Electroactive Monolayers

DOI: 10.1002/ange.200800166

An Electroactive Catalytic Dynamic Substrate that Immobilizes and Releases Patterned Ligands, Proteins, and Cells**

*Eugene W. L. Chan, Sungjin Park, and Muhammad N. Yousaf**

The ability to spatially and temporally control the attachment and detachment of molecules and cells on solid supports is important to research areas ranging from fundamental cell biology to biomaterial development and heterogeneous catalysis.[1–5] Various approaches have been developed to promote or inhibit cell attachment by altering the macroscopic properties of the materials in situ, including mechanical stretching,[6–8] photochemical illumination,[9–11] electrochemical modulation,[12,13] and thermal activation.[14–16] Alternatively, other strategies have focused on manipulating the cell–surface interactions at the molecular level by incorporating specific chemistries that can alter ligand presentation for attached cell culture through a noninvasive external switch.[17–23] To access more sophisticated and complex cell behavior studies, a surface strategy that can dynamically modulate attached cell culture at the molecular level catalytically, with spatial and temporal control in patterns and gradients, would greatly extend the utility of these model surfaces for a variety of cell motility, cell signaling, and cell–cell communication studies. These surfaces may also lead to the development of renewable surfaces for synthetic organic chemistry applications ranging from new solid-phase peptide synthesis resins to heterogeneous catalysis.

Herein, we report an electroactive quinone-terminated self-assembled monolayer (SAM) on gold that captures and subsequently releases ligands, proteins, and cells in situ through an electrochemical potential. We also show that the surface is catalytic for multiple rounds of immobilization and release that are pH dependent. From a synthetic organic chemistry perspective, a clean and quantitative functional-group transformation occurs from an oxyamine group to a primary alcohol upon mild electrochemically applied potential. Furthermore, by extending this strategy with a photochemical approach, we demonstrate the immobilization and release of peptide ligands that mediate cell attachment in defined gradient patterns on inert surfaces.

[*] Dr. E. W. L. Chan, S. Park, Prof. M. N. Yousaf
Department of Chemistry and The Carolina Center for Genome Science
University of North Carolina at Chapel Hill
Chapel Hill, NC 27599-3290 (USA)
Fax: (+1) 919-962-2388
E-mail: mnyousaf@email.unc.edu
Homepage: http://www.chem.unc.edu/people/faculty/yousafmn/mnyindex.ht

[**] This work was supported by the Carolina Center for Cancer Nanotechnology Excellence, The National Cancer Institute, and the Burroughs Wellcome Foundation (Interface Career Award).

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.200800166.

Our approach is based on a redox-active hydroquinone-terminated SAM that can be electrochemically oxidized to the corresponding quinone (Figure 1). The resulting quinone

