

## GENERATING ENCODED COMPOUND LIBRARIES FOR FABRICATING MICROARRAYS AS A HIGH-THROUGHPUT PROTEIN LIGAND DISCOVERY PLATFORM

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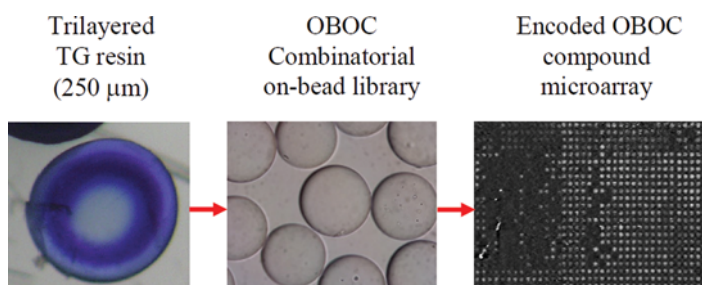
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### GRAPHICAL ABSTRACT



**Abstract** We demonstrate an effective method for generating libraries of encoded compounds for fabricating large compound microarrays on solid supports. This method is based on one-bead, one-compound synthesis and employs a novel trilayer bead-partition

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*scheme that ensures sufficient quantity of synthesized compounds releasable from each bead for compound microarray fabrication in high-throughput protein–ligand discovery assays.*

*[Supplementary materials are available for this article. Go to the publisher's online edition of Synthetic Communications® for the following free supplemental resource(s): Full experimental and spectral details.]*

**Keywords** Chemical microarray; high-throughput screening; one-bead–one-compound (OBOC) synthesis; protein–ligand binding assay

## INTRODUCTION

Combinatorial chemistry and high-throughput screening (HTS) assays are among the major approaches in studies of protein–ligand interaction and in drug discovery. The “one-bead–one-compound” (OBOC) combinatorial synthesis<sup>[1]</sup> uses the “split-mix” approach to synthesize thousands to millions of compounds on individual TentaGel (TG) beads, each having a unique chemical entity. On-bead screening assays have been used to identify ligands from OBOC libraries for biological targets such as enzyme substrates and inhibitors,<sup>[2–4]</sup> cell surface receptors,<sup>[5,6]</sup> and artificial enzymes.<sup>[7,8]</sup> Other assay platforms have also been used to screen molecular targets in high-throughput fashion.<sup>[9,10]</sup> These platforms include microarray technique (tissue<sup>[11]</sup>/cellular,<sup>[12]</sup> DNA,<sup>[13,14]</sup> protein,<sup>[15,16]</sup> peptide, and small molecules<sup>[17–19]</sup>), liquid chromatography–mass spectrometry,<sup>[20]</sup> microfluidics,<sup>[21,22]</sup> and sensor-based technologies.<sup>[23]</sup>

Small-molecule microarrays (a.k.a. chemical microarrays) with thousands of compounds immobilized on a glass slide have been used to screen compound libraries for protein ligands.<sup>[19,24]</sup> Binding reactions of a protein probe with chemical microarrays are typically detected with fluorescence-based sensors.<sup>[25,26]</sup> However, it is known that labeling protein probes with extrinsic fluorescent molecules for the purpose of detection can change intrinsic characteristics of protein–ligand interaction significantly.<sup>[27,28]</sup> Furthermore, endpoint fluorescence data such as images of reacted microarrays contain little information on kinetic rate constants, and the reliability of endpoint data as measures of binding affinity depends on reaction conditions and postreaction processing.<sup>[29]</sup> Recently Zhu and coworkers developed an ellipsometry-based scanning microscope for label-free microarray detection. They demonstrated that such a microscope was capable of simultaneously detecting 10,000 protein–compound reactions in real time on a small molecule microarray.<sup>[27,29–34]</sup> Combining OBOC synthesis with small-molecule microarrays offers an enabling platform of generating and screening structurally diverse compound libraries for ligands with desirable functionality.<sup>[35]</sup>

To achieve such a feat, one has to meet a number of challenges: (1) compounds need a common molecular handle, away from the functional portions of the compounds, for efficient anchoring on a functionalized solid support; (2) individual compounds need to be placed in wells of microtiter plates for microarray fabrication; (3) releasable compounds from a single bead need to be in the range of 0.5 nanomole (nmol) for fabrication of compound microarrays with sufficiently high compound coverage.<sup>[35]</sup> In this article, we report a successful effort in meeting these challenges.

