Combining Microfluidic Networks and Peptide Arrays for Multi-Enzyme Assays

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The development of assays that measure enzyme activities underlies much work in cell biology, clinical diagnostics, and drug discovery. Recent technical advances in microfluidic networks (μFNs) and in biochip microarrays have separately contributed to the development of assays that require few manipulations and that can simultaneously measure large numbers of activities. Microfluidic networks require small sample volumes and can integrate several sample processing steps on a single platform,1 while biochips allow multiple assays to be performed on a single sample.2 This paper reports a strategy for combining μFNs and biochip arrays to assay multiple enzyme activities in a sample.

Microfluidic networks have most commonly been used to perform homogeneous phase assays,3 but several recent examples have addressed solid-phase format assays.4 These examples have emphasized immunoassays to detect, or quantitate, analytes. The use of μFN to assay multiple enzymatic activities is much less common, in part, because the labeling protocols required to identify these activities add several additional steps to the assays.

We demonstrate the multi-analyte assay with a set of peptides that are selective substrates for a panel of kinase and phosphatase enzymes (Table 1).5 The peptides contain a terminal cysteine residue, which permits immobilization to a self-assembled monolayer presenting maleimide groups.6 To prepare the peptide arrays, we used a well-established “criss-cross” procedure for patterning.7 A poly(dimethylsiloxane) (PDMS) stamp having six parallel channels in negative relief (500 μm width, 50 μm height) was applied to the monolayer,8 and separate aqueous solutions each containing a different peptide (0.2 mM) were flowed into the channels in contact with the monolayer for 30 min (Figure 1A). The channels were emptied, and the stamp was removed, washed, and reapplied to the monolayer in a perpendicular orientation so that each channel intersected each of the six immobilized peptides. Solutions containing each of the six kinases were next flowed over the monolayers for 1 h at 150 nL/min.9 We then removed the stamp and used MALDI-TOF MS to analyze the 36 regions of the monolayer corresponding to treatment of each peptide with each of the kinases.10,11 Spectra corresponding to regions where peptides had been immobilized but not exposed to kinases showed expected peaks for cysteine-mediated immobilization of the peptide substrate (Figure 1B, i). Only regions containing peptides that were exposed to specific kinases showed peaks corresponding to phosphorylation (iii).

Figure 2A summarizes the specificity of each kinase for the panel of peptides.12 The kinases showed the expected specificities toward the peptides. For example, the related kinases Abl and Src (and PKA and PKC) show a slight cross-reactivity for their peptide substrates.5

We next illustrate that the peptide arrays can be used to identify enzyme activities in a mixture of kinases. Using the technique described above, we assayed kinase and inhibitor activities simul-

Table 1. Peptide Substrates Used in Enzyme Assays

<table>
<thead>
<tr>
<th>Peptides</th>
<th>Enzymes</th>
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<tbody>
<tr>
<td>P1</td>
<td>Ac-AIYAAPFKGC-NH₂</td>
</tr>
<tr>
<td>P2</td>
<td>Ac-EEIYGEFEAKKC-NH₂</td>
</tr>
<tr>
<td>P3</td>
<td>Ac-CKRRALS(p)VASLPGL-NH₂</td>
</tr>
<tr>
<td>P4</td>
<td>Ac-TGLPSGPFGC-NH₂</td>
</tr>
<tr>
<td>P5</td>
<td>Ac-LRRASLG-NH₂</td>
</tr>
<tr>
<td>P6</td>
<td>Ac-AKIQASFRGHMARKKGC-NH₂</td>
</tr>
<tr>
<td>P7</td>
<td>Ac-RKRSRAEC-NH₂</td>
</tr>
<tr>
<td>PP1</td>
<td>Ac-AIY(p)AAPFKGNC-NH₂</td>
</tr>
<tr>
<td>PP5</td>
<td>Ac-LRRAS(p)LGC-NH₂</td>
</tr>
</tbody>
</table>

Figure 1. (A) This work describes the use of a μFN to prepare a peptide array and treat the array with samples that contain kinase and phosphatase activities. (B) Mass spectrometry is used to analyze the immobilized peptides. Spectra show maleimide-terminated alkanethiols before (i) and after (ii) reaction with a cysteine-terminated peptide. Treatment of the peptide with a kinase results in phosphorylation, with a mass increase of 80 D (iii).

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small molecule inhibitors prevented phosphorylation of the P1 and P6 substrates (lane K6). These results demonstrate that the multi-analyte assays accurately report on the presence of kinase activities in samples containing multiple enzymes, and that the assays are effective for evaluating selective inhibitors of these kinases.

Finally, we show that the multi-analyte assay described above is well-suited for analysis of cell extracts. We used human K562 cells that overexpress the oncogenic Bcr-Abl fusion protein, resulting in tyrosine hyperphosphorylation of numerous cellular substrates.\(^{16}\) Cell extracts were prepared and supplemented with ATP (0.2 mM). We prepared a peptide array containing three kinase substrates (P1, P2, and P5) that report on tyrosine and Ser/Thr kinase activities and three phosphorylated peptides (P3, P1P, and P5P) that report on phosphate activities.\(^{17}\)

We assayed a series of cell cultures and small molecule inhibitors. The first lane (Figure 2C, L1) contained an extract from normal K562 cells cultured with Gleevec (50 µM L2). Mass spectra from this lane reveal that Abl kinase activity is inhibited in samples containing multiple enzymes, and that the assays are denaturing in previous work,\(^{5,11,20}\) into a single platform represents a significant advance in bioanalytical microsystems and will be useful in chemical biology and applied biology programs.

Acknowledgment. This work was supported by the NSF-MRSEC and used the microfluidics and protein expression facilities.

Supporting Information Available: Details for fabrication of µFNs, preparation of SAMs, enzyme assays, mass spectrometric analysis, and cell extract preparation. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(5) See Supporting Information for references on substrate sequences.
(8) Phosphorylation of immobilized peptides is determined from the relative intensities of substrate and product peaks in MALDI MS spectra and assumes a linear response of peak intensity and amount of peptide.
(10) To MALDI MS analysis, the substrate (2.5 cm long by 2.5 cm wide) was treated with 2,4,6-trihydroxyacetophenone (THAP, 30 µL, 10 mg mL\(^{-1}\) in acetone).
(12) Mass spectrometry to be used to directly determine the modification of immobilized peptides. The MS detection also allows different classes of enzymatic activities to be assayed with a single protocol. The combination of these characteristics, which have individually been demonstrated in previous work,\(^{5,11,20}\) into a single platform represents a significant advance in bioanalytical microsystems and will be useful in chemical biology and applied biology programs.