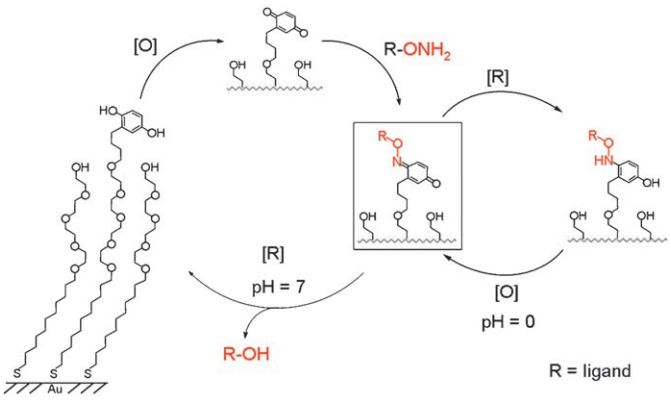


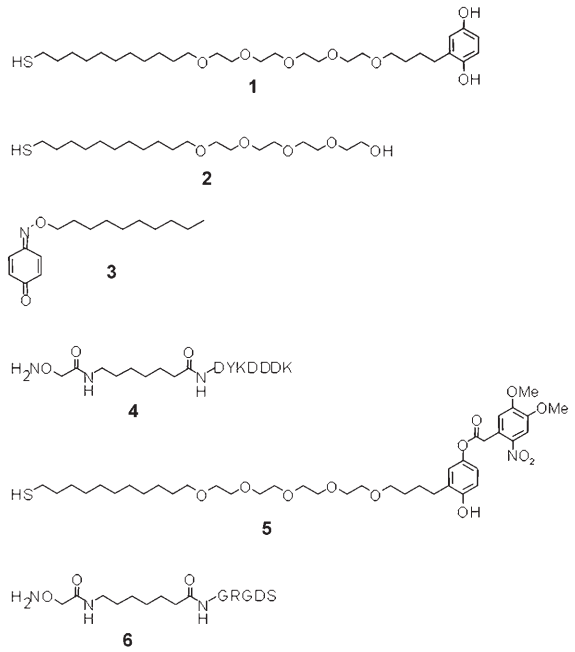
Figure 1. Interfacial reaction between soluble oxyamine and quinone-terminated SAMs. Electrochemical oxidation [O] of the mixed monolayers presenting hydroquinone and tetraethylene glycol groups converts the hydroquinone into the corresponding quinone. This quinone then reacts selectively with a soluble oxyamine-tagged ligand ($R\text{-}ONH_2$) to give the redox-active oxime conjugate on the surface. The oxime is chemically stable and undergoes reversible redox coupling in $HClO_4$ (1 M, pH 0). Electrochemical reduction [R] of the monolayer in buffer solution at pH > 0 spontaneously reverts the oxime to the hydroquinone by release of the surface-bound ligand as a hydroxy group.

monolayer permits the selective coupling of soluble oxyamine ligands to the surface via an oxime conjugate. The oxime conjugate is chemically stable at pH 1–14, but upon application of an applied potential it can undergo a subsequent reversible redox reaction at low pH (1M $HClO_4$, pH 0) with the ligand still covalently bound. However, electrochemical reduction of the monolayer at a higher pH value (phosphate-buffered saline (PBS), pH 7) reverts the oxime to the hydroquinone and releases the ligand from the surface. The oxyamine terminal group on the ligand is converted to a hydroxy group and the surface is regenerated to the catalytic hydroquinone form for subsequent immobilization and release.

This strategy possesses several unique features that are important in the preparation of model substrates for modulating cell attachment. First, the immobilization and subsequent release of ligands is controlled by mild electrochemical potentials under physiological conditions (PBS, pH 7), and therefore permits the modulation of ligands in the presence of attached cell culture.[24] Second, the electroactive quinone and oxime monolayers permit quantitative characterization of the

extent of ligand immobilization and release by cyclic voltammetry (CV).[25] Third, oxyamine moieties can be introduced into a variety of ligands through straightforward solution- and solid-phase synthesis to further extend the utility of these model substrates for other biological studies.[26] A potentially wide range of soluble aminooxy-terminated ligands can be coupled to the quinone surface by the oximation reaction, and subsequently released from the surface electrochemically. Finally, the hydroquinone surface acts as a catalyst for the immobilization and release of ligands and also provides a route for a mild functional-group transformation of oxyamine groups to hydroxy groups. Because the surface is renewable, this approach greatly simplifies the preparation of monolayer substrates for both presenting biologically active ligands and dynamically modulating the activity of immobilized ligands.

We first prepared monolayer surfaces presenting both hydroquinone (**1**) and tetraethylene glycol (**2**) groups (Scheme 1), and characterized the interfacial immobilization



Scheme 1. Surface groups used in this study.

and subsequent release of aminooxyacetic acid by CV. Figure 2A shows the cyclic voltammograms of a mixed monolayer presenting both the hydroquinone and tetraethylene glycol groups (1:1) in 1 M $HClO_4$ and PBS. The hydroquinone undergoes a reversible two-electron, two-proton process to give the corresponding quinone at different redox potentials in the two buffer solutions. Selective immobilization of soluble aminooxyacetic acid (0.2 M in H_2O, 2 h) to the quinone monolayer resulted in the formation of a redox-active oxime on the surface. The changes in the redox-active peaks for the cyclic voltammogram in 1 M $HClO_4$ correspond to the oxidation and reduction of the oxime monolayer (Figure 2B). The oxime is stable in 1 M $HClO_4$ and can be cycled between the oxidized and reduced forms at least 50 times with no change in the voltammograms.