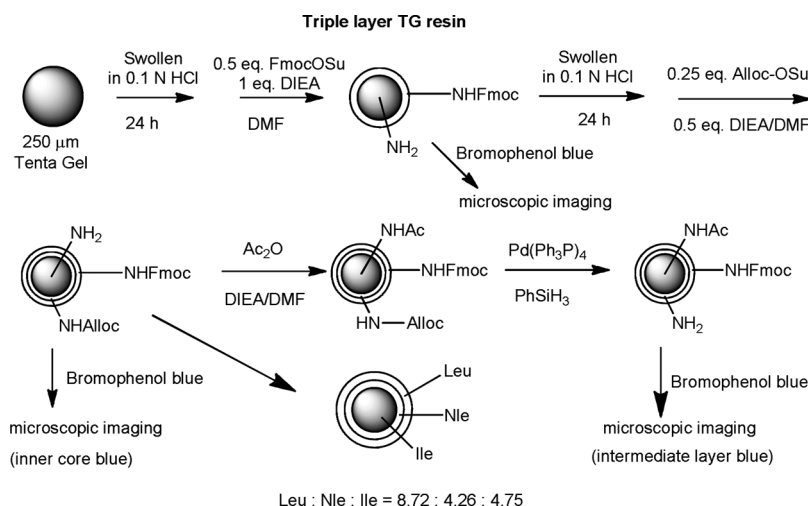
Our strategy is an OBOC synthesis scheme on *trilayered* beads. OBOC libraries are typically synthesized on bilayered beads of 90  $\mu\text{m}$  in diameter and thus with a

loading capacity of 0.1 nmol. The outer layer with nominal 50% of the bead volume is used for compound synthesis and the interior core with the remaining bead volume is used for coding-tag synthesis. As a result the compounds released from a single bead are roughly 50 picomoles (pmol). In 10  $\mu$ L solvent, the solution has a concentration of 5  $\mu$ M, one order of magnitude below what is needed to immobilize sufficient compounds on a solid surface to capture a layer of protein probes. To solve this problem, we used beads of 250  $\mu$ m in diameter and thus with a loading capacity of 1.5 nmol. The challenge with using large beads in multiple-step OBOC synthesis is that slow mass transport of chemicals through large beads easily traps truncated chemicals inside the beads, thus causing contamination when “compounds” are finally released. To resolve the mass transport challenge, we employed the scheme of partitioning 250- $\mu$ m TG beads into three layers with the interior core blocked with acetyl in the early phase of the synthesis and using the two outer layers for encoder and compound synthesis.

## EXPERIMENTAL

### Preparation of Trilayered TentaGel Beads

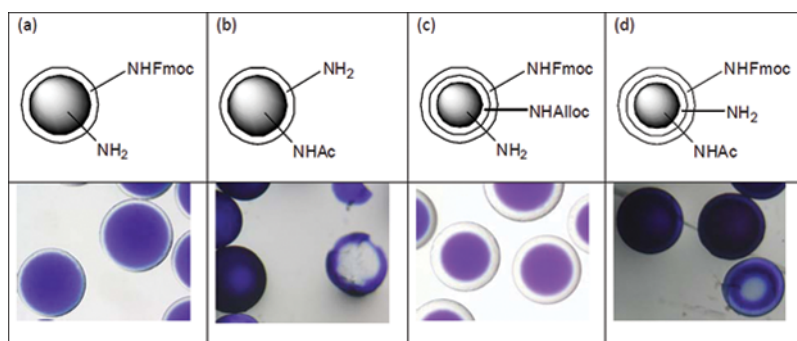
We used a procedure modified from the previously published methods (see Scheme 1).<sup>[36–38]</sup> We first soaked 100 mg of 250  $\mu$ m TG beads (Rapp Polymere GmbH, Tübingen, Germany) in 0.1 M HCl aqueous solution at room temperature for 24 h. The HCl solution was then removed by vacuum filtration and the beads were washed three times with ddH<sub>2</sub>O. We added Fmoc protection groups to the outer layer by adding N-(9-fluorenylmethoxycarbonyloxy) succinimide (Fmoc-OSu, 0.5 equiv. to the bead substitution) and N,N-diisopropylethylamine (DIEA, 1 equiv.) in 2 mL of N,N-dimethylformamide (DMF) to the beads and agitating the mixture for 4 h. The beads were then collected by vacuum filtration and washed with dichloromethane



**Scheme 1.** Procedures for preparation of trilayered TentaGel beads. Nle, norleucine; Ile, isoleucine.

(DCM) and dimethylformanice (DMF). We next soaked the beads again in 0.1 M HCl for 24 h. The HCl solution was removed afterward by vacuum filtration and the beads were washed three times with ddH<sub>2</sub>O. We added Alloc protection groups to the intermediate layer by mixing the beads with Alloc-OSu (0.25 equiv.) and DIEA (0.5 equiv.) in 2 mL of DMF and agitating the mixture for 2 h. The beads were then collected by vacuum filtration and washed with DCM and DMF. The remaining free amino groups in the interior core of the beads were permanently blocked with acetic anhydride (2 equiv.) and DIEA (4 equiv.) in DMF.

