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Redox-Switchable Surface for Controlling Peptide Structure

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Supporting Information

ABSTRACT: A general surface chemistry strategy is described for the development of a new switchable material. The method modulates a surface-immobilized-molecules structure by using two orthogonal "click" reactions based on Huisgen cycloaddition and oxime chemistry, where the oxime linkage is redox active and switchable. We demonstrate this strategy by developing a noninvasive, biocompatible, *in situ* surface chemistry that is able to modulate the affinity of a cell-adhesive peptide to cell integrin receptors to study dynamic cell adhesion and cell migration in real time and as a new hide-and-reveal strategy for application in new types of smart biofouling biomaterials.

Switchable materials are important for a wide range of applications in polymer science, molecular electronics, nanotechnology, and biology.[1−5] These materials contain chemical groups or linkages that can be altered with physical stimuli to produce interesting properties and dynamic functions. Important examples of recently developed stimuli-responsive materials include surfaces with electronically actuated hydrophobicity,[6] switchable hydrogels that selectively release therapeutic agents,[7] self-assembled monolayer (SAM) surfaces that employ ultraviolet light to actuate small-molecule conformation,[8] and polymers that selectively bind and release cells due to changes in swelling caused by mild temperature fluctuations.[9]

Cells live in a complex and dynamic environment and receive a multitude of extracellular input cues ranging from physical stimuli to small molecules and hormones that are then processed for a range of output signaling and cell behaviors. In particular, the extracellular matrix (ECM) environment cells occupy provides a dynamic scaffold that is able to continually remodel itself and elicits a range of cell functions. To study how cells respond to a dynamic and evolving environment, model substrates based on materials that can undergo molecular remodeling or conformational shifts in response to noninvasive triggers are required to provide novel strategies for stimulating and controlling biological functions both *in vitro* and *in vivo*.[10]

Herein a general strategy for switching an immobilized-molecules structure on a surface is described based on two orthogonal "click" reactions. We demonstrate this strategy by developing a redox-responsive chemistry that is able to switch the activity of a cell-adhesive peptide (RGD) presented to cells. The method relies on two orthogonal click reactions based on Huisgen cycloaddition and benzoquinone−oxime chemistry where the oxime linkage is redox active and switchable. This is an innovative application of molecular switches to alter small-molecule structures on surfaces via a redox-responsive orthogonal and biologically noninvasive electrochemical cue. The dynamic and switchable covalent chemistry demonstrated herein is general and may potentially be applied to hiding-and-revealing pharmaceuticals or small molecules for cell biology assays.

Electroactive SAMs on gold have been extensively characterized and developed for a range of fundamental studies of electrode processes and as a platform for biomaterial applications.[11−17] We recently introduced an electrochemical "click" methodology for immobilizing and releasing ligands to and from a surface. The method is based on the conjugation of oxyamine (OA)-terminated ligands on hydroquinone (HQ)-terminated SAMs.[18,19] The HQ group permits an electrochemical activation and click reaction to occur with OA-terminated small molecules that can be precisely monitored and actuated with cyclic voltammetry (CV). Although HQ lacks a carbonyl, it can be oxidized to the benzoquinone (BQ) form (essentially a redox activation to ketone groups) and reacted with OA to form a stable quinone−oxime linkage (Q_{ox}).[20] When Q_{ox} is reduced, cleavage of Q_{ox} releases a primary alcohol and regenerates HQ. The methodology has several inherent advantages: facile incorporation of OA into biomolecules for subsequent conjugation, an ability to conjugate and release ligands under biological conditions (pH 7.4, 37 °C), and precise electrochemical control of redox state for switchable immobilization and release.

