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Small-molecule microarrays: Development of novel linkers and an efficient detection method for bound proteins

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Abstract—Novel isocyanate and diazoketone linkers possessing polyoxypropylenediamine as a spacer for small-molecule microrray are developed. White light interferometry is introduced to detect bound proteins on the glass slides without using chemically modified proteins.

Drug discovery process generally involves the analyses of the hundreds or thousands of small organic molecules to identify drug leads.[1] However, the identification of active molecules for a target is relatively time consuming. In order to accelerate pre-lead molecule identification process, large groups of diverse molecules (or library) have been synthesized via a split-pool combinatorial chemistry and biological assays for these molecules have been adapted for high-throughput-screening (HTS) assays.[2] Although efficient solution assay systems have been developed, analyses of the results are very tedious and often produce false positive results caused by interactions of by-products generated in the sequence of library productions with components of assay systems. In addition, because a large number of library molecules synthesized on polymer-support have not been aimed to specific targets,[3] HTS of these molecules requires detecting lower affinity molecules against a variety of target proteins. On the other hand, HTS based on polymer-supported systems can be applied to screen lower affinity molecules when an excess amount of target proteins was used.[4] We have been developing small-molecule microarray (SMM) which is a binding-based assay that allows a large number of proteins to be screened against large collection of small molecules in a time-efficient mannar.[5] In this method compounds are immobilized on glass slides, and each slide can then be proved with target proteins. Binding events can generally be detected by fluorescence-linked assay. Significantly, immobilized library molecules on glass slides can be reused multiple times after extensive washing of glass surface. In general, SMM technology has been applied to a primary screening of drug discovery process to reduce large libraries (i.e., >10,000 of molecules) to smaller subsets and identify target proteins for novel library molecules.[6] However, current SMM protocols remain in which several issues need to be improved: (1) in order to screen low affinity molecules, SMM desired to be utilized with relatively high concentrations of target proteins (vide supra), this procedure, depending upon the nature of proteins, increases the chance of non-specific binding of target proteins to small molecules; thus, the detection of lower affinity molecules is difficult due to the high background, (2) reliable site-specific immobilization methods of library molecules on glass slides (printing) are necessary that can be performed with a high-precision robotic equipment under ambient atmosphere, and (3) due to a lowered reactivity and/or instability of the chemically modified proteins (i.e., fluorescent proteins), there is a significant advantage that can be analyzed via chemically unmodified proteins. We now wish to report novel linkers to improve site-specific immobilization of library molecules and the probing/washing steps by introduction of polyethylene glycol spacer on the glass slides, and an efficient detection method to identify bound proteins via white light interferometry.

Polyethylene glycol (PEG) has been used in numerous biomedically motivated systems to aid in minimization of protein adsorption and cell adhesion.[7] Furthermore,

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the longer spacers are better able to minimize intramolecular interactions and to keep the distance between the glass surface and the linker to allow interaction effectively with the target proteins. In order to anchor a variety length of PEGs on the amine glass slide **1**, we decided to utilize commercially available polyoxypropylenediamines (Jaffamines). Jaffamine-148 (**2a**), -600 (**2b**), -900 (**2c**), and -2000 (**2d**) could be anchored to **1** with 1,3-benzenedisulfonyl chloride (**3**) in the presence of $^{i}Pr_2NEt$ to afford the glass slides possessing polyoxypropylene-mono free amines **4a**, **4b**, **4c**, and **4d** (base slides). These amine terminals can be derivatized with the bifunctional molecules to provide a variety of functional groups that have a potential to immobilize the small organic molecules. We chose phenyl isocyanate as a versatile SMM linker to print the molecules possessing alcohol, amine, and other reactive functional groups.[8] The base slides **4a–d** were reacted with 1,4-phenyl diisocyanate (**5**) in THF to provide the slides **6a–d** as shown in Scheme 1.[9] Thus, unmodified polyoxypropylenediamines could efficiently be incorporated on the amine-glass slide without using a protection–deprotection strategy.[10]

