Anal. Chem. **2004,** *76,* 3923−3929

A Method for Connecting Solution-Phase Enzyme Activity Assays with Immobilized Format Analysis by Mass Spectrometry

Dal-Hee Min, Woon-Seok Yeo, and Milan Mrksich*

Department of Chemistry and Institute for Biophysical Dynamics, The University of Chicago, 5735 South Ellis Avenue, Chicago, Illinois 60637

This paper reports an enzyme activity assay that combines the assets of both homogeneous and solid-phase formats. In this method, enzyme reactions are carried out in solution using substrates that are tagged with an immobilization reagent that allows the substrates to be selectively immobilized to self-assembled monolayers (SAMs), for direct analysis by matrix assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry (MS). As a model enzyme reaction, this work examined the transfer of a methyl group from *S*-adenosyl-L-methionine (AdoMet) to an arginine side chain of a peptide substrate by the enzyme protein arginine methyltransferase 1 (RMT1). A cysteine-terminated peptide substrate was methylated by RMT1 in solution and then applied to a maleimide-presenting SAM to give selective immobilization of the peptide. Time-dependent analysis of methylation using MALDI-TOFMS clearly showed that both the presence and relative amount of the two reaction products—the mono- and dimethylated peptides—can be conveniently evaluated. This assay strategy is rapid, takes advantage of solution-phase assay conditions, avoids the use of labels and complicated purification steps, and is applicable to multianalyte analyses.

A major theme in analytical chemistry is the development of biochemical assays that are rapid, simple, sensitive, and compatible with immobilized-array formats. Assays that measure protein−protein binding interactions and enzymatic activities continue to be important in basic research, drug discovery, and diagnostics.[1] The strategies that are now used to measure biochemical activities can be divided in two classes: homogeneous and solid-phase formats.[2,3] The former, which measures activities in solution, often uses fluorescence resonance energy transfer[4,5] or fluorescence polarization[6,7] strategies to monitor binding events. The latter measures activities at a solid−liquid interface and begins with a solid surface to which a protein binding partner or enzyme substrate is immobilized.[8−10]

These two formats each offer a combination of advantages and limitations and in practice many users have a strong preference for one of the two formats. The homogeneous format has the main advantage that the assay is performed in a solution environment that can be made to closely mimic the normal environment for the enzyme and also avoid perturbations from linking enzymes or substrates to a surface. However, this format is not well-suited to multiplexing assays to several analytes in a single sample, and it often requires preparatory steps to isolate (or partially purify) the analyte from the reaction mixture prior to analysis. Immobilized format assays, by contrast, are experimentally simple, avoid purification steps, and allow hundreds of assays to be performed on each sample through the use of patterned arrays. Further, recent work shows that immobilized format assays can be well-suited for detection by mass spectrometry methods,[11−13] enabling the direct analyses of activities without the use of labels. A concern with this format, though, is that many biomolecules, when immobilized, are partially or completely inactive and therefore incompatible with the solid-phase format (Figure 1A). This loss of activity can arise from denaturation of the immobilized biomolecule, immobilization in a nonproductive orientation, or steric interactions with the substrate.[14−16] We report here a strategy for combining the assets of both homogeneous and solid-phase formats to give assays that are rapid, allow for multianalyte analysis, and avoid the use of labels to report the enzyme activity.

Our strategy is based on a sequence of (i) performing a solution-phase assay of the enzyme with a tagged substrate, (ii) selectively capturing the substrate by reaction of the tag with a

* To whom correspondence should be addressed. Fax: (773)702-0805. E-mail: mmrksich@uchicago.edu.

(1) Cooper, M. A. *Curr. Opin. Pharmacol.* **2003**, *3*, 557−562.
(2) Sundberg, S. A. *Curr. Opin. Biotechnol.* **2000**, *11*, 47−53.
(3) Howbrook, D. N.; van der Valk, A. M.; O'Shaughnessy, M. C.; Sarker, D. K.; Baker, S. C.; Lloyd, A. W. *Drug Discov. Today* **2003**, *8*, 642−651.
(4) Pollok, B. A.; Heim, R. *Trends Cell. Biol.* **1999**, *9*, 57−60.
(5) Fattori, D.; Urbani, A.; Brunetti, M.; Ingenito, R.; Pessi, A.; Prendergast, K.; Narjes, F.; Matassa, V. G.; De Francesco, R.; Steinkuhler, C. *J. Biol. Chem.* **2000**, *275*, 15106−15113.
(6) Chen, X.; Levine, L.; Kwok, P.-Y. *Genome Res.* **1999**, *9*, 492−498.
(7) Levine, L. M.; Michener, M. L.; Toth, M. V.; Holwerda, B. C. *Anal. Biochem.* **1997**, *247*, 83−88.
(8) Houseman, B. T.; Huh, J. H.; Kron, S. J.; Mrksich, M. *Nat. Biotechnol.* **2002**, *20*, 270−274.
(9) Zhu, H.; Bilgin, M.; Bangham, R.; Hall, D.; Casamayor, A.; Bertone, P.; Lan, N.; Jansen, R.; Bidlingmaier, S.; Houfek, T.; Mitchell, T.; Miller, P.; Dean, R. A.; Gerstein, M.; Snyder, M. *Science* **2001**, *293*, 2101−2105.
(10) MacBeath, G.; Schreiber, S. L. *Science* **2000**, *289*, 1760−1763.
(11) Su, J.; Mrksich, M. *Langmuir* **2003**, *19*, 4867−4870.
(12) Su, J.; Mrksich, M. *Angew. Chem., Int. Ed.* **2002**, *41*, 4715−4718.
(13) Min, D.-H.; Tang, W.-J.; Mrksich, M. *Nat. Biotechnol.*, in press.
(14) Lee, Y.-S.; Mrksich, M. *Trends Biotechnol.* **2002**, *20*, S14−18.
(15) Haab, B. B.; Dunham, M. J.; Brown, P. O. *Genome Biol.* **2001**, *2*, 1−13.
(16) Johnson, C. P.; Jensen, I. E.; Prakasam, A.; Vijayendran, R.; Leckband, D. *Bioconjugate Chem.* **2003**, *14*, 974−978.