Peptides, small molecules, carbohydrates, and oligonucleotides are routinely functionalized with both Huisgen cycloaddition and oxime conjugation chemistry (alkyne, azide, ketone, OA), are readily incorporated into many biomolecules, and have been shown to undergo mutually orthogonal chemoselective reactions.[21−26] In order to generate a switchable surface that replicates dynamic ECM functions, such as revealing adhesive proteins on connective tissue in response to injury, a bifunctional redox-active HQ molecule was synthesized containing an azide linkage for orthogonal Huisgen and oxime click ligations (Scheme 1). Huisgen azide/alkyne cycloaddition chemistry was chosen as an orthogonal and complementary chemoselective strategy to the benzoquinone−oxime chemistry both for the minimal steric size of the azide and alkyne moieties and for the broad and facile applicability of the chemical coupling methodology.[27]

A synthetic scheme was designed to incorporate azide and HQ functional groups in close proximity into an alkanethiol. The synthesis is modular and provides a general framework for future modification of the system for alternative applications (e.g., SAMs on indium tin oxide, replacement of azide). The synthesis of **7**, an alkanethiol containing a tetra(ethylene glycol) linker, a

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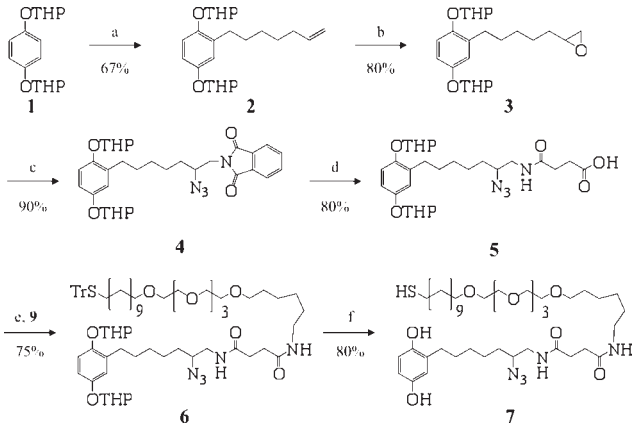
Scheme 1. Synthesis of a Redox-Active Bifunctional and Orthoganol Molecule (7) for Huisgen and Oxime Click Conjugation[a]



[a] Reagents and conditions: (a) i, *tert*-butyllithium, THF, 0 °C; ii, 1-bromoheptene, HMPA, 3 h. (b) MCPBA, bicarbonate, dichloromethane (DCM), 3 h, 25 °C. (c) i, bicarbonate, phthalimide, 100 °C, 24 h; ii, mesityl chloride, 10 equiv of triethylamine, THF, 1 h 25 °C; iii, DMF, sodium azide, 110 °C, 3 days. (d) i, hydrazine, methanol, 50 °C, 12 h; ii, THF, succinic anhydride, 30 min, 25 °C. (e) DMF, HBTU, diisopropylethylamine, **9**, 2 h, 25 °C. (f) DCM, trifluoroacetic acid, triethylsilane, 30 min, 25 °C.

secondary azide (for Huisgen cycloaddition), and a HQ is described. A branching point for placing the two reactive functional groups, HQ and azide, was introduced by ortho-lithiation of **1** and reaction with 1-bromoheptene to produce olefin **2**. Epoxidation of olefin **2** with *m*-chloroperoxybenzoic acid (MCPBA) afforded **3** in high yield. Nucleophilic ring opening of the epoxide with sodium phthalimide, transformation of the resulting secondary alcohol to a mesylate, and substitution with sodium azide provided **4** in a 90% yield. Reaction of **4** with excess hydrazine in methanol yielded the free amine, which was reacted with succinic anhydride **5** and then coupled to **9** with HBTU and DIEA to yield **6**. Subsequent deprotection in DCM/TFA/TIPS yielded the target product **7** in near-quantitative yield. By creating a SAM containing two clickable functional handles (7) via electrochemically controlled click oxime chemistry and Huisgen cycloaddition, a general strategy for immobilizing and switchably controlling ligand presentation on SAM surfaces was achieved (Figure 1).[27]

Small molecules and peptides were synthesized to contain both OA and alkyne coupling groups to react with the dual chemoselective HQ and azide functional groups presented on the SAM surface. This strategy allows for the change of structure of bound molecules on a surface by the general immobilization, activation, conjugation, and release of the oxime bond.