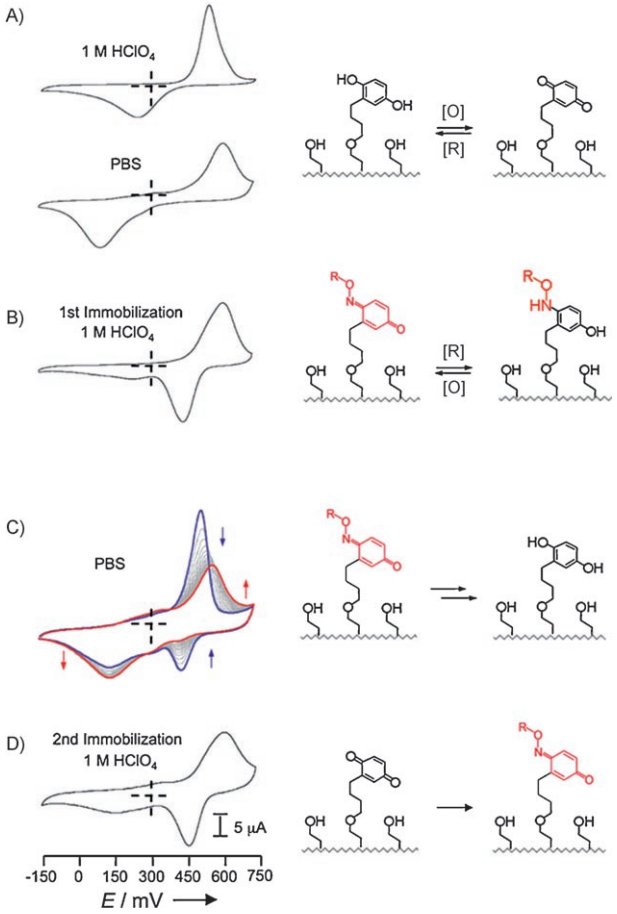


Figure 2. Electrochemical characterization of the reaction between the quinone monolayer and soluble aminooxyacetic acid by CV. A) The hydroquinone-terminated monolayer undergoes electrochemical oxidation [O] at 539 and 600 mV and reduction [R] at 322 and 95 mV in 1 M $HClO_4$ and PBS (pH 7.4), respectively. B) The quinone monolayer reacts with soluble aminooxyacetic acid to form the chemically stable oxime on the surface. The oxime undergoes reversible redox coupling at 624 and 484 mV in 1 M $HClO_4$. C) Consecutive cyclic voltammograms of the oxime monolayer in PBS show the breakdown of the oxime, release of ligand, and formation of the hydroquinone in situ. D) The resulting hydroquinone is electrochemically oxidized to the quinone. Subsequent immobilization of the monolayer with soluble aminooxyacetic acid regenerates the oxime conjugate on the surface. All voltammograms were recorded at a scan rate of 50 $mV s^{-1}$; the intersection of the crosshairs represents zero current.

When the same electrochemical experiment was repeated in PBS, consecutive voltammograms showed a decrease in peak currents for the oxime monolayer, and an increase in peak currents corresponding to the hydroquinone–quinone redox couples (Figure 2C). This result suggests that the oxime undergoes electrochemical reduction to the corresponding hydroquinone by selectively releasing the ligand under physiological conditions (pH 7). To verify that the resulting peaks are characteristics of the oxidation of the hydroquinone and reduction of the quinone, we reimmobilized soluble aminooxyacetic acid (0.2 M in water, 2 h) on the monolayer. A cyclic voltammogram showed diagnostic peaks at 624 and 484 mV in 1 M $HClO_4$, and confirmed the oxime conjugate on the surface (Figure 2D).

To determine the chemical nature of the ligand released from the surface, we synthesized a soluble oxime conjugate (**3**) by reacting benzoquinone with *n*-decanyl oxyamine. The oxime was chemically reduced in 1 M ascorbic acid. Characterization by ^{1}H NMR and IR spectroscopy identified the released ligand as *n*-decanol. This result suggests that electrochemical reduction of the oxime monolayer in PBS regenerates the hydroquinone by releasing the ligand as a hydroxy moiety. X-ray photoelectron spectroscopy of the surface after release shows no nitrogen present on the surface. We believe that nitrogen in the form of ammonia (NH_3) is also released during the electrochemical reduction, but have not yet been able to identify this species.

To further characterize the kinetics of oxime degradation in PBS, we examined the cyclic voltammograms obtained for the interfacial reaction. Figure 3 shows a plot of the normalized peak currents at 425 mV versus time for the data that correspond to the loss of oxime on the surface illustrated in Figure 2C. The data were fitted to an exponential decay [Eq. (1)] to give a pseudo-first-order rate constant of 0.01 s^{-1} in PBS, where I_t is the peak current at time t, I_0 is the initial peak current, and I_f is the residual nonfaradaic current.