To check the effectiveness of the trilayer partition scheme, we conjugated different amino acids (prior to acetylation of the core) to each layer for quantitative analysis: (1) the interior core was conjugated with Boc-Isoleucine(Ile)-OH (2 equiv.) in the presence of 1-hydroxybenzotriazole (HOBt) and diisopropylcarbodiimide (DIC, 2 equiv.) in DMF; (2) then, the outer layer was conjugated with Boc-Leu-OH after removal of Fmoc protection groups with 25% of 4-methylpiperidine in DMF; (3) at the end, the intermediate layer was conjugated with Boc-Norleucine(Nle)-OH after removal of Alloc protection groups via Pd(PhP<sub>3</sub>)<sub>4</sub> in DCM. Upon removal of Boc protecting groups with TFA/DCM, the amino acids (Leu, Nle, and Ile) were cleaved from the trilayered beads via Edman degradation and analyzed on a peptide micro-sequencer to quantify the relative amount of amino acids in each layer. The similar structures of these three amino acids allow us to quantify the ratio of the volume of different layers. The quantitative ratio of amino acids cleaved from trilayered beads was found to be Leu/Nle/Ile = 8.72:4.26:4.75, very close to our design ratio of 2:1:1 (see the text and Fig. S1 in Supporting Information). For visual inspection of trilayered partition, we used bromophenol blue in ethanol to stain the region of the beads with free amino groups. As shown in Fig. 1, staining bilayered and trilayered beads revealed clear edges separating the layers. The trilayered beads in Fig. 1(c) have both the outer layer and the intermediate layer protected by Fmoc and Alloc, thus showing thicker white “layer” than the ones for the bilayered beads (Fig. 1(a)). The equatorial section of a trilayered bead in Fig. 1(d) reveals the three distinct layers.



**Figure 1.** Bilayered [(a) and (b)] and trilayered TentaGel beads [(c) and (d)] stained with bromophenol blue. The layered structures were fabricated using the method described in the main text. Because of the staining of the outer layers of the beads shown in panels (b) and (d), the interior layer or layers are not visible to the camera. Therefore, one bead was cut to show the interior layer(s). The interior structures of the uncut beads are the same. (Figure is provided in color online.)

### One-Bead Synthesis of Control Compounds

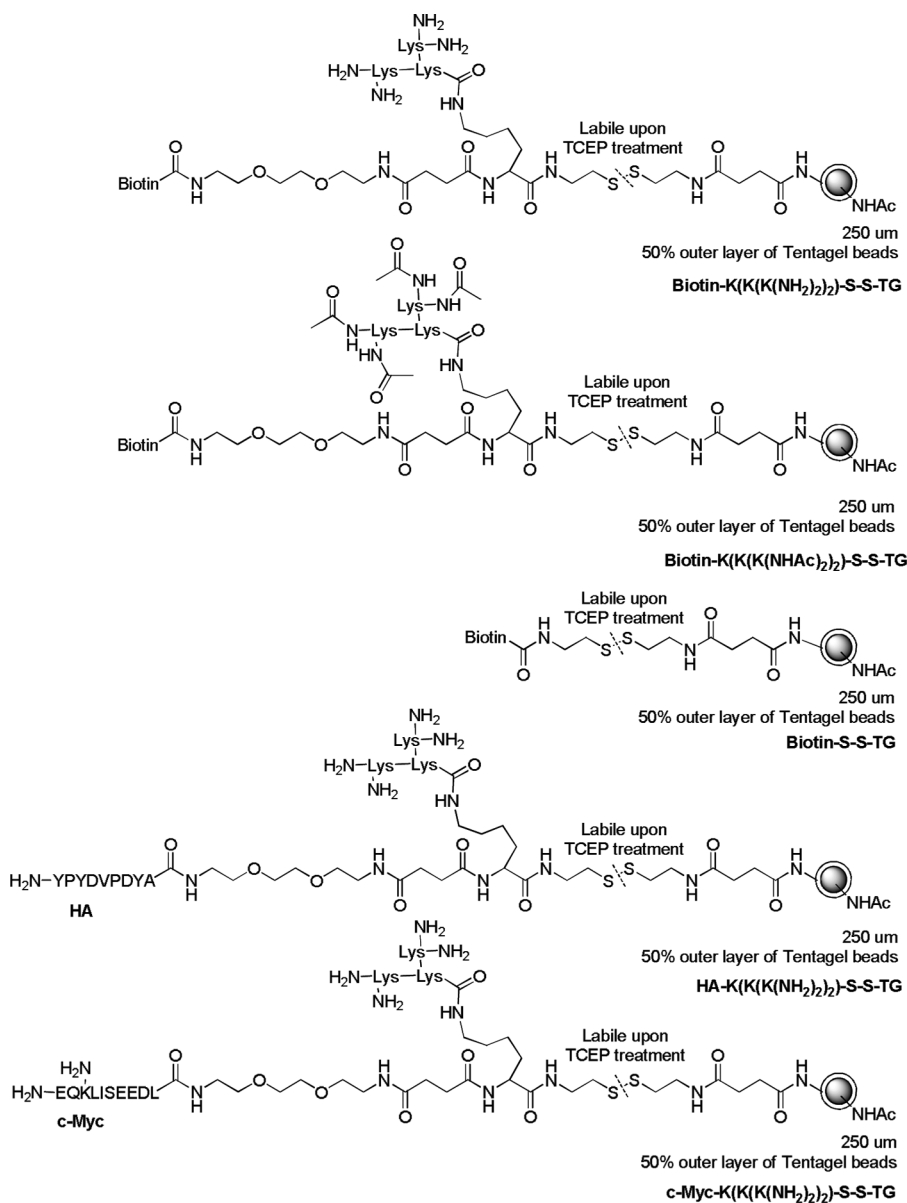
Using the Fmoc peptide chemistry and DIC/HOBt as coupling reagents, we carried out a sequence of solid-phase reactions to conjugate control compounds (biotin and peptides) and a branched poly-lysine residue (common handle) on tri-layered beads. We first conjugated a spacer molecule containing a disulfide bond onto the outer layer of the beads and then coupled an Fmoc-Lys(Alloc)-OH (an orthogonally protected lysine) to the spacer. Upon removal of the Fmoc protecting group via 4-methylpiperidine in DMF,<sup>[39]</sup> we coupled a short polyethylene glycol (PEG, a flexible linker molecule) to “separate” the region for common handle synthesis (i.e., the Alloc-protected site) from the region for compound synthesis. For control compounds, we conjugated biotin, HA (YPYDVPDYA) and c-Myc (EQKLISEEDL) in steps to the PEG linker using the DIC/HOBt coupling Fmoc chemistry.