10.1021/ac049816z CCC: $27.50
Published on Web 05/20/2004

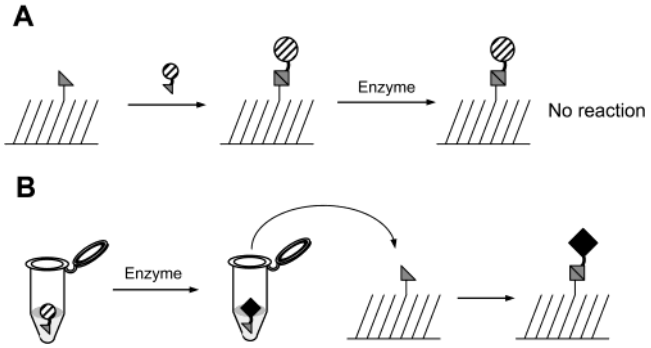

Figure 1. The basis for a "pull down" assay format that combines enzyme reactions in the solution phase with mass spectrometric detection in the immobilized format. (A) In a standard immobilized format assay, a substrate for an enzyme (circle) is immobilized to a surface and then treated with an enzyme to effect a reaction. In some cases, however, the immobilized biomolecules are inactive. (B) In the pull down format, an enzymatic reaction is carried out in a solution, and the reaction mixture is subsequently applied to a surface. The reaction product (square) is specifically immobilized, and reaction profiles are afforded by mass spectrometry without labels and purification steps.

solid phase, and (iii) analyzing the solid phase by MS to determine the extent of enzyme modification to the substrate (Figure 1B). A variety of immobilization tags can be employed, provided that the immobilization reactions are selective for the tagged substrate, rapid, and proceed in high yield.[17−25] One important aspect of this strategy is that both the substrate and the product of enzymatic modification should undergo immobilization with comparable rates. This requirement ensures that the ratio of modified and unmodified substrates on the surface is representative of the ratio in solution. In this work, we use as the solid-phase SAMs presenting maleimide groups among tri(ethylene glycol) groups.[18] Maleimide groups provide for selective immobilization of cysteine-containing peptides by way of the specific conjugation reaction with the sulfhydryl group on a cysteine side chain, provided that other thiol species are absent in the reaction mixture. At the end of the solution-phase enzyme reaction, a small volume (typically, 500 nL) is applied to the monolayers, resulting in selective immobilization of the substrate and the product of enzymatic reaction. After rinsing, matrix is applied to the monolayer, which is then analyzed by MALDI-TOFMS, a technique we refer to as SAMDI (Self-Assembled Monolayers for MALDI). Because most enzymatic reactions result in molecular weight changes of substrates—including kinases, proteases, and functional group transferases—this biochemical assay system is applicable to a wide range of enzymes.

To illustrate the utility of this pull down assay format, we used the enzyme protein arginine methyltransferase 1 (RMT1). RMT1 transfers methyl groups from *S*-adenosyl-L-methionine (AdoMet) to arginine side chains of proteins.[26,27] RMT1 is abundant in the nuclei of cells and is involved in regulating the nuclear export of substrate proteins including mRNA-binding proteins.[28,29] Most RMT1 substrates contain consensus sequences based on the RGG or RXR tripeptide. It has been shown that RMT1 substrates isolated in vivo are usually completely or nearly completely dimethylated.[26] Current assays of RMT1 activity rely on the use of antibodies, isotopically labeled AdoMet, and/or liquid chromatography to measure the methylation of the substrate peptide.[26,29,30] Hence, unlike many assays of kinase and protease activity, assays of RMT1 remain tedious and time-consuming, making this a good system for demonstrating the attributes of the pull down format assay. Another property that makes the enzyme a good candidate for this study is that RMT1 adds two methyl groups to the peptide substrate, raising an important question of the relative activities of the nonmethylated and monomethylated peptides toward enzyme action. While conventional assays have difficulty in resolving the relative rates for the two methyl transfer reactions, we show that the SAMDI strategy can measure both species.

EXPERIMENTAL SECTION

Materials. *S*-Adenosyl-L-methionine (AdoMet) was purchased from Sigma (St. Louis, MO). The plasmid pGEX-2T-RMT1, which encodes GST-RMT1 was provided by Professor Steven Clarke, Department of Chemistry and Biochemistry, University of California, Los Angeles. The peptide substrate, GGRGGFGC, was prepared on Fmoc-Rink amide MBHA resin (AnaSpec. Inc., San Jose, CA) using an ABI 430A peptide synthesizer and Fmoc solid-phase peptide synthesis methods in the Protein Core Facility at the University of Chicago. 2,4,6-Trihydroxyacetophenone was purchased from Aldrich Chemical Co. (Milwaukee, WI). The microscope cover glasses and other reagents were purchased from Fisher Scientific (Pittsburgh, PA).