In order to test the ability of new slides **6a–d** in Scheme 1, the molecules for which specific protein receptors are available such as digitoxin, corticosterone, Aktide-2t, NEMO-binding domain binding peptide, and biotin (2-hydroxyethyl)amide were printed with a robotic microarray.[11] These immobilized molecules onto the glass slides **6a–d** were probed with known binding proteins.[12] We have observed a general trend from the number of experiments with PEG-linked slides **6a–d** that shorter-PEG-linked slide **6a** generates higher background; these observations can be attributed to the stronger interactions of proteins to shorter spacer of the glass slide surface. On the other hand, the same experiments with **6b–d** exhibited significantly lower background that indicates more hydrophilic nature of the slide surface of **6b–d** resists protein adhesion.[13] Because slides **6b–d** equally work in many cases, we decided to use **6b** possessing Jaffamine-600 as a spacer for further studies.[14]

To confirm effectiveness of **6b**, we conducted the cross-reactivity test with thyroid hormones. Thyroid hormones, L-thyroxine (T4) and triiodo-L-thyronine (T3), were printed onto the slide **6b** and both immobilized slides were probed with the antibody, anti-T3.[15] In these experiments, we did not observe cross-reactivity between T4 and anti-T3 on phenyl isocyanate linker **6b**. However, the same experiments with the other linkers such as acyl chloride-functionalized glass slide **7** exhibited considerable cross-reactivity. These cross-reactivity tests indicated that the phenyl isocyanate linker could be immobilized selectively with more reactive functional group (–NH_2 over phenol-OH) and the binding domains of T3 and T4 could contact with anti-T3 and -T4, respectively. However, far more reactive linker **7** afforded non-selective coupling products that resulted in unspecific interactions with proteins (Fig. 1).

Because the number of combinatorial library productions has relied on phenols and carboxylic acids so as to attach to the polymer-support, we next investigated phenol and carboxylic acid selective linker. Diazoketones are generally stable under physiological conditions and known to react selectively with the molecules possessing acidic proton with or without the catalysts depending upon the acidity-nucleophilicity balance of the molecules.[16] The base slide **4b** was transformed into the diazoketone-derived glass slide **8** as illustrated in Scheme 1.[17] To examine the reactivity of the diazoketone slide **8**, a series of biotin derivatives and biotin in

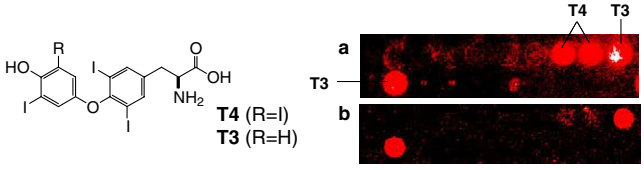
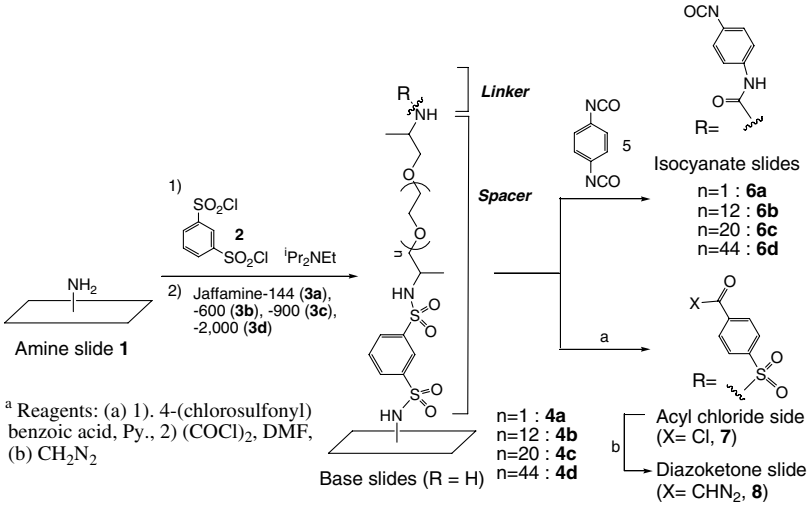


Figure 1. (a) Immobilized T4 and T3 onto the acylchloride slide **7**, and the slide was probed with anti-T4-FITC conjugate. (b) The same experiment with the phenyl isocyanate slide **6b**.