### On-Bead Synthesis of a Branched Poly-Lysine Residue as the Common Handle for Anchoring on Solid Surfaces

There are two main strategies to immobilize small molecules onto a solid support for microarray fabrication. One is direct immobilization through a reaction of intrinsic reactive residues on the molecule with the functionalized surface (e.g., amine, epoxy, aldehyde, isocyanate, or streptavidin) without or with photo-initiated cross-linking.<sup>[40,41]</sup> Another is indirect immobilization through the reaction of active residue(s) on a common handle such as biotin or a carrier molecule such as bovine serum albumin (BSA) to which the molecule is conjugated.<sup>[27,30,32,33,42–44]</sup> During OBOC synthesis, it is sensible and advantageous to add a common handle to compounds so that (1) the handles uniformly tether compounds to a functionalized solid surface and (2) functional residues on compounds are kept intact and away from the solid surface for subsequent binding assays. We chose a branched poly-lysine residue as the common handle for anchoring compounds on epoxy-coated glass surface. Multiple primary amine residues on such a handle enable immobilization of OBOC synthesized compounds on epoxy-coated solid surface with a uniformly high efficiency and spare intrinsic amine residues on compounds by competing effectively with the latter for epoxy groups on the solid surface. Furthermore, by separating the synthesized compound from the branched poly-lysine residue with a spacer molecule, positive charges, albeit screened under aqueous condition, on the remaining lysine groups (i.e., not used in reaction with surface-bound epoxide groups) are not expected to affect the binding of positively charged proteins to the compounds. They may, however, cause nonspecific binding of negatively charged proteins to an extent. In those cases, one can always reduce the number of lysine groups so that there are no unused lysine groups after reaction with surface-bound epoxide groups.

For synthesis of branched poly-lysine on control compounds, we removed the Fmoc group on the N-terminal of HA or c-Myc and protected it with (Boc)<sub>2</sub>O. This step was skipped for biotin-conjugated beads. We next removed the Alloc protection group with (Ph<sub>3</sub>P)<sub>4</sub>Pd in DCM and coupled Fmoc-Lys(Fmoc)-OH to the side chain of the lysine. After removal of Fmoc group again, we coupled two more Fmoc-Lys(Fmoc)-OH molecules. Then the Fmoc groups were removed again to unveil a branched poly-lysine handle with four primary amine residues. Finally we treated

the beads with reagent K (TFA/phenol/H<sub>2</sub>O/thioanisole/triisopropylsilane = 10:0.75:0.5:0.5:0.25) to globally remove the protecting groups on the peptide side chains.<sup>[45]</sup> The structures of compound-handle conjugates are shown in Scheme 2. For negative control, some of the beads were further treated with acetic anhydride overnight to block the primary amine residues on the branched poly-lysine.