Expression of RMT1. GST-RMT1 was expressed and purified as described.[26] Briefly, transformed *E. coli.* BL21(DE3) pLysS were grown at 37 °C until $A_{600\ nm}$ reached 0.5. Expression of GST-RMT1 was induced by addition of isopropyl-1-thio-β-D-galactopyranoside (0.5 mM) for 3 h. After centrifugation at 5000 rpm at 4 °C, bacteria pellets were resuspended in lysis buffer (PBS containing 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 5% glycerol, 0.5% Triton X-100, and 1 mM phenylmethylsulfonyl fluoride). Resuspended cells were sonicated, and cell debris was removed after centrifugation at 10 000 rpm at 4 °C. GST-RMT1 was purified from cleared cell lysate with glutathione-sepharose beads. The molecular weight and purity of expressed GST-RMT1 were determined by SDS−PAGE gel electrophoresis. Purified GST-RMT1 was concentrated using a centrifugal filtration device (Centriplus, cutoff; 10 kDa). The concentration of GST-RMT1 was determined by measuring

(17) Banks, P. R.; Paquette, D. M. *Bioconjugate Chem.* **1995**, *6*, 447−458.
(18) Houseman, B. T.; Gawalt, E. S.; Mrksich, M. *Langmuir* **2003**, *19*, 1522−1531.
(19) Chaves, F.; Calvo, J. C.; Carvajal, C.; Rivera, Z.; Ramirez, L.; Pinto, M.; Trujillo, M.; Guzman, F.; Patarroyo, M. E. *J. Pept. Res.* **2001**, *58*, 307−316.
(20) Kwon, Y.; Mrksich, M. *J. Am. Chem. Soc.* **2002**, *124*, 806−812.
(21) Yousaf, M. N.; Mrksich, M. *J. Am. Chem. Soc.* **1999**, *121*, 4286−4287.
(22) Yan, M.; Cai, S. X.; Wybourne, M. N.; Keana, J. F. W. *Bioconjugate Chem.* **1994**, *5*, 151−157.
(23) Wang, Q.; Chan, T. R.; Hilgraf, R.; Fokin, V. V.; Sharpless, K. B.; Finn, M. G. *J. Am. Chem. Soc.* **2003**, *125*, 3192−3193.
(24) Soellner, M. B.; Dickson, K. A.; Nilsson, B. L.; Raines, R. T. *J. Am. Chem. Soc.* **2003**, *125*, 11790−11791.
(25) Nilsson, B. L.; Kiessling, L. L.; Raines, R. T. *Org. Lett.* **2001**, *3*, 9−12.
(26) Gary, J. D.; Lin, W.-J.; Yang, M. C.; Herschman, H. R.; Clarke, S. *J. Biol. Chem.* **1996**, *271*, 12585−12594.
(27) Weiss, V. H.; McBride, A. E.; Soriano, M. A.; Filman, D. J.; Silver, P. A.; Hogle, J. M. *Nat. Struct. Biol.* **2000**, *7*, 1165−1171.
(28) Henry, M. F.; Silver, P. A. *Mol. Cell. Biol.* **1996**, *16*, 3668−3678.
(29) McBride, A. E.; Weiss, V. H.; Kim, H. K.; Hogle, J. M.; Silver, P. A. *J. Biol. Chem.* **2000**, *275*, 3128−3136.
(30) Hyun, Y.-L.; Lew, D. B.; Park, S. H.; Kim, C.-W.; Paik, W. K.; Kim, S. *Biochem. J.* **2000**, *348*, 573−578.

absorbance of the solution at 280 nm (ϵ_{280} = 84 000 $M^{-1}cm^{-1}$). GST-RMT1 was stored at −20 °C in pH 8.0 Tris buffer containing 10% glycerol.

RMT1 Reactions. AdoMet (3 μL, 5 mM) was added to a solution of GST-RMT1 (5 μL, 20 μM). The enzyme reaction was initiated by addition of the substrate (1 μL, 5 mM) to this mixture, followed by distilled water to bring the final volume to 10 μL and final concentrations of GST-RMT1, AdoMet, and peptide substrate to 10 μM, 1.5 mM, and 0.5 mM, respectively. The reaction was incubated at 37 °C, and aliquots (0.6 μL) of the reaction mixture were removed for MS analyses at various times ranging from 40 to 440 min.

Preparation of Gold Substrates. Glass coverslips were cleaned in Piranha solution (sulfuric acid:30% hydrogen peroxide = 70:30—WARNING: Piranha solution is highly corrosive and reactive. Handle with caution.) for 30 min, washed with deionized water and ethanol, and dried under a nitrogen stream. Titanium (5 nm) and then gold (15 nm) were evaporated onto the glass coverslips using an electron beam evaporator (Thermionics VE-100) at a rate of 0.2−0.4 nm/s and at a pressure of 9 $\times 10^{-7}$ Torr.

Preparation of SAMs Presenting Maleimide Groups. The maleimide-presenting SAMs were prepared as described previously.[18] Briefly, gold-coated coverslips were immersed in an ethanolic solution of maleimide-terminated disulfide and tri(ethylene glycol)-terminated disulfide in a ratio of 3:7 for 5 h (total concentration of disulfide was 1 mM.). The substrate was rinsed with ethanol and dried under a stream of nitrogen.

Immobilization of Substrate in Pull Down Assay. A volume of 0.6 μL of the reaction mixture described above was removed and transferred onto the maleimide-presenting SAMs at each time point and incubated at 37 °C with complete immobilization of the cysteine-terminated peptide within 20 min. The drops of reaction mixture were applied to the monolayer within engraved circles (2 mm in diameter) to control the spreading of the drop to a constant area. Following immobilization of peptides, monolayers were rinsed with distilled water, 10 μM hydrochloric acid solution, distilled water, and ethanol. Monolayers were then treated with matrix (2,4,6-trihydroxyacetophenone, 20 μL, 3 mg/mL in methanol), dried, and analyzed by MALDI-TOFMS to obtain a mass spectrum for each circular region. Matrix-treated monolayers were stable at room temperature for several days. Complete peptide immobilization was confirmed by the disappearance of peaks corresponding to maleimide-terminated disulfide.

Mass Spectrometry. Mass analysis was performed using a Voyager DE-PRO Biospectrometry mass spectrometer (Applied Biosystems, Framingham, MA). A 337 nm nitrogen laser was used as a desorption−ionization source, and all the spectra were acquired with 20 kV accelerating voltage using reflector mode in positive ions. The extraction delay time was 100 ns, and 800 laser shots were applied for each spectrum. All spectra were obtained by moving the laser beam to several different spots within a circle.

