Microarrays

Immobilization of Natural Products on Glass Slides by Using a Photoaffinity Reaction and the Detection of Protein–Small-Molecule Interactions**

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Small molecules, especially from natural resources, are an important source of bioprobes which are useful in the study of protein function and cell signaling networks.[1] The identification of target proteins for small molecules which modulate particular biological process has not only provided new insights in biology,[2] but has also provided chemists and pharmacologists with the opportunity to create new synthetic molecules which efficiently regulate specific protein–protein interactions and biological processes.[3] Using a split-pool strategy, for chemical library synthesis, and biochemical technology, high-throughput processes for creating and identifying synthetic ligands for any protein have recently been developed.[4] Printing small molecules and carbohydrates as microarrays on glass slides or on other solid surfaces has been reported as one of the most frequently used approaches to date.[4a–d,5] A key aspect of such approaches is the immobilization of small molecules on solid surfaces; recently, mild and selective coupling reactions have been developed which include a Michael addition,[4a,5c] the addition of alcohol to silyl chloride,[4b] 1,3-dipolar cycloaddition,[5b] and a Diels–Alder reaction.[5d] Very recently, Schreiber and co-workers developed diazobenzilidene-functionalized glass slides and used these slides for the covalent capture of phenols as well as of other compounds containing functional groups of comparable acidity.[4g]

However, there remain some drawbacks to using these selective coupling approaches. Small molecules have to possess certain functional groups to be attached to solid surfaces. Combinatorial chemical libraries prepared by solid-phase synthesis inherently have such functional groups, but small-molecule libraries derived from natural resources contain a variety of structures and functional groups which are not always compatible with the selective coupling process. Even though the small molecules could be introduced onto the solid surfaces, a certain area of the small molecule faces towards the solid surface, and therefore some of the immobilized compounds will not come into contact with the relevant binding proteins. As it is difficult to predict the binding domain of small molecules that interact with unknown proteins, this poses a major drawback to such studies, especially when the goal is to discover new target proteins for small molecules by using microarray technology. To overcome these drawbacks, we describe herein a "non-selective" universal coupling method that enables the introduction of a variety of small molecules to glass slides by using a photoaffinity reaction. The small molecules introduced onto the glass slide by this method have the inherent ability to interact with their binding proteins.

Photoaffinity labeling has been used for investigating ligand–receptor interactions and analyzing binding sites within receptor molecules.[6] Photoreactive groups introduced to ligands are promoted upon UV irradiation to highly reactive species, which in turn bind irreversibly to the receptor at the interaction site. These species, in many cases aryl nitrenes and carbenes, are so reactive that they can insert into a heteroatom–hydrogen and even into a carbon–hydrogen bond. Recently, photoinduced cross-linking reactions using an aryl nitrene[7a] and an aryl carbene[7b] have been reported to anchor proteins on the solid supports. We expected that these photoinduced reactions could be used to immobilize not only proteins but also various types of small molecules on glass slides. Our strategy is summarized in Figure 1. Small-molecule solutions are first printed on glass slides, which are coated by a photoreactive group. The slides are then dried to remove the solvent used in the printing

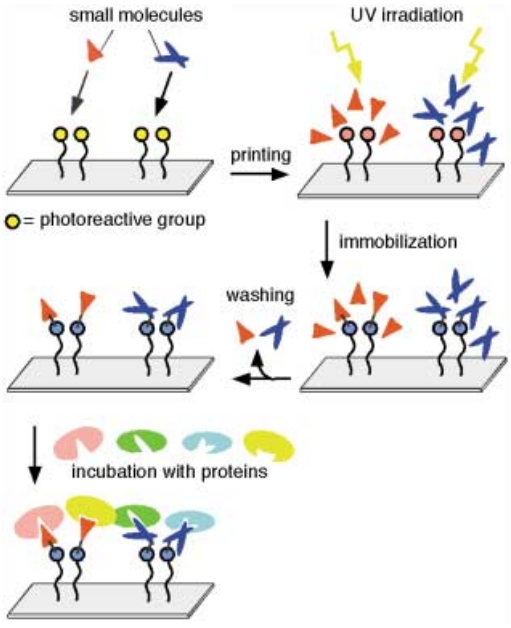

Figure 1. Immobilization of small molecules using a photoaffinity reaction.