Mixed SAMs composed of **7** and **8** were constructed by immersing freshly evaporated gold surfaces in 1 mM total mixed alkanethiol solution in ethanol (Scheme 2). To confirm the availability of both the HQ and azide functionalities to react selectively with their chemical partners, model alkyne- and OA-terminated ligands were immobilized onto the SAM surface. Ethynyl ferrocene was immobilized to a SAM surface composed of 20 mol % **7** and 80 mol % **8** with standard ascorbic acid/$CuSO_4$ Huisgen cycloaddition conditions and characterized by CV (Supporting Information (SI) Figure S1).[28]

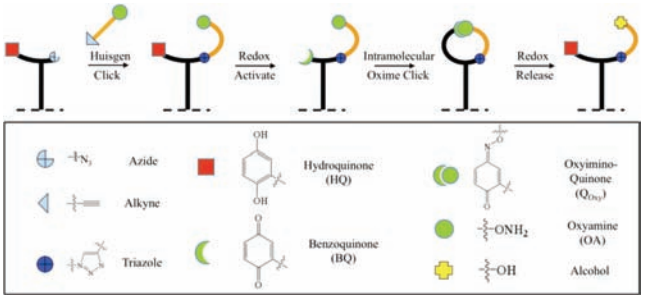
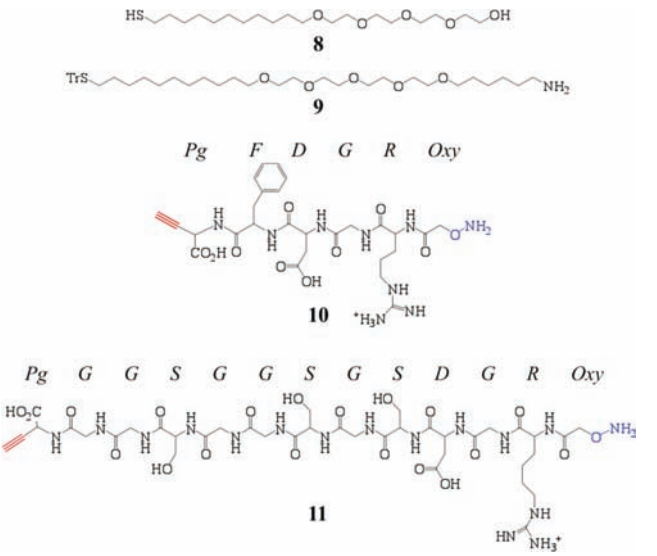


Figure 1. Illustration of a methodology switching small-molecule conformations on surfaces. Small molecules containing both alkyne and oxyamine functional groups are first immobilized onto the surface via Huisgen 1,3-cycloaddition. Conversion of HQ to BQ occurs via a mild oxidative potential. BQ then reacts with the OA group on the ligand for an intramolecular cyclization via oxime chemistry. Upon application of a reducing potential to the substrate, the oxime bond is cleaved, regenerating the HQ and producing the linearized ligand. Each step is monitored and controlled by electrochemistry since each molecule (HQ, BQ, Q_{ox}) has a distinct and diagnostic CV signature.

Scheme 2. Bifunctional Peptides and Small Molecules Used for Characterizing Surfaces and for Generating Smart Bio-interfacial Surfaces for Studies of Cell Behavior



Further confirmation of ferrocene immobilization was obtained by MALDI mass spectrometry of the surface (SI, Figure S3). In a separate experiment, BQ was reacted with methoxylamine to form Q_{ox}, which upon application of −100 mV reducing potential regenerated the HQ with release of methanol (Figure S1). To confirm the mutual compatibility of the azide and HQ, solution-phase experiments demonstrating two-step immobilization, activation, conjugation, and release were performed and monitored by UV−vis spectroscopy. The addition of $CuSO_4$ to the solution after Huisgen cycloaddition oxidized the HQ group to BQ, promoting cyclization via oxime bond formation (SI, Figure S2). Reduction of Q_{ox} to HQ resulted in release of the alcohol product.

For cell adhesion studies we used the well-known cell-adhesive RGD peptide as a dynamic ligand. The affinity and specificity of RGD peptides for cell integrin receptors are strongly dependent on the conformation of the peptide backbone, and many studies