Quantitative Analysis. For quantitation, the mass spectra were taken using the conditions described above. The relative amount of each component was calculated by measuring the relative intensities (relative amount of DSx = $I_{DSx}/(I_{DS1} + I_{DS2} + I_{DS3})$) of each molecular ion peaks (M^+).

HPLC-Based Assay. RMT1 reactions were carried out using the same conditions as described above, but with a total reaction volume of 250 μL. For each time point, an aliquot (40 μL) of the reaction mixture was removed and quenched by adding 10% trifluoroacetic acid (5 μL). The sample (20 μL) was analyzed by high-performance liquid chromatography (C18 reverse phase column, Varians, 5 micron spherical particles, 4.6 × 250 mm). The chromatography used a first buffer (0.1% trifluoroacetic acid in water) to elute for 10 min at a rate of 1 mL/min and then used gradients with a second buffer (0.1% trifluoroacetic acid and 0.9% water in acetonitrile) as follows: 10−30 min, increasing from 0 to 50%; 30−33 min, increasing from 50 to 100%; 33−37 min, 100%; 37−40 min, decreasing from 100 to 0%; 40−45 min, 0%. Retention times were approximately 21.9 min for substrate peptide, 22.0 min for monomethylated product, and 22.1 min for dimethylated product, respectively. For quantitation, the relative amount of each component was calculated by integrating the areas of peaks.

RESULTS AND DISCUSSION

Preparation and Properties of Monolayers Presenting Maleimide Groups. Maleimide-presenting SAMs were prepared by immersing gold-coated coverslips in an ethanolic solution of a maleimide-terminated disulfide and a tri(ethylene glycol)-terminated disulfide as described previously.[18] The maleimide group serves as a handle to covalently immobilize peptides terminated in a cysteine residue. The reaction between a thiol and maleimide is well suited for selective immobilization because the conjugation reaction is fast and selective in the absence of other thiol-containing small molecules. The use of SAMs on gold as substrates in bioanalytical applications offers several other advantages. The density of active groups on the monolayer is easily adjusted by changing the ratio of molecules in the solution from which the SAM is prepared. SAMs on gold also provide uniform presentation of ligand in a regular environment, giving a uniform activity of the immobilized ligands and enabling quantitative assays of biological activities. SAMs are also compatible with several important detection methods including fluorescence,[31] radioactivity,[8] surface plasmon resonance (SPR),[31,32] and mass spectrometry (MS).[11−13] Further, dynamic properties can be added to afford active substrates that are able to manipulate, in real-time, ligand−receptor interactions at the interface.[21,33−37] The monolayers provide a flexible platform that has been used in assays with immobilized peptides, carbohydrates, and proteins.[8,12,13,18,31,32] Overall, these benefits make SAMs an attractive choice for engineering biochips for applications in enzyme assays and chemical screening.[13,14]

Assay Design. The scheme for the RMT1-mediated methylation assay is shown in Figure 2. We used the peptide GGRGGFGC as a substrate for methylation by RMT1. This peptide contains the consensus arginine methylation site. We first evaluated a solid-phase format assay and began by immobilizing the

(31) Houseman, B. T.; Mrksich, M. *Chem. Biol.* **2002**, *9*, 443−454.
(32) Hodneland, C. D.; Lee, Y.-S.; Min, D.-H.; Mrksich, M. *Proc. Natl. Acad. Sci. U.S.A.* **2002**, *99*, 5048−5052.
(33) Yeo, W.-S.; Hodneland, C. D.; Mrksich, M. *ChemBioChem* **2001**, *2*, 590−593.
(34) Yeo, W.-S.; Yousaf, M. N.; Mrksich, M. *J. Am. Chem. Soc.* **2003**, *125*, 14994−14995.
(35) Yousaf, M. N.; Houseman, B. T.; Mrksich, M. *Proc. Natl. Acad. Sci. U.S.A.* **2001**, *98*, 5992−5996.
(36) Yousaf, M. N.; Houseman, B. T.; Mrksich, M. *Angew. Chem., Int. Ed.* **2001**, *40*, 1093−1096.
(37) Hodneland, C. D.; Mrksich, M. *J. Am. Chem. Soc.* **2000**, *122*, 4235−4236.