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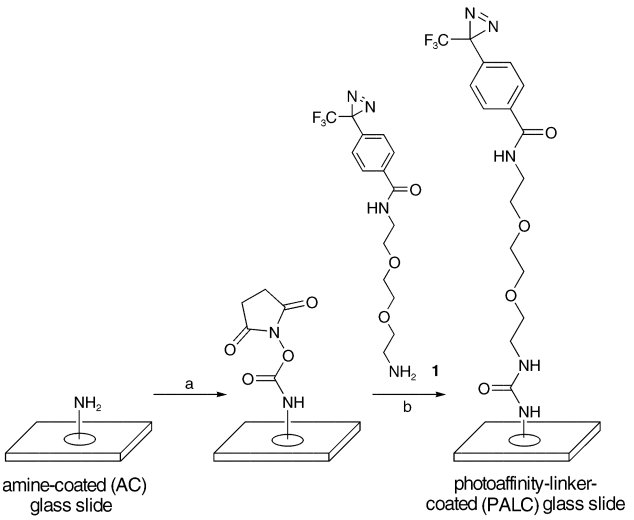
process. Irradiation of the resulting slides activates the photoreactive group on the slides, generating highly reactive species that can insert into or add to proximate small molecules. The immobilized small molecules remain on the glass slides after washing, and the washed slides can be used to detect the interactions between proteins and small molecules by incubating the slides with labeled proteins. Immobilization should occur in a functional-group-independent manner; using this method, some, but not all, of the immobilized small molecules are expected to retain binding affinity towards all of the possible binding proteins. As discussed above, this is important when discovering new target proteins for each small molecule.

To test this concept, we prepared photoaffinity linker **1** from *N-tert*-butoxycarbonyl-2,2′-ethylenedioxybis(ethylamine)[8] and 4-[3-(trifluoromethyl)-3*H*-diazirin-3-yl]benzoic acid,[9] and introduced it onto amine-coated (AC) glass slides (Scheme 1). A variety of photoaffinity reagents was screened,



Scheme 1. The introduction of a photoaffinity linker on an amine-coated glass slide. Reagents and conditions: a) immersed in a solution of *N,N*-disuccinimidyl carbonate (1.0 M) and diisopropylethylamine (1.0 M) in DMF, RT, overnight, then washed, b) immersed in a solution of **1** (100 mM) and diisopropylethylamine (500 mM) in DMF, RT, overnight, then washed.

and the 4-[3-(trifluoromethyl)-3*H*-diazirin-3-yl]benzoyl group was found to be most suitable for our purposes. The AC slides were first treated with *N,N*-disuccinimidyl carbonate to yield mixed carbonate on the slides. The resultant succinimidyl slides were then treated with **1** to give photoaffinity linker-coated (PALC) glass slides.

Rhodamine B (RhoB) and biotin solutions in DMSO were printed in quadruplicate at various concentrations (100, 10, 1, 0.1, and 0.01 mM) on both PALC and AC surfaces (Figure 2a), and the slides were dried in vacuo to remove DMSO. After the printed surface of a slide was irradiated at 365 nm for 30 min, it was washed successively with organic solvents and water. The slide was then probed with streptavidin–Alexa$_{633}$ conjugate in reaction buffer (see Supporting Information), washed with TBST buffer, and scanned for

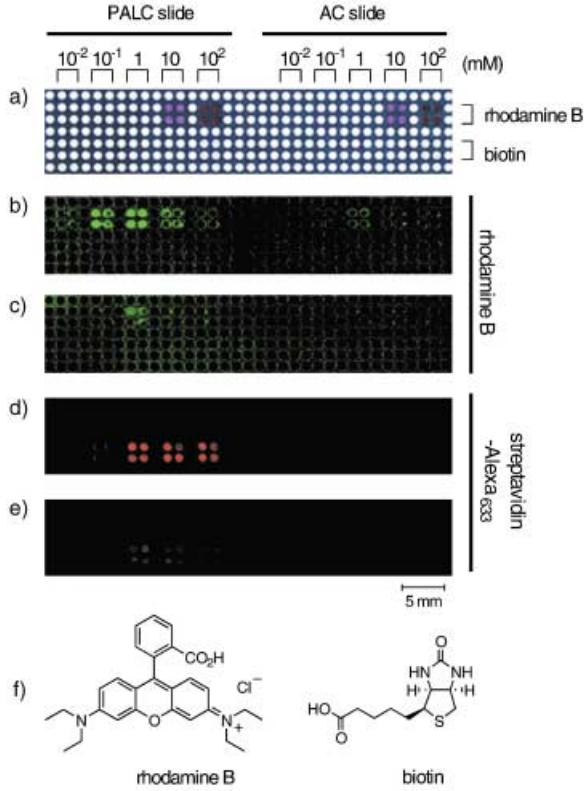


Figure 2. Immobilized small molecules on PALC and AC slides and the detection of the biotin–streptavidin interaction. a) Image of PALC and AC slides on which rhodamine B and biotin solution were printed in quadruplicate. The concentration of printed solution decreases 10-fold with each interval from the right to the left, as indicated. b)–e) Fluorescent images: After the slides were irradiated at 365 nm (irradiation time: 30 min for b and d, 7 min for c and e), they were washed successively with organic solvents and water. The resultant slides were incubated with streptavidin–Alexa$_{633}$ conjugate (3.7 μg mL^{-1}), washed, and scanned for rhodamine (b and c) and Alexa$_{633}$ fluorescence (d and e). f) Structure of rhodamine B and biotin.