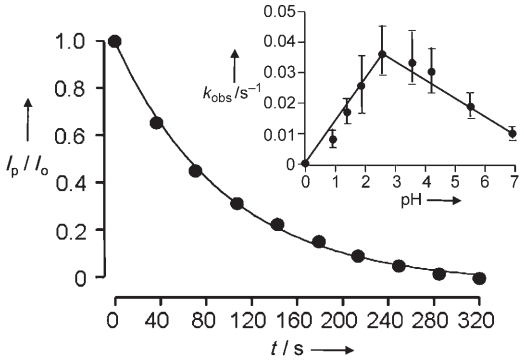


Figure 3. Normalized peak currents (I_p/I_o) at 425 mV versus time for the consecutive cyclic voltammograms shown in Figure 2C. Inset: Plot of pseudo-first-order rate constants versus pH, which shows the relative rates of oxime degradation in various buffer solutions.

$$I_t = I_f + (I_0 - I_f)\exp(-k't) \quad (1)$$

We repeated the same electrochemical experiment in various buffer solutions to determine the effect of pH on the rate of oxime degradation and therefore release from the surface. Figure 3 (inset) shows a plot of the observed first-order rate constants (k') versus pH. The data show a pH dependence on the rate of oxime degradation. Interestingly, the observed first-order rate constants increase from pH 0 to 3, and then decrease from pH 3 to 7. Although the reaction mechanism for the oxime degradation is complex and remains unclear, a break in the observed first-order rate constants at pH 3 suggests a change in the rate-determining step at this buffer pH. Note that the oxime conjugate is stable in these pH ranges and only becomes unstable, and therefore releases, upon application of a reductive potential, thus enhancing its ability for dynamic surface applications.

To demonstrate the utility of this methodology for the release of biological ligands, we used the association of FLAG antibody to a monolayer presenting surface-immobilized FLAG peptides.[27] We prepared a mixed monolayer presenting 1% hydroquinone groups and 99% tetraethylene glycol groups. A high percentage of background tetraethylene glycol density ensures that the monolayer surface completely resists nonspecific protein adsorption.[28] The hydroquinone monolayer was electrochemically oxidized at 750 mV for 10 s in PBS to give the corresponding quinone. We next immobilized an aminooxy-terminated FLAG peptide (**4**) onto the monolayer (0.1 M solution in PBS, 4 h). Association of FLAG antibody to the peptide-immobilized monolayer substrate was characterized by surface plasmon resonance (SPR) spectroscopy.

Figure 4 (top) shows the SPR sensorgram for the binding of anti-FLAG (0.1 mg mL^{-1}) onto the monolayer presenting immobilized FLAG peptides. When the peptide-functionalized monolayer substrate was electrochemically reduced at

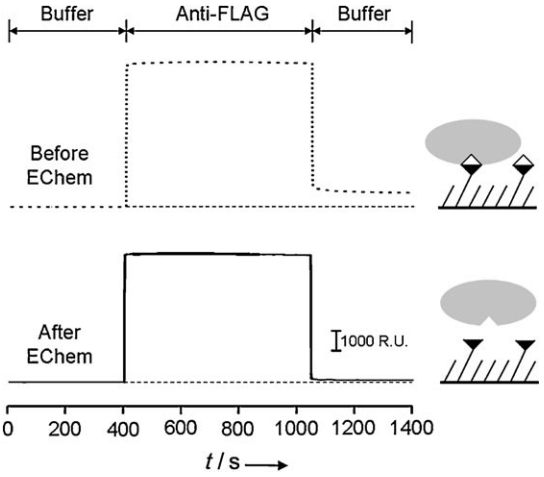


Figure 4. SPR spectroscopy demonstrating the association and release of anti-FLAG. A monolayer presenting surface-immobilized FLAG peptide through oxime conjugation before (top) and after (bottom) electrochemical (EChem) reduction, which releases the bound peptide. The 700 R.U. in anti-FLAG binding from the baseline before release of peptide shows biospecific association of the anti-FLAG. After release of the peptide no anti-FLAG is bound to the surface.

−50 mV for 5 min in PBS causing release of peptide, a SPR sensorgram showed that the anti-FLAG did not bind to the surface (Figure 4, bottom). This result confirmed that electrochemical reduction of the monolayer released the immobilized FLAG peptide ligands from the surface through oxime degradation.