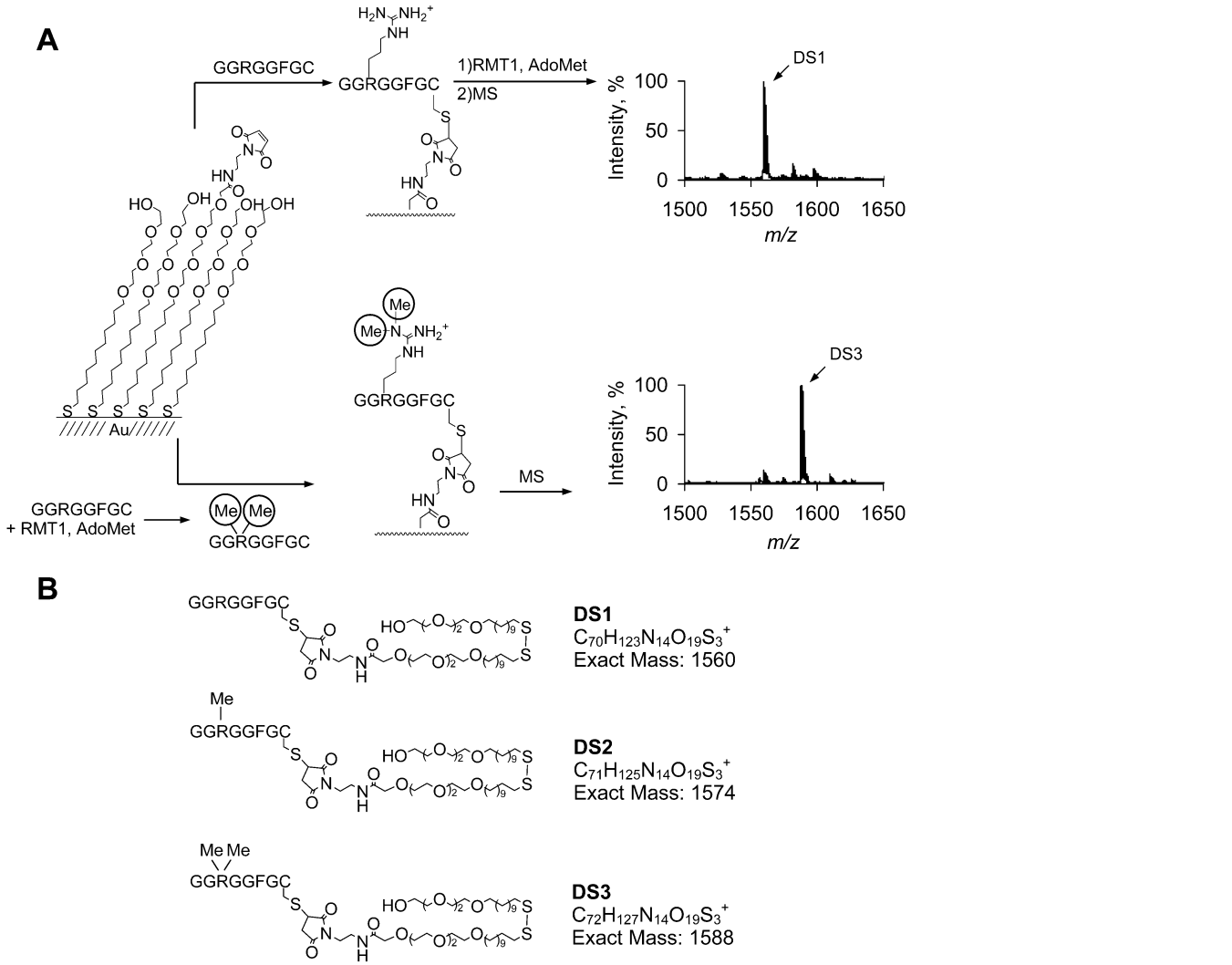

Figure 2. Scheme for assaying the RMT1-mediated methylation of peptides. (A) A substrate peptide was immobilized to a monolayer presenting maleimide group by a selective conjugation reaction between the cysteine side chain of the peptide and the maleimide groups of the monolayer. Treatment of the monolayer with RMT1 and analysis by MALDI-TOFMS showed that the peptide was unreactive to the enzyme (top). The methylation reaction was then carried out in solution, and the reaction mixture was applied directly to the monolayer. Only cysteine-containing peptides were immobilized to the monolayer, and analysis by MS showed that the peptide was completely dimethylated (bottom). (B) Molecular structures and calculated masses of analytes for the RMT1 assay are shown.

cysteine-terminated peptide on the maleimide-presenting monolayer. This monolayer was treated with RMT1 (10 μM) and AdoMet (1.5 mM), incubated at 37 °C for 7 h, and analyzed by MALDI-TOFMS. The MS spectrum of this monolayer revealed a peak at a mass-to-charge ratio of 1560, corresponding to an unsymmetrical disulfide derived from a peptide-terminated alkanethiol and a glycol-terminated alkanethiol (Figure 2A, top and Figure 2B, DS1). We did not observe any peaks that corresponded to a methylated form of the peptide, which implies that the immobilized peptide does not serve as a good substrate for RMT1. We next performed the same assay using the pull down format. The enzyme reaction was first carried out in solution. A reaction mixture containing RMT1 (10 μM), substrate peptide (0.5 mM), and cofactor AdoMet (1.5 mM) was prepared and incubated 37 °C for 7 h. Next, the reaction mixture was applied to a maleimide-presenting monolayer to immobilize cysteine-terminated peptides. Characterization of the surface by MALDI-TOFMS showed that only the target peptide underwent immobilization to the monolayer. Further, the peptide was methylated by RMT1, as revealed by a mass peak (m/z 1588) corresponding to the dimethylated peptide (Figure 2A, bottom and Figure 2B, DS3). This example establishes that RMT1 activity can be measured using the pull down format with MALDI-TOFMS detection.

Application to Kinetic Studies. We next applied the pull down assay to characterize the kinetic profile for methylation of the peptide by RMT1. The methylation reaction was first carried out in solution (10 μL) by mixing all the required components including RMT1 (10 μM), AdoMet (1.5 mM), substrate peptide (0.5 mM), and Tris buffer (5 mM, pH 8.0) in a micro test tube. Aliquots of the reaction mixture (0.6 μL) were transferred to designated areas on a maleimide-presenting monolayer at various times and incubated in a humidified chamber to immobilize peptides. Prior to the immobilization reactions, the monolayer substrate was mechanically etched to give an array of circular grooves, which served to define the location and sizes of the regions to which the peptides immobilize. In this way, each circular region contained peptides that were immobilized after a specific time of reaction in solution. The immobilization reactions

were completed rapidly, so that the ratio of peptide intermediates on the SAM is representative of that in solution at the time of immobilization. As a benefit of the fast conjugation of thiols to maleimides, other considerations to preserve shape and volume of the spotted solutions, such as addition of glycerol to the solution, were not required.[38] After the immobilization reaction, the monolayer was rinsed with water, hydrochloric acid (0.01 mM), water, and absolute ethanol and then dried. Preparation of a clean surface is critical for obtaining satisfactory mass spectra because salts and other surface adsorbents reduce peak intensities. A methanolic solution of a matrix, 2,4,6-trihydroxyacetophenone (3 mg/mL), was applied to the substrate and allowed to dry on the flat surface. Slow evaporation of methanol from the surface in a semiclosed chamber favored homogeneous distribution of the matrix on the entire surface. We optimized the instrumental parameters for acquiring mass spectra from the monolayer and found that an accelerating voltage of 20 kV, operating in reflector mode, and extraction delay time of 100 ns were effective. Within the circles, we observed a homogeneous response when multiple spots in the same circular region were analyzed, which is consistent with the well-defined structure of SAMs. Conventional sample preparations, such as dried-droplet methods, often produce heterogeneous crystallization of analyte and matrix and consequently require locating "sweet spots" to obtain satisfactory spectra. In our system, monolayers provide homogeneous densities of analyte over an entire surface and hence give reproducible and consistent mass spectra.