Scheme 1. Surface modifications of amine slide for SMM.

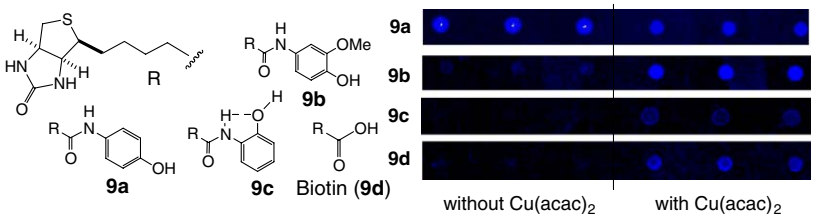


Figure 2. (a) Immobilized biotin derivatives onto the diazoketone slide **8**, and the slide was probed with streptavidin–FITC conjugate.

DMF were printed in the presence and absence of Cu(acac)$_2$. Probing of these slides with FITC–streptavidin conjugate provided the illuminated slides. The alkanols could not immobilize in the presence or absence of Cu(acac)$_2$. *p*-Substituted phenol derivative **9b** could be printed even in the absence of the catalyst. Significantly, the diazoketone slide could immobilize *o*-substituted phenol derivative **9c** in which phenol-OH forms strong hydrogen bonding with amide hydrogen in the presence of Cu(acac)$_2$.[18] Biotin that represents a carboxylic acid could be printed with Cu(acac)$_2$ but not without the catalyst (Fig. 2).

To address the requirement of using chemically unmodified proteins in the probing step, we have introduced white light interferometric measurements that would directly identify bound proteins on the glass slide. To prove effectiveness of white light interferometric measurements[19] in SMM, the biotin derivative and digoxin were printed on the isocyanate slides **6b**. Probing of these slides with both unmodified streptavidin proteins (~55 kDa) and its FITC conjugate provided the illuminated slide obtained by microarray scanners (**a**) and 3D image obtained by interferometry (**b**) as depicted in Figure 3.[20] Microarray spots on both slides showed similar relative quantitation and morphology at identical place. Therefore, we concluded that white light interferometry could be applicable to bound protein detection in SMM.[21] To the best of our knowledge this is the first application of white light interferometry to detect bound proteins on the glass slides.

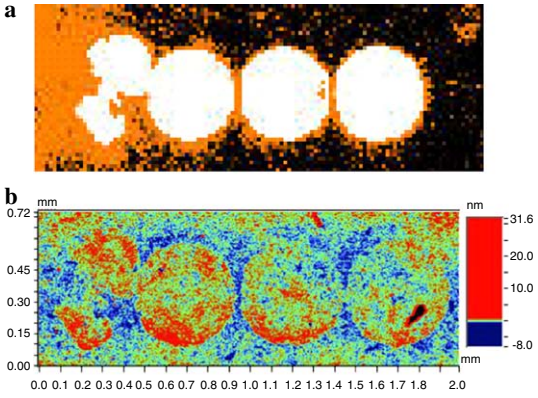


Figure 3. Biotin derivative printed on the slide **6b** and proved with streptavidin or its FITC conjugate. (a) Fluorescent microarray, (b) the bound protein was detected by white light interferometry.

Thus, we have developed novel phenyl isocyanate and diazoketone linkers that can minimize non-specific binding of proteins. These linkers were validated with a number of well-known pairs of the organic molecules and specific binding proteins. These slides serve as a complementary manner: the phenyl isocyanate linker captures alcohols, amines, and presumably many other nucleophilic functional groups such as thiols, whereas the diazoketone linker captures phenols and carboxylic acids. Since electrophilic nature of carbene complex, the diazoketone slide would be utilized in immobilizing the molecules possessing multiple bonds and enolizable ketones. We have introduced white light interferometry to detect bound proteins on the glass slides. Because fast and efficient bound protein analyses are hampered by a number of ill-characterized chemically modified proteins, therefore, a detection method with fluorescent-free proteins is a significant advantage over fluorescent-coupled microarrays; a large number of characterized or uncharacterized proteins can be applied to screen against large sets of small molecules.