RhoB and Alexa$_{633}$ fluorescence by using a DNA scope IV fluorescence slide scanner (Biomedical Photometrics Inc., Waterloo, ON, Canada). The results are shown in Figure 2b and 2d. Strong RhoB and Alexa$_{633}$ fluorescence was observed only on the PALC slides. Note that the spots where 100 and 10 mM RhoB were printed showed weak fluorescence signals relative to 1 and 0.1 mM areas. When 100 and 10 mM solutions of RhoB were printed on the slides, the printed areas were deep red owing to the high concentration of RhoB (Figure 2a). Therefore, it is probable that UV light did not pass through the RhoB layer, and the photoaffinity reaction did not occur efficiently in these areas. The fluorescence intensity of RhoB and Alexa$_{633}$ depended on the irradiation time (30 min for Figure 2b and d; 7 min for Figure 2c and e). Furthermore, when the compounds were washed off without irradiation, no RhoB and Alexa$_{633}$ fluorescence was observed after the probe with streptavidin–Alexa$_{633}$ conjugate (data not shown). These results strongly suggest that RhoB and biotin were immobilized by a photoaffinity reaction.

We applied this approach for immobilizing a variety of natural products on the PALC slides to observe specific

interactions between small molecules and proteins (Figure 3). Digoxin is a cardiac steroidal glycoside that is recognized by mouse monoclonal antibody DI-22 (Sigma). A DI-22 clone is also known to show cross-reactivity with digoxigenin (strong) and digitoxin (< 5 %), and almost no cross-reactivity has been observed with progesterone and hydrocortisone (< 0.1 %).[10] The immunosuppressive agent FK506 and rapamycin are known to bind their target protein FKBP12.[2c,11] Also, immunosuppressant cyclic peptide cyclosporin A is recognized by its monoclonal antibody CSZ.22 (HyTest). These molecules were immobilized on a PALC slide, and the resultant slide was probed with a solution of labeled proteins and scanned. All the immobilized molecules were successfully recognized in a specific manner by their protein targets. This is the first example in which complex natural products have been immobilized on a solid surface as arrays in a uniform process.

Because the spots that contained steroids exhibited a similar fluorescent signal (Figure 3c), we prepared a "steroid array" to determine whether or not other steroids could interact with the DI-22 clone on the slide (Figure 4). Five