Figure 4 shows the mass spectra that were obtained from each region of the monolayer, where each region presented peptide that was treated with RMT1 for a specific time. Spectra are shown for reaction times ranging from 0 to 7 h. The panel of spectra shows that the substrate peptide (m/z 1560, DS1) was consumed during the course of the reaction. Concurrent with consumption of the substrate peptide, peaks for the monomethylated peptide (m/z 1574, DS2) and dimethylated peptide (m/z 1588, DS3) increased in intensity during the reaction. The spectra show that the changes in concentration of peptide species can be followed over the course of the reaction, indicating that the assay method can provide a kinetic profile of the enzyme activity. It is notable that the monomethylated peptide is present at a low concentration throughout the reaction, showing that this peptide is a more active substrate for RMT1 than the parent peptide. We quantitated the relative amount of the three peptides in each spectra to obtain plots for the time-dependent change in concentration for each species (Figure 5A). These data show that the monomethylated peptide increased to approximately 10% of the total peptide and remained constant until it dropped at 300 min as the substrate peptide was depleted. With longer times of reaction, the methylated peptide was completely converted to the dimethylated form. The reaction profile obtained using the pull down format confirms that this method allows for quantitative analysis of enzymatic reactions over time. However, differences in ionization and detection efficiencies of DS1, DS2, and DS3 during MS analysis have to be considered for more accurate quantitative evaluation of enzyme activities.

(38) In other biochemical assay systems, the addition of glycerol to the reaction mixture lowered the enzyme activities. See: Gosalia, D. N.; Diamond, S. L. *Proc. Natl. Acad. Sci. U.S.A.* **2003**, *100*, 8721−8726.

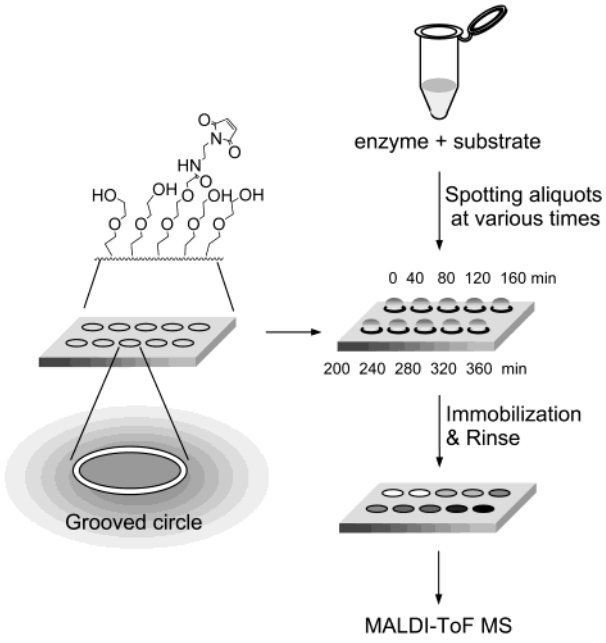

Figure 3. Scheme for obtaining kinetic information of the RMT1 assays using a "pull down" method. The enzymatic reaction was carried out in a homogeneous solution by mixing all the required components in a reaction vessel. Aliquots of the reaction mixture were then transferred to designated areas on a maleimide-presenting monolayer at various times to give selective immobilization of the peptide. The monolayer was rinsed, treated with matrix, and analyzed by MALDI-TOFMS directly.

To verify that our method provides data that agree with conventional solution-phase assay methods, we characterized the enzymatic activity of RMT1 in solution. The RMT1 reactions were performed in the same conditions as used in pull down assay. The experiment used a reaction volume of 250 μL, in contrast to the 10 μL volume required by the SAMDI assay. For each time point, a reaction volume (40 μL) was removed, quenched with trifluoroacetic acid, and analyzed by high-performance liquid chromatography. We quantitated the relative amount of three peptides in each chromatogram—by integrating the areas under the peaks—and plotted the relative concentrations as a function of reaction time (Figure 5B). The graph shows a similar kinetic profile to that obtained with the pull down assay. This result illustrates that the SAMDI method can be used in place of a conventional solution-phase assay. The data also show the better resolution and reproducibility intrinsic to the MS method.

Comparison with Other MS-Based Methods. There have been many reports of using MS methods to measure biochemical activities.[39−47] The method we describe here compares favorably

(39) Kevala, J. H.; Kim, H.-Y. *Anal. Biochem.* **2001**, *292*, 130−138.
(40) Sato, H.; Yamada, N.; Shimba, N.; Takahara, Y. *Anal. Biochem.* **2000**, *281*, 68−76.
(41) Trummal, K.; Vija, H.; Subbi, J.; Siigur, J. *Biochim. Biophys. Acta* **2000**, *1476*, 331−336.
(42) Galletti, P.; Ingrosso, D.; Manna, C.; Sica, F.; Capasso, S.; Pucci, P.; Marino, G. *Eur. J. Biochem.* **1988**, *177*, 233−239.
(43) Schmid, D. G.; Majer, F.; Kupke, T.; Jung, G. *Rapid Commun. Mass Spectrom.* **2002**, *16*, 1779−1784.
(44) Houston, C. T.; Taylor, W. P.; Widlanski, T. S.; Reilly, J. P. *Anal. Chem.* **2000**, *72*, 3311−3319.
(45) van Breemen, R. B.; Davis, R. G. *Anal. Chem.* **1992**, *64*, 2233−2237.
(46) Wu, J.; Takayama, S.; Wong, C.-H.; Siuzdak, G. *Chem. Biol.* **1997**, *4*, 653−657.