Acknowledgments

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8. For a utility of polymer-supported isocyanate as a reagent scavenger; see: Galaffu, N.; Bradley, M. *Tetrahedron Lett.* **2005**, *46*, 859.
9. *General Synthesis of Isocyanate Slide:* Amine slides **1** (reactive amines: ~0.06 μmol/slide) were placed in a microscopic stain dish and covered with CH_2Cl_2. Into the reaction vessel 1,3-benzenedisulfonyl chloride (5 equivalents) and $^iPr_2NEt$ (1 equivalent) were added. After 12 h, the slides were washed with CH_2Cl_2, THF, and CH_2Cl_2. The benzenesulfonyl chloride slides were placed in a microscopic stain dish and CH_2Cl_2 was added. Into the reaction vessel Jeffamine (10 equivalents) was added. After 6 h, the slides were washed with THF–water, THF, and EtOAc to provide the base slides. These slides were dried under high vacuum. The base slides were placed in a microscopic stain dish and THF was added. Into the reaction vessel 1,4-phenyl diisocyanate (5 equivalents) was added. After 12 h, the slides were washed with THF and CH_2Cl_2. The generated isocyanate slides, **6a**–**d**, were stored in a glove box.
10. A mono-protected (*N*-Boc) shorter PEG-diamine has been utilized for the modification of the polymer surface.
11. A microarray was printed (1 nL spot volumes in quadruplicate) wells containing the standard molecules at 5 mM (in DMF).
12. Bio-Rad microarray chip reader was used.
13. Surface of the slides **6a**–**d** has a contact angle (θ) of 110°, 75°, 58°, 55°, and 53°, respectively (for water). Thus, hydrophilicity of slide surface was dramatically improved by introduction of Jeffamine-600 or higher. A higher surface energy retains water molecules on the surface of glass slide. This surface profile is very important to the probing with target proteins and washing unbound proteins on the glass surface. For quantification of the hydrophobicity/hydrophilicity of polymer surface by contact angle measurement, see: Augsburg, A.; Grundke, K.; Pöschel, K.; Jacobasch, H. J.; Neuman, A. W. *Acta Polymerica* **1998**, *49*, 417.
14. SMMs with other modified slides have suffered from low signal-to-noise (S/N) ratio. In our hands, SMMs using silyl chloride and succinyl chloride linkers exhibited S/N ratio of 1.5. On the other hand, SMMs using 6b ~ d generally exhibited S/N ratio of over 6.1.
15. Cross-reactivity of monoclonal anti-tyroxine (anti-T4) against 3,3′,5-triiodo-L-thyronine (T3) is less than 1%, see. Schall, R. F., Jr.; Fraser, A. S.; Hansen, H. W.; Kern, C. W.; Teneso, H. *J. Clin. Chem.* **1978**, *24*, 1801.
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17. Synthesis of the diazoketone slide **8**: The base slides **4c** (reactive groups: considered as ~0.06 μmol/slide) were placed in a microscopic stain dish and covered with CH_2Cl_2. Into the reaction vessel 4-(chlorosulfonyl)benzoic acid (5 equivalents) and pyridine (10 equivalents) were added. The reaction mixture was stored for 12 h. The benzoic acid slides were washed with THF–1 N HCl (10/1), THF–water (3/1), THF, and EtOAc, and dried under high vacuum for 12 h. The benzoic acid slides were placed in a microscopic stain dish and CH_2Cl_2 was added. Into the vessel $(COCl)_2$ (20 equivalents) was added and stored for 12 h at rt. The acyl chloride slides were washed with CH_2Cl_2 and dried under high vacuum. These are placed in a microscopic stain dish and added THF, and then freshly generated CH_2N_2 in Et_2O (excess) was added. After 12 h, the slides were washed with THF and EtOAc. The diazoketone slides **8** can be left at rt for over 5 months in the dark without noticeable deterioration.
18. The biotin derivative **9c** was unable to print on the other slides such as **6b** and **7**.
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20. Lateral Scanning White Light Interferometry (Veeco) was used in this study.
21. Detection limit of bound proteins by white-light interferometry is a combination between the efficiency of printing small molecules and the size of target proteins. Scope and limitations of white-light interferometry method in SMM are currently under investigation.