**Scheme 2.** Chemical structures of biotin-K(K(K(NH<sub>2</sub>)<sub>2</sub>)<sub>2</sub>)-S-S-TG, biotin-K(K(K(NHAc)<sub>2</sub>)<sub>2</sub>)-S-S-TG, biotin-S-S-TG, HA-K(K(K(NH<sub>2</sub>)<sub>2</sub>)<sub>2</sub>)-S-S-TG, and c-Myc-K(K(K(NH<sub>2</sub>)<sub>2</sub>)<sub>2</sub>)-S-S-TG synthesized on the outer layers of trilayered 250-μm TG beads.

### Synthesis of an OBOC Compound Library with Branched Poly-Lysine Handles

After removal of Boc protection groups,<sup>[5]</sup> the interior cores of orthogonally protected, trilayered beads (Fmoc-Alloc-Boc) were permanently blocked first by acetic anhydride. Then the Fmoc protection groups in the outer layer were removed with 20% piperidine in DMF (instead of 4-methylpiperidine) and Fmoc-protected disulfide-bond spacers were conjugated to the layer. After removal of Fmoc group again, we coupled Fmoc-Lys(Dde)-OH to the spacers. After removal of Fmoc group still again, we added the short PEG linkers to the  $\alpha$ -amino of the lysine, followed by Boc anhydride protection of the N-terminal of the PEG linker after removal of Fmoc group. The Alloc protection groups in the intermediate layer for encoding were removed by the palladium chemistry, followed by conjugation of a methionine cleavable linker.<sup>[35,37,38]</sup> The methionine cleavable linker consists of methionine, 4-bromophenyl- $\beta$ -amino acid, arginine, and PEG. After conjugation of methionine and 4-bromophenyl- $\beta$ -amino acid using HOBt/DIC chemistry, the Boc protecting group on the outer layer was removed with 50% TFA in DCM and then protected with Alloc-OSu to spare the Pbf protecting group on arginine. The latter was subsequently coupled to the  $\beta$ -amino group of 4-bromophenyl- $\beta$ -amino acid in the intermediate layer after removal of Fmoc group. Finally a PEG flexible linker was conjugated to the N-terminal of arginine after removal of Fmoc group.

Following Scheme 3, we then synthesized 10,800 encoded OBOC compounds at N-terminals of the PEG linkers. The compounds consisted of dipeptides (see Table S1 in Supporting Information) with the N-terminals conjugated with different carboxylic acids (Table 1); the encoders consisted of tripeptides (Table S1). The synthetic procedure is briefly as follows: Upon deprotection of PEG linkers in both the outer layer (Alloc protected) and the intermediate layer (Fmoc protected) via  $\text{Pd}(\text{Ph}_3\text{P})_4$  treatment and 20% piperidine treatments subsequently, the beads were split into 30 columns. Each column reacted with one of the 30 amino acids in Table 1 at the  $X_1$  position. After the reaction, the beads were mixed, deprotected, split again into 30 columns, and each column again reacted with the 30 amino acids at  $X_2$  (listed in Table S1 in Supporting Information). After the second reaction, the beads were mixed, deprotected once again, and then soaked in 0.1 M HCl for 24 h. Afterward the outer layers were protected with Fmoc-OSu in DMF, and the amino residues in the intermediate layer were blocked with Alloc-OSu. Next the Fmoc groups in the outer layers of the beads were removed and the beads were split into 12 columns. The N-terminals of the dipeptides in the outer layers of the beads in a column were capped with one of the 12 carboxylic acids listed in Table 1 to complete the synthesis for the present study.

To finish encoder synthesis, the beads were kept in the respective columns and Alloc groups on the dipeptides in the intermediate layers were deprotected. One of 12 different amino acids was conjugated to the dipeptides in a column to form unique tripeptides as encoders. The N-terminals of the tripeptides were blocked with Boc anhydride.

To add branched poly-lysine residues as the last step of the OBOC library synthesis, the Dde groups in the outer layer were removed with 2% hydrazine in DMF, followed by Fmoc-Lys(Fmoc)-OH coupling reactions as described earlier in



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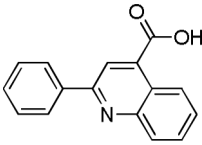
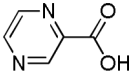
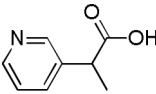
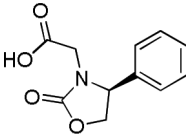
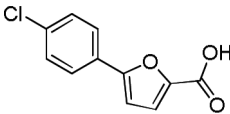
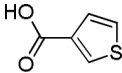
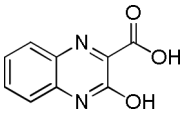
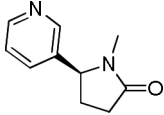
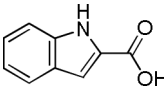
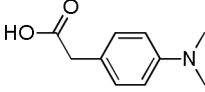
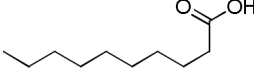
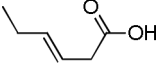
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**Table 1.** Twelve carboxylic acids for building block R

