective ligation reaction developed in this effort will find many applications in the preparation of bioconjugates, such as neoglycopeptides, peptide–nucleic acid conjugates, and tagged peptides.

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