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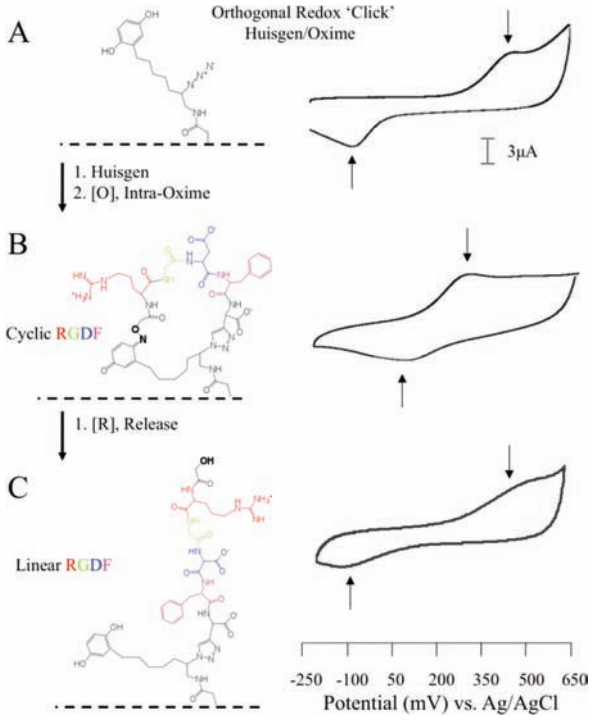

Figure 2. Electrochemical monitoring and control of cyclization and linearization of an immobilized cell-adhesive RGD peptide containing terminal alkyne and OA functional groups. (A) The bifunctional SAM contains a HQ group and an azide group. HQ-to-BQ interconversion can be monitored by CV (−95 mV, 405 mV). (B) Upon reaction with the bifunctional RGD peptide **10** followed by oxidation [O], a cyclic RGD is formed. The cyclic RGD is redox active and shows diagnostic Q_{ox} redox peaks (55 mV, 310 mV). (C) The cyclic RGD can then be linearized by cleavage of the Q_{ox} by application of a mild reductive [R] potential at physiological conditions (pH 7.2, 37 °C). The cleaved linear peptide regenerates HQ, which has distinct HQ-to-BQ CV peaks.

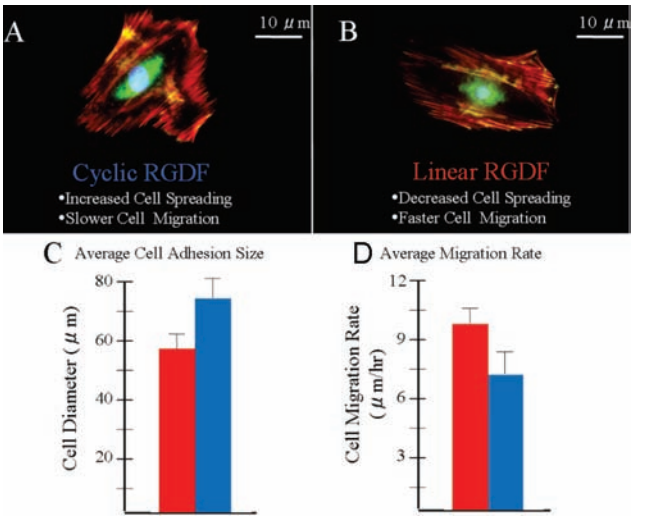

Figure 3. Switchable control of the RGDF peptide structure for cell-adhesion and migration studies. (A,B) Representative images of stained cells on the linear and cyclic RGDF switchable surfaces. (C,D) Cell spreading and migration rates can be controlled by dynamic switching of RGD peptide structure. On the more adhesive cyclic RGD surface (blue), cells have larger diameters and slower migration rates. On the less adhesive linear RGD surface (red), cells have smaller diameters and migrate faster. The control of RGD peptide structure *in situ* in the presence of adhered cells allows for real-time study of cell behavior.

have examined and compared linear and cyclic RGD structures in solution through competitive binding assays.[29,30]

Cyclic RGD peptides, in general, have a higher binding affinity (nM) for cell integrins than linear RGD peptides (μM). To demonstrate the immobilization of bifunctional dynamic RGD peptides onto a SAM surface, a Pth-RGDF-Pg peptide was synthesized by standard solid-phase peptide synthesis and installed via Huisgen cycloaddition to the surface. Although the phthalimide protecting group for the OA is not necessary for immobilization, it enables better control over oxidative activation of the HQ. MALDI analysis of the surface showed complete conversion of azide-terminated monolayer to peptide-functionalized monolayer (SI, Figure 4). Phthalimide deprotection in dilute hydrazine in methanol provided the free OA group. The surface was then placed in PBS (pH 7.2) solution and oxidized at 650 mV to promote conjugation via oxime formation. Reduction at −250 mV resulted in oxime release (Figure 2 and SI, Figure S2).