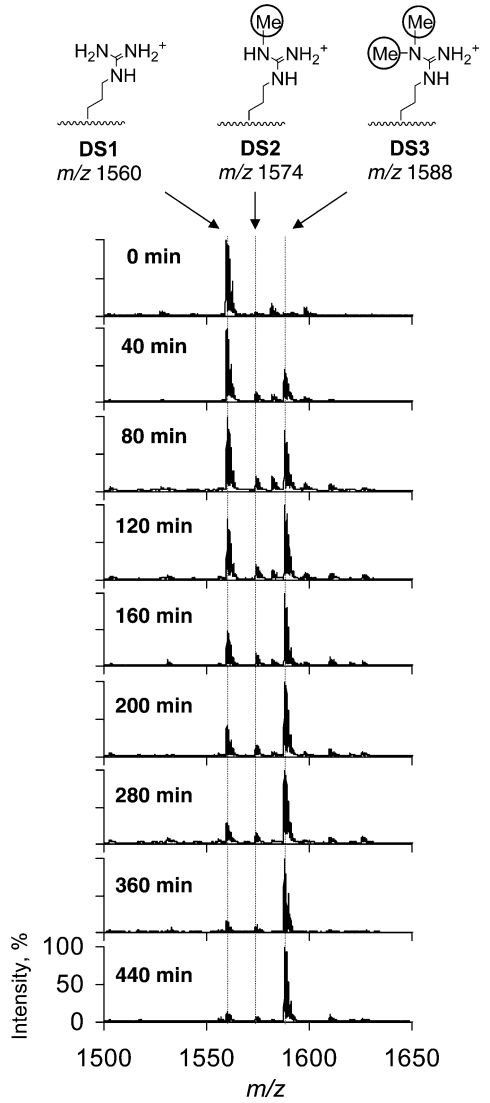

Figure 4. The time-dependent MALDI-TOFMS analysis for the methylation of peptide substrate by RMT1 obtained as described in Figure 3. Spectra show that the substrate peptide peak decreases with time, and the dimethylated product peak increases accordingly. The monomethylated product peak increases initially to a steady-state concentration and then decreases as the reaction proceeds.

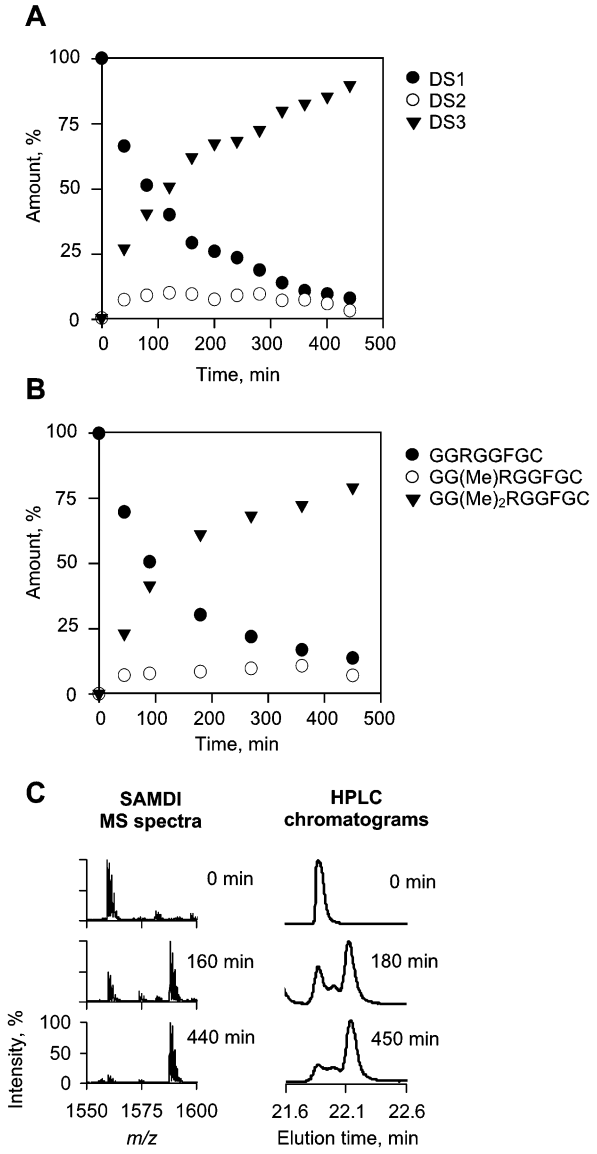

Figure 5. The kinetic profiles for the enzymatic reactions. (A) The amount of each analyte was determined by measuring the heights of individual peaks in the mass spectra in Figure 4 and is plotted versus time for each analyte. The enzymatic dimethylation shows saturation behavior. The amount of monomethylated peptide increases at the beginning of the reaction and remains constant until it drops at 300 min as the substrate peptide is depleted. (B) The enzymatic reaction was performed in the solution phase, and the reaction progress was followed by high-performance liquid chromatography. The amount of three peptides is plotted versus reaction time. The data show a similar kinetic profile to that of Figure 5A. (C) The representative chromatograms (right panel) and MS spectra (left panel) were shown for comparison.

with these existing MS formats. One of these formats combines liquid chromatography with a MS detector (LC-MS) and relies on a chromatographic separation to purify analytes prior to detection by MS.[39] The SAMDI method described in this paper, by contrast, eliminates the purification steps by employing a specific and rapid conjugation reaction of a cysteine-terminated peptide to the maleimide-terminated SAM. More importantly, the LC-MS and related formats require solution-phase samples and therefore are not directly compatible with the use of immobilized arrays. Of the established MS methods, MALDI-TOFMS is most appropriate for analysis of biopolymers but still requires the use of sample preparation protocols to remove salt and extraneous molecules from the sample. The pull down assay format we describe in this paper avoids these steps by harnessing a specific conjugation reaction for selectively retaining the analyte of interest from the homogeneous reaction mixture. After the immobilization reaction is complete, a simple rinsing step serves to purify the analyte. Further, the monolayers of alkanethiolates on gold serve as excellent substrates in MALDI-TOFMS and give clear and easily interpreted spectra.[11−13]

The biochemical assay method described here has several characteristics that make it a valuable addition to the biochemical assay formats that are currently in wide use. Most importantly, the pull down format provides a strategy for harnessing the desirable aspects of both homogeneous and solid-phase formats.