Compound	Formula
2-Phenyl-4-quinolinecarboxylic acid	
2-Pyrazinecarboxylic acid	
3-Pyridine propionic acid	
(S)-(+)-2-Oxo-4-phenyl-3-oxazolidineacetic acid	
5-(4-Chlorophenyl)-2-furoic acid	
3-Thiophenecarboxylic acid	
3-Hydroxy-2-quinoxalinecarboxylic acid	
Trans-4-cotinine carboxylic acid	
Indole-2-carboxylic acid	
4-(Dimethylamino)phenylacetic acid	
Decanoic acid	
Trans-3-hexenoic acid	

Holliston, MA). The first microarray (microarray A) was designed to examine immobilization efficiencies of small molecule compounds with and without branched poly-lysine handles on an epoxy-coated glass surface. It consists of biotin-K(K(K(NH<sub>2</sub>)<sub>2</sub>)<sub>2</sub>)-SH, biotin-K(K(K(NHAc)<sub>2</sub>)<sub>2</sub>)-SH, and biotin-SH, released respectively from biotin-K(K(K(NH<sub>2</sub>)<sub>2</sub>)<sub>2</sub>)-S-S-TG bead, biotin-K(K(K(NHAc)<sub>2</sub>)<sub>2</sub>)-S-S-TG bead, and biotin-S-S-TG bead (see Scheme 3 for their structures). We released the compounds by incubating individual bead in each well of a microtiter plate overnight in 10  $\mu$ L of 2 mM tris (2-carboxyethyl) phosphine (TCEP, Thermo Fisher Scientific, Rockford, IL) dissolved in either DMF or ddH<sub>2</sub>O.<sup>[49]</sup> The concentration of the compounds in the cleaving buffer was estimated to be 75  $\mu$ M (0.75 nmol in 10  $\mu$ L), assuming that the loading capacity in the outer layer of the bead was fully utilized in synthesis and subsequent release. The actual concentration was likely to be less than 75  $\mu$ M. The compound solution was further diluted to estimated 50  $\mu$ M, 25  $\mu$ M, 12.5  $\mu$ M, and 6.25  $\mu$ M in DMF. The diluted solutions were each printed in quadruplet on an epoxy-coated glass slide to form microarray A.

### Fabrication of the Encoded OBOC Compound Microarray

After the synthesis, the beads tended to stick together at room temperature under dry conditions. We treated the beads with 100 mM SDS for 30 min and lyophilized the beads after draining SDS. After this treatment the beads were well separated at room temperature, ready for transfer to microtiter plates. Then 10,800 beads were individually distributed into 384-well microtiter plates with a custom-built bead placement device (see Fig. S4 in Supporting Information). The device deposited 384 individual beads into 384 wells of a microtiter plate in one transfer. Compounds on each bead was released by adding to each well 10  $\mu$ L of 2 mM TCEP and 100 mM NaHCO<sub>3</sub> in a mixture of DMF and H<sub>2</sub>O (1:1 v/v) and incubating overnight. The concentration of each compound solution was estimated to be 40  $\mu$ M. We fabricated a large microarray (microarray D) consisting of 9,216 OBOC compounds on an epoxy functionalized glass slide by printing each compound *once* for high throughput. We also printed bovine serum albumin (BSA, 8  $\mu$ M) along the perimeter as control features. The entire microarray has a total of 9,849 targets with 67 rows and 147 columns.

After printing, a microarray-bearing glass slide was assembled into a fluidic assembly and washed with 1 $\times$ phosphate buffer saline (PBS, pH 7.4) to remove excess printed materials. The microarray was then incubated in a bovine serum albumin (BSA) solution (8  $\mu$ M for 30 min, the so-called BSA blocking treatment). The BSA molecules bound to the remaining free epoxy groups on the epoxy-coated glass surface, in the printed region if the printed compound did not cover the surface completely as well as in the unprinted region.

### Ellipsometry-Based Scanning Microscope, Oblique-Incidence Reflectivity Difference (OI-RD) Optical Scanner for Label-Free Characterization of OBOC Compound Microarrays