We next extended this methodology to cell biological applications by releasing peptide ligands that support cell adhesion in patterns on monolayer surfaces. To prepare surfaces that can capture and release patterned cells, we introduced a photochemical strategy that permitted the selective immobilization and release of ligands in defined patterns. Monolayers presenting nitroveratryloxycarbonyl (NVOC) hydroquinone groups undergo photochemical

deprotection to reveal the hydroquinone in the selected region upon UV illumination through a photomask. This photochemical approach permits the patterning of a variety of soluble oxyamines onto the quinone monolayer.[29]

Figure 5 shows the photopatterning of attached cell culture on surface-immobilized RGD gradients, and subsequent release of the patterned cells by an electrochemical potential.[30] To demonstrate that electrochemical treatment of the monolayer is noncytotoxic to the attached cell culture, we used microcontact printing (μCp) to pattern hexadecanethiols that promote adhesion of another subset of cells through hydrophobic interactions on the same substrate.[31]

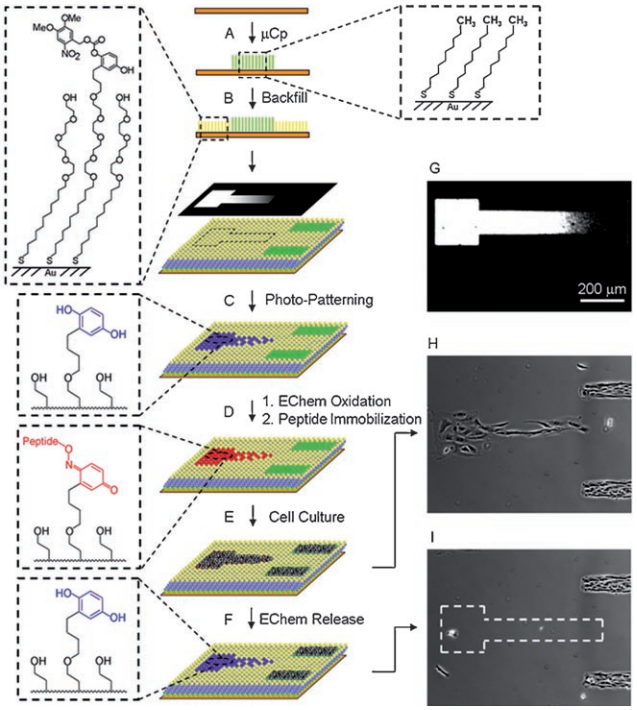


Figure 5. Electrochemical release of photopatterned cells adhered to RGD gradient SAMs. A) Hexadecanethiols were microcontact printed to generate hydrophobic line patterns on the gold-coated glass substrate. B) The remaining bare gold region was backfilled with a mixed monolayer presenting both the NVOC hydroquinone and tetraethylene glycol groups. C) UV illumination through a photomask deprotected the NVOC groups to reveal the hydroquinone in select regions on the monolayer surface. D) The substrate was oxidized to convert the hydroquinone to the corresponding quinone. Addition of soluble RGD-oxyamine installs the peptide on the quinone monolayer through oxime formation. The resulting peptide oxime conjugate alters the inert photopattern area to biospecific cell adhesive. E) Addition of fibroblasts to the monolayer substrate resulted in cells adhering to both microcontact-printed and photopatterned regions. F) Mild electrochemical reduction of the gold substrate causes selective release of cells from only the RGD-defined gradient, whereas cells attached to the hydrophobic SAMs remain adherent. G) Micrograph of a photomask with a gradient used in the preparation of the photopatterned RGD peptide ligands. H) Image showing patterned fibroblasts on a RGD gradient and on microcontact-printed line patterns. I) Electrochemical reduction of the monolayer leads to cell detachment on the gradient by release of the peptide ligand, while cells patterned on hydrophobic lines remain attached.

After the hexadecanethiols were printed in line patterns, the remaining bare gold region was backfilled with a mixed monolayer presenting 1% NVOC-protected hydroquinone (**5**) and 99% tetraethylene glycol groups. A photomask consisting of gradient patterns was placed in direct contact with the monolayer. Subsequent UV illumination of the monolayer through a photomask revealed the hydroquinone in select regions on the surface. The substrate was electrochemically oxidized and then treated with soluble RGD oxyamine (0.1 M, 4 h) to form the corresponding peptide oxime conjugate on the surface. Swiss 3T3 fibroblasts were added to the resulting substrate and attached exclusively to both the microcontact-printed region (hydrophobic and therefore cell-adhesive) and the photoactivated gradient region of the monolayer (presenting adhesive RGD peptides; Figure 5H).