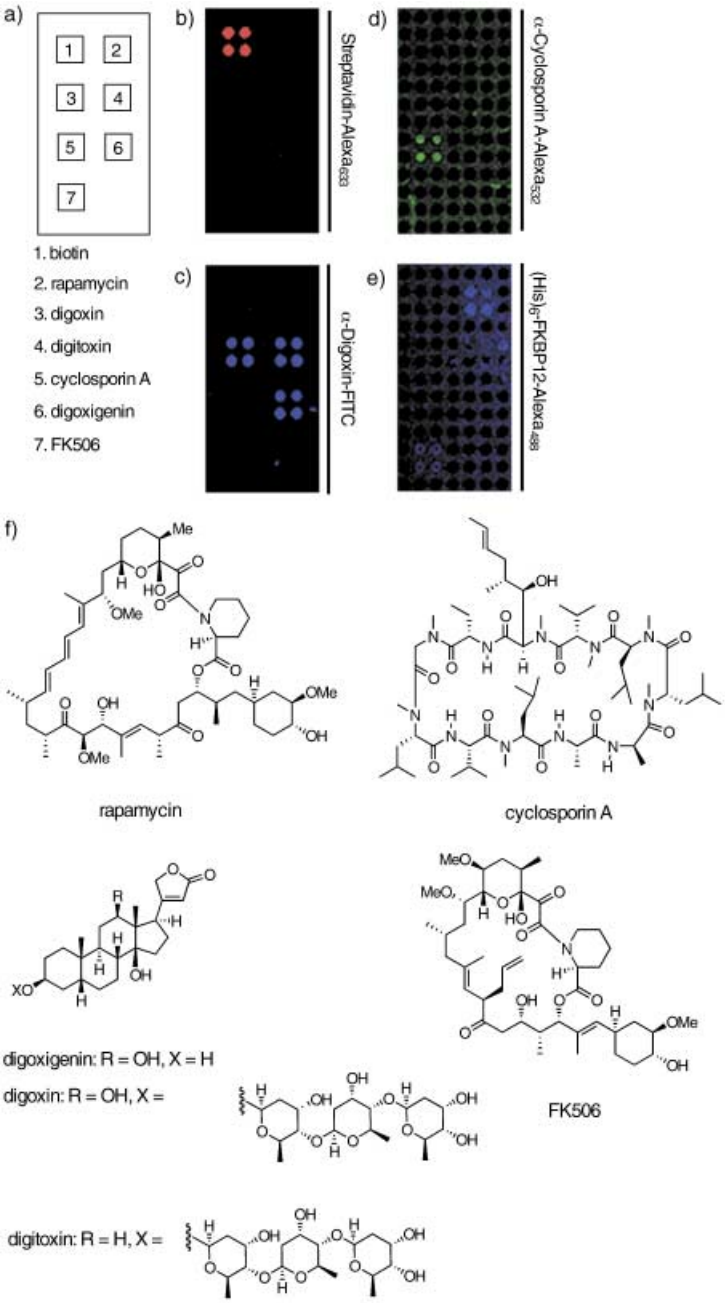


Figure 3. The detection of specific interactions between small molecules and proteins. a) Each slide was printed with 10 mM solutions of small molecules according to the pattern illustrated. b)–e) Fluorescent images: b) probed with streptavidin–$Alexa_{633}$ conjugate (5 μg mL^{-1}), c) probed with anti-digoxin DI-22-FITC conjugate (150 μg mL^{-1}), d) probed with anti-cyclosporin A CSZ.22–$Alexa_{532}$ conjugate (10 μg mL^{-1}) and $(His)_6$-FKBP12–$Alexa_{488}$ conjugate (10 μg mL^{-1}), and then scanned for $Alexa_{532}$; e) probed with anti-cyclosporin A CSZ.22–$Alexa_{532}$ conjugate (10 μg mL^{-1}) and $(His)_6$-FKBP12–$Alexa_{488}$ conjugate (10 μg mL^{-1}), and then scanned for $Alexa_{488}$. f) Structures of the test compounds.

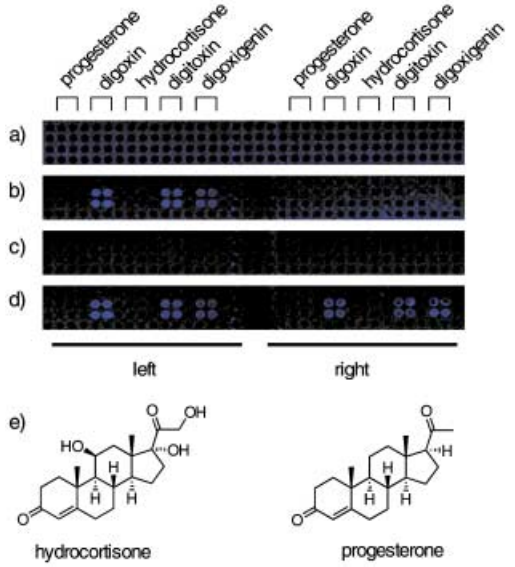


Figure 4. Steroid arrays: 10 mM solutions of five different steroids were printed on a PALC slide. a) Background, b) fluorescent image after the probe with anti-digoxin DI-22-FITC conjugate (150 μg mL^{-1}) in the presence (right) or absence (left) of digoxin (10 μM), c) fluorescent image of the slide stripped with stripping buffer (see text), d) fluorescent image of the slide reprobed with anti-digoxin DI-22-FITC conjugate (150 μg mL^{-1}), e) structures of hydrocortisone and progesterone; digoxin, digitoxin, and digoxigenin are depicted in Figure 3 f.

steroidal compounds were immobilized on the slide and probed with DI-22-FITC conjugate. As expected, spots containing progesterone and hydrocortisone had no FITC fluorescence, demonstrating that DI-22 did not interact with these compounds (Figure 4b, left). In the presence of digoxin (10 μM), no fluorescence was observed, showing that fluorescence on the slides is the result of specific interactions between steroids and antibody (Figure 4b, right). According to these observations, it appears that this type of natural products array could be useful for testing the antigen specificity of an antibody. In addition, it should be noted that the captured antibody was stripped by stripping buffer (62.5 mM Tris·HCl, pH 6.8, 100 mM β-mercaptoethanol, 2 % SDS) at 50 °C for 1 h (Figure 4c), and the stripped slide was then available for reuse (Figure 4d).

In summary, we prepared PALC slides and, using a photoaffinity reaction, we immobilized structurally distinct

small molecules on these slides. The immobilized molecules were shown to retain specific binding activity towards their binding proteins. Importantly, this immobilization procedure does not require a specific functional group, and can be applied for use with a diverse small-molecule library.

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