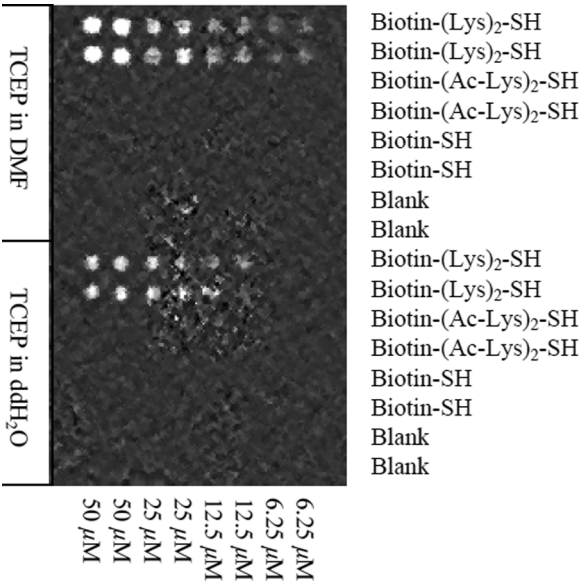
The OI-RD scanning microscope for detection of chemical microarrays has been described previously.<sup>[27,30–34]</sup> The microscope measures the reflectivity change due to a molecular layer on a solid surface with a surface mass density  $\Gamma$  (gm/cm<sup>2</sup>),

$$\Delta_P - \Delta_S \cong \frac{(-i)4\pi\sqrt{\epsilon_s}\epsilon_0}{(\epsilon_s - \epsilon_0)(\epsilon_s/\epsilon_0 - \cot^2 \theta)} \frac{(\epsilon_d - \epsilon_0)(\epsilon_d - \epsilon_s)}{\epsilon_d} \left( \frac{\Gamma}{\lambda\rho} \right) \tag{1}$$

$\theta$  is the incidence angle of the illumination laser beam at  $\lambda = 532\text{nm}$ .  $\epsilon_0$ ,  $\epsilon_d$ , and  $\epsilon_s$  are the optical dielectric constants of the glass slide, the molecular layer, and the aqueous ambient.<sup>[34]</sup>  $\rho = 1.35\text{g/cm}^3$  is the volume mass density of globular proteins.

**Amine Residues Are Needed for Compound Immobilization on Epoxy-Functionalized Glass Surface**

Microarray A of printed biotin-K(K(K(NH<sub>2</sub>)<sub>2</sub>)<sub>2</sub>)-SH, biotin-K(K(K(NHAc)<sub>2</sub>)<sub>2</sub>)-SH, and biotin-SH was incubated in a solution of F<sub>ab</sub> fragments of mouse anti-biotin antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) at 87 nM in 1 × PBS for 1 hour. Figure 2 shows the change in OI-RD image after the incubation. Anti-biotin antibodies molecules were not captured at the locations where biotin-K(K(K(NHAc)<sub>2</sub>)<sub>2</sub>)-SH and biotin-SH were printed, indicating that neither compounds were immobilized on the glass surface for lack of free amine residues. Anti-biotin antibodies were captured at the locations where biotin-K(K(K(NH<sub>2</sub>)<sub>2</sub>)<sub>2</sub>)-SH was printed, confirming that biotin molecules conjugated with branched poly-lysine handles were immobilized through amine residues on the handles. The amount of the anti-biotin antibody molecules captured by biotin-K(K(K(NH<sub>2</sub>)<sub>2</sub>)<sub>2</sub>)-SH cleaved off with 2 mM TCEP in DMF was twice as much



**Figure 2.** Change in OI-RD image of microarray A consisting of printed biotin-K(K(K(NH<sub>2</sub>)<sub>2</sub>)<sub>2</sub>)-SH, biotin-K(K(K(NHAc)<sub>2</sub>)<sub>2</sub>)-SH, and biotin-SH after incubation in a solution of F<sub>ab</sub> fragments of mouse anti-biotin IgG at 87 nM in 1 × PBS for 1 h. The compounds were cleaved from single beads by 2 mM TCEP dissolved in DMF or in ddH<sub>2</sub>O and printed at concentrations of 50 μM (columns 1 and 2), 25 μM (columns 3 and 4), 12.5 μM (columns 5 and 6), and 6.25 μM (columns 7 and 8).

as the amount captured by biotin-K(K(K(NH<sub>2</sub>)<sub>2</sub>)<sub>2</sub>)-SH cleaved off with 2 mM TCEP in ddH<sub>2</sub>O. At the compound concentration of estimated 50  $\mu$ M in DMF, the anti-biotin antibody molecules captured by the immobilized biotin-K(K(K(NH<sub>2</sub>)<sub>2</sub>)<sub>2</sub>)-SH cover 1/3 of the printed area.

### Synthesis/Release/Immobilization Efficiency of 9,000 OBOC Library Compounds with Poly-Lysine Handles on Epoxy-Coated Glass Slide Printed at 40 $\mu$ m

We used microarray D with 9,216 printed targets to further examine the overall synthesis–release–immobilization efficiency of OBOC compounds for capturing protein probes from a solution. All compounds were released with the optimized cleaving buffer consisting of 2 mM TCEP and 100 mM NaHCO<sub>3</sub> in a mixture of DMF and H<sub>2</sub>O (1:1 v/v) (see Supporting Information). The printed microarray was washed in 1  $\times$  PBS and then incubated in a BSA solution at 8  $\mu$ M so that the surface region without immobilized compounds was covered with BSA molecules.