To selectively release the surface-immobilized RGD ligands, a reductive potential of −50 mV was applied for 1 min in serum-free medium. Cells patterned on the gradient began to adopt a more rounded morphology and then detached from the surface. Figure 5I shows a phase-contrast image of patterned cells after the electrochemical treatment. Cells on the gradient pattern were released, whereas cells on the microcontact-printed hydrophobic lines remained attached. This result confirmed that electrochemical reduction of the monolayer released the RGD ligands in situ, and therefore caused the cells to detach from the otherwise inert surface as a result of a lack of surface-bound adhesive RGD ligands. We also showed the release of patterned cells on gradients overlapping with microcontact-printed hydrophobic regions, to demonstrate that this methodology can also be used for the spatial and temporal control of cell–cell interactions and co-cultures (see the Supporting Information).

In conclusion, we have developed a general methodology to immobilize oxyamine ligands on an electroactive quinone monolayer, and subsequently release the same ligands from the surface to regenerate the hydroquinone monolayer by electrochemical reduction. The redox activity between the quinone and oxime groups permits characterization of each step of the interfacial immobilization and release quantitatively by CV in real time. The hydroquinone surface is catalytic, can perform several rounds of immobilization and release of ligands, and also converts the oxyamine functional group to a hydroxy group by a mild electrochemical potential. This selective functional-group transformation may be used for applications ranging from solid-phase peptide synthesis and heterogeneous catalysis to chemical-based sensor amplification. Furthermore, we have extended this methodology to modulate the activity of immobilized peptide ligands to promote or inhibit the selective binding of protein. Finally, by combining this electrochemical strategy with a photochemical approach, we have demonstrated the immobilization and subsequent release of peptide ligands that mediate cell attachment in defined gradient patterns on inert surfaces. The examples demonstrated herein present a molecular level of control over ligand presentation for modulating cell behavior on model surfaces. We believe this methodology will provide a broad range of tailored substrates for new

fundamental studies in attached cell culture and applications in synthetic chemistry and biotechnology.[32]

Received: January 12, 2008
Revised: April 16, 2008
Published online: June 20, 2008

Keywords: cell adhesion · electrochemistry · heterogeneous catalysis · monolayers · redox chemistry