To determine whether on-surface *in situ* peptide conformation changes would affect cell behavior, cells were seeded on 1% dynamic peptide surfaces initially presenting cyclic RGDF. An *in situ* electrochemical activation in the presence of cells switched the peptide structure to the linear RGDF, and cell behavior was observed via time-lapse microscopy (Figure 3 and Scheme 2). On cyclized RGDF, cells were observed to bind more strongly to the surface, as evidenced by a 25% greater surface adhesion diameter, and showed a 35% slower rate of migration compared to cells on linear RGDF. These observations are consistent with cell behavior observed on separate linear and cyclic RGD surfaces in previous work.[31]

Staining of cell cytoskeleton and focal adhesions with phalloidin and anti-vinculin showed that cells on cyclic RGDF peptide exhibited a more intricately networked system of actin filaments and more focal adhesions than cells on linear RGDF. As controls, a scrambled GRFD peptide was immobilized, in which cells did not adhere or bound very weakly and did not migrate on the surface due to an inability of integrin receptors to recognize the peptide sequence.

Finally, to demonstrate the broad applicability of the methodology as a dynamic biomaterial, an elongated RGDS peptide (**11**) was immobilized onto the surface (Scheme 2). This strategy allows for the substrate to become selectively adhesive and nonadhesive to cell attachment via an electrochemical hide-and-reveal. Spacer residues (Gly-Gly-Ser-Gly-Gly-Ser-Gly) were included to separate the biospecific RGDS sequence from the C-terminal end of the peptide, so that upon oxidation, the N-terminal OA functional group reacts to form Q_{ox}, thereby shielding the RGDS sequence from cell integrin receptors (Figure 4). When the oxime is cleaved, the RGDS sequence is released and becomes available to bind cell integrin receptors. Cells did not adhere to cyclized peptide on surfaces presenting 0.1% peptide **11** but did adhere to surfaces presenting the revealed linear peptide, as shown in Figure 4. Importantly, the cell adhesion profile is dependent on ligand density and shows that cells are able to differentiate between hidden and revealed RGDS peptides. These results indicate future potential of this method to perform biomolecule hide-and-reveal for applications ranging from fundamental studies of cell adhesion and dynamic microarrays to wound healing and metastasis assays.

We have designed a redox-switchable surface that is able to alter small-molecule structure through a redox-responsive linkage. The strategy is based on the immobilization of a

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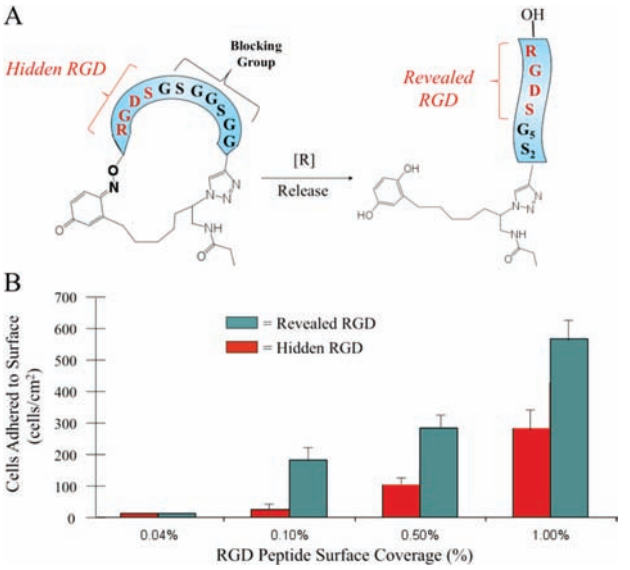

Figure 4. Dynamic hide-and-reveal ligand strategy for controlling substrate adhesiveness for biospecific cell attachment. (A) A cyclized RGDS peptide containing a blocking sequence does not permit cell adhesion (left). Reductive cleavage of the oxime bond reveals the adhesive RGDS peptide for cell adhesion (right). (B) Cell adhesion profiles on surfaces presenting