From OI-RD images acquired before and after the BSA blocking treatment, we determined the surface coverage  $\Theta$  of immobilized compounds as follows. As described in Refs. 30 and 31, the difference between the optical signal after BSA blocking and the signal before BSA blocking from the printed region is a linear function of the compound coverage [i.e.,  $s_{\text{printed}}(\Theta) = \gamma(1 - \Theta)$ ]. The difference in optical signal from the unprinted region (between printed spots) is simply given by  $s_{\text{unprinted}} = \gamma$ . As a result, the compound coverage  $\Theta$  is computed as

$$\Theta = 1 - \frac{s_{\text{printed}}(\Theta)}{s_{\text{unprinted}}} \quad (2)$$

In Fig. 3, we display the coverage of 9,216 OBOC compounds immobilized on the epoxy-coated glass surface. Except for the spots that are completely missing, the compound coverage is quite uniform both within a spot (of a given compound) and from spot to spot for different compounds. The average coverage is 50% as deduced from Eq. (2). The uniformly high coverage for most of 9,216 OBOC compounds validates the overall high efficiency from synthesis through cleavage to



**Figure 3.** Surface coverage of 9,216 printed OBOC compounds on an epoxy-coated glass slide, deduced from the change in OI-RD image of microarray D after BSA blocking treatment and Eq. (2) in the main text. The average coverage in each spot is close to 50% of the printed area.

immobilization of our trilayered OBOC synthesis scheme for high-throughput microarray fabrication and detection. The missing spots were mostly due to imperfect bead transfer with the custom bead placement device.

Eleven proteins from human plasma (Sigma-Aldrich, St. Louis, MO) were flowed over OBOC microarrays, sequentially (human holo-transferrin, plasminogen from human plasma, human IgM purified immunoglobulin, thyroxine-binding globulin from human plasma, fibrinogen from human plasma,  $\sigma_2$ -macroglobulin from human plasma, hemoglobin A<sub>0</sub>, haptoglobin from human plasma, complement C9 from human serum, retinol binding protein from human urine and human complement C7). Fibrinogen from human plasma shows strong binding to every compound immobilized on glass slide, hemoglobin A<sub>0</sub> shows weak binding to every compound, whereas the others show no binding to any compound. Figure 4 shows the differential OI-RD image of the OBOC compound microarray interaction with 0.1  $\mu$ M fibrinogen in 1  $\times$  PBS buffer. The OI-RD changes resulting from fibrinogen captured on each spot corresponds to the change of a full monolayer protein with a size comparable to that of BSA. The binding pattern of fibrinogen with OBOC compound microarray resembles the target coverage pattern of immobilized compounds on surface. Although no compound was found to bind any of 11 human plasma proteins with specificity, nonspecific binding of fibrinogen and hemoglobin with OBOC compounds confirms the successful fabrication of OBOC compound microarray with enough of the compounds immobilized on surface, ready for high-throughput, high-speed screening and drug discovery.

In summary we demonstrated that one-bead-one-compound (OBOC) split synthesis on 250- $\mu$ m, trilayered beads is an effective scheme for generating encoded libraries with sufficient single-bead compound release for chemical microarray fabrication. By synthesizing branched poly-lysine residues through PEG linkers to OBOC compounds as the common surface-anchoring handles, the OBOC compounds can be uniformly immobilized on an epoxy-functionalized solid surface with high efficiency. Branched poly-lysine residues preserve the functional integrity of most immobilized compounds in subsequent binding assays. The combination of such an OBOC synthesis scheme with small molecule microarrays detected by a label-free optical scanning microscope is a highly effective tool for drug lead discovery, protein capture, and other molecular biology studies.



**Figure 4.** Differential OI-RD image of OBOC compound microarray after interaction with fibrinogen from human plasma at a probe concentration of 0.1  $\mu$ M.

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## SUPPORTING INFORMATION

We experimentally characterized the volume ratio of trilayered beads by peptide sequencing analysis of amino acids Leu, Nle and Ile, that were conjugated to the outer layer, the intermediate layer, and the interior core, respectively (see Fig. S1). We also found, as expected, that branched poly-lysine handles competed effectively with amine residues on peptide compounds for site-specific immobilization (Fig. S2). Furthermore, we experimented with nine different buffers for compound release from trilayered beads and subsequent compound microarray fabrication on epoxy-functionalized glass surface and found the optimal buffer for the intended purpose (see Fig. S3). We used the optimal buffer in fabrication of the large OBOC compound microarray (microarray D, Fig. 3). Finally we designed and made a single-bead placement device for distributing 384 beads into 384 wells of a microtiter plate (Fig. S4) and used it in fabrication of the OBOC compound microarray (microarray D).

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