[1] S. R. Khetani, S. N. Bhatia, *Curr. Opin. Biotechnol.* **2006**, *17*, 524 – 531.
[2] R. Langer, D. A. Tirrell, *Nature* **2004**, *428*, 487 – 492.
[3] U. Hersel, C. Dahmen, H. Kessler, *Biomaterials* **2003**, *24*, 4385 – 4415.
[4] J. A. Hubbell, *Curr. Opin. Biotechnol.* **1999**, *10*, 123 – 129.
[5] M. Mrksich, *Chem. Soc. Rev.* **1995**, *10*, 73 – 78.
[6] G. Bao, S. Suresh, *Nat. Mater.* **2003**, *2*, 715 – 725.
[7] J. L. Tan, J. Tien, D. M. Pirone, D. S. Gray, K. Bhadriraju, C. S. Chen, *Proc. Natl. Acad. Sci. USA* **2003**, *100*, 1484 – 1489.
[8] N. Q. Balaban, U. S. Schwarz, D. Riveline, P. Goichberg, G. Tzur, I. Sabany, D. Mahalu, S. Safran, A. Bershadsky, L. Addadi, B. Geiger, *Nat. Cell Biol.* **2001**, *3*, 466 – 472.
[9] L. A. Haines, K. Rajapopal, B. Ozbas, D. A. Salick, D. J. Pochan, J. P. Schneider, *J. Am. Chem. Soc.* **2005**, *127*, 17025 – 17029.
[10] D. L. Elbert, J. A. Hubbell, *Biomacromolecules* **2001**, *2*, 430 – 441.
[11] D. L. Hern, J. A. Hubbell, *J. Biomed. Mater. Res.* **1998**, *39*, 266 – 276.
[12] X. Jiang, D. A. Bruzewicz, A. P. Wong, M. Piel, G. M. Whitesides, *Proc. Natl. Acad. Sci. USA* **2005**, *102*, 975 – 978.
[13] X. Jiang, R. Ferrigno, M. Mrksich, G. M. Whitesides, *J. Am. Chem. Soc.* **2003**, *125*, 883 – 886.
[14] T. Okano, N. Yamata, J. Sakai, Y. Sakurai, *Biomaterials* **1995**, *16*, 297 – 303.
[15] T. O. Collier, J. M. Anderson, A. Kikuchi, T. J. Okano, *J. Biomed. Mater. Res.* **2002**, *59*, 136 – 143.
[16] T. Shimizu, M. Yamato, A. Kikuchi, T. Okano, *Biomaterials* **2003**, *24*, 2309 – 2316.
[17] M. Mrksich, *Curr. Opin. Chem. Biol.* **2002**, *6*, 794 – 797.
[18] W.-S. Yeo, M. Mrksich, *Langmuir* **2006**, *22*, 10816 – 10820.
[19] W.-S. Yeo, M. Mrksich, *Adv. Mater.* **2004**, *16*, 1352 – 1356.
[20] W.-S. Yeo, C. D. Hodneland, M. Mrksich, *ChemBioChem* **2001**, *2*, 590 – 593.
[21] W.-S. Yeo, M. N. Yousaf, M. Mrksich, *J. Am. Chem. Soc.* **2003**, *125*, 14994 – 14995.
[22] M. N. Yousaf, B. T. Houseman, M. Mrksich, *Angew. Chem.* **2001**, *113*, 1127 – 1130; *Angew. Chem. Int. Ed.* **2001**, *40*, 1093 – 1096; .
[23] M. N. Yousaf, B. T. Houseman, M. Mrksich, *Proc. Natl. Acad. Sci. USA* **2001**, *98*, 5992 – 5996.
[24] a) D. K. Hoover, E.-J. Lee, E. W. L. Chan, M. N. Yousaf, *ChemBioChem* **2007**, *8*, 1920 – 1923; b) D. K. Hoover, E. W. L. Chan, M. N. Yousaf, *J. Am. Chem. Soc.* **2008**, *130*, 3280 – 3281.
[25] a) E. W. L. Chan, M. N. Yousaf, *J. Am. Chem. Soc.* **2006**, *128*, 15542 – 15546; b) E. W. L. Chan, M. N. Yousaf, *ChemPhysChem* **2007**, *8*, 1469 – 1472.
[26] T. Horn, B. B. Lee, K. A. Dill, R. N. Zuckermann, *Bioconjugate Chem.* **2004**, *15*, 428 – 435.
[27] FLAG peptide is an eight amino acid (DYKDDDDK) fusion tag commonly used for immunoaffinity chromatography: see A. Einhauer, A. J. Jungbauer, *J. Biochem. Biophys. Methods* **2001**, *49*, 455 – 465.
[28] K. L. Prime, G. M. Whitesides, *J. Am. Chem. Soc.* **1993**, *115*, 10714 – 10721.
[29] a) W. S. Dillmore, M. N. Yousaf, M. Mrksich, *Langmuir* **2004**, *20*, 7223 – 7231; b) N. P. Westcott, M. N. Yousaf, *Langmuir* **2008**, *24*, 2261 – 2265.
[30] The RGD peptide is found in the central binding domain of the extracellular matrix protein fibronectin and is known to facilitate adhesion through cell-surface integrin receptors: see E. Ruoslahti, *Annu. Rev. Cell Dev. Biol.* **1996**, *12*, 697 – 748.
[31] Polydimethylsiloxane stamps were fabricated according to the literature: see Y. Xia, J. Tien, D. Qin, G. M. Whitesides, *Langmuir* **1996**, *12*, 4033 – 4038.
[32] a) L. O. Hodgson, E. W. L. Chan, K. M. Hahn, M. N. Yousaf, *J. Am. Chem. Soc.* **2007**, *129*, 9264 – 9265; b) D. G. Barrett, M. N. Yousaf, *Angew. Chem.* **2007**, *119*, 7581 – 7583; *Angew. Chem. Int. Ed.* **2007**, *46*, 7437 – 7439; c) E. W. L. Chan, M. N. Yousaf, *Angew. Chem.* **2007**, *119*, 3955 – 3958; *Angew. Chem. Int. Ed.* **2007**, *46*, 3881 – 3884.