(47) Lin, Q.; Jiang, F.; Schultz, P. G.; Gray, N. S. *J. Am. Chem. Soc.* **2001**, *123*, 11608−11613.

The former is important because certain activities are difficult to assay when one of the reagents is immobilized to a solid phase, either because the underlying surface prevents the biochemical interaction with soluble proteins or because the surface leads to a loss of activity of the immobilized species. Examples of activities that are difficult to measure in the solid-phase format include membrane-bound enzymes (i.e., enzymes involved in the glycosylation of proteins) and activities associated with large multiprotein complexes (i.e., the proteasome).[48,49] At the same time, the immobilization of a substrate to a self-assembled monolayer allows the straightforward use of MALDI-TOFMS to directly characterize the substrate for enzymatic modification. The ability to selectively capture the substrate from solution to the monolayer enables both advantages to be realized in the assay. The use of selective immobilization chemistries means that contaminants are rinsed away from the surface, giving a simple and rapid process for preparing the analyte for detection by MS. For all of these reasons, this immobilization strategy avoids the need for separation steps (chromatography, extraction, etc.) to purify the analyte, which is often the tedious step in many MS-based assay formats. Finally, this strategy is applicable to assays of a broad range of enzymatic activities because it gives a direct detection on the basis of changes in mass of the substrate. For this reason, the SAMDI platform can be applied to a panel of different enzymatic activities. This generality is in contrast to the use of current assays, which require different platforms for measuring distinct enzymatic activities.

The use of SAMs of alkanethiolates on gold offers many opportunities for elaborating the pull down assay for additional applications. The use of two or more immobilization chemistries that are not cross-reactive, for example, should allow the simultaneous assaying of multiple enzyme activities in the same sample provided that the masses of the analytes are resolved. The method should also be extendable to the use of proteins as substrates of enzyme activity. A recently reported method for protein immobilization—based on the covalent capture of cutinase fusion proteins[32,50,51]—offers a selective and stable immobilization of protein from mixtures and meets the requirements for the immobilization tags in the pull down format. Further, the monolayers are effective as substrates in attached cell culture and therefore could be used to evaluate enzymatic activities associated with the cell surface.[52−54] Finally, dynamic properties can be incorporated into the monolayers, allowing the surface to be electrochemically switched to either activate the surface for immobilization of tagged substrates or to release immobilized substrates.[33−37] The former offers an opportunity to simplify the pull down assay by performing the homogeneous reaction in a solution that is in contact with the monolayer and then electrically activating the monolayer to initiate the immobilization reaction.

We believe that the SAMDI method will be useful for additional biological applications. Recently, we reported a chemical screening method to identify inhibitors for anthrax lethal factor.[13] In that work we immobilized a peptide substrate for the enzyme lethal factor to a SAM and then screened 10 000 small molecules to find inhibitors of the enzyme. SAMDI was used to resolve spots on the chip wherein peptides were either cleaved or protected by the protease. For broader application to enzyme activity assays and for applications in proteomics, it will be useful to directly observe proteins that are immobilized to the monolayer. We have reported two examples that showed SAMDI could be used to detect proteins that had bound to ligands presented on a monolayer.[12,55] We are currently developing this system to allow for direct digestion of the bound proteins and therefore identification of the protein.

CONCLUSIONS

This work demonstrates a flexible strategy for performing biochemical assays without the use of labels. The method is characterized by performing the enzyme reaction in a homogeneous solution and then carrying out a selective immobilization of the substrate to permit detection by MALDI-TOFMS. By using as a model reaction the methylation of arginine by RMT1, we show that the SAMDI pull down format assay provides quantitative information on enzyme activities. We believe that the simplicity inherent to this assay and the widespread availability of commercial instruments for MALDI-TOFMS will make this method a useful addition to bioanalytical chemistry.

ACKNOWLEDGMENT

This work was supported by the National Science Foundation. D.-H.M. is grateful to the Burroughs Wellcome Fund for a graduate fellowship in the cross-disciplinary training program in biophysical dynamics and biocomplexity. We are grateful to Professor Steven G. Clarke (UCLA) for providing the plasmid encoding the RMT1 enzyme.

Received for review February 3, 2004. Accepted April 21, 2004.

AC049816Z

(48) Dricu, A.; Kanter, L.; Wang, M.; Nilsson, G.; Hjertman, M.; Wejde, J.; Larsson, O. *Glycobiology* **1999**, *9*, 571−579.
(49) MacFarlane, M.; Merrison, W.; Bratton, S. B.; Cohen, G. M. *J. Biol. Chem.* **2002**, *277*, 36611−36616.
(50) Murphy, W. L.; Mercurius, K. O.; Koide, S.; Mrksich, M. *Langmuir* **2004**, *20*, 1026−1030.
(51) Shen, Y.; Lee, Y.-S.; Soelaiman, S.; Bergson, P.; Lu, D.; Chen, A.; Beckingham, K.; Grabarek, Z.; Mrksich, M.; Tang, W.-J. *EMBO J.* **2002**, *21*, 6721−6732.
(52) Houseman, B. T.; Mrksich, M. *Biomaterials* **2001**, *22*, 943−955.
(53) Kato, M.; Mrksich, M. *Biochemistry* **2004**, *43*, 2699−2707.
(54) Kato, M.; Mrksich, M. *J. Am. Chem. Soc.* **2004**, *126*, 6504−6505.
(55) Yeo, W.-S.; Mrksich, M. *Adv. Mater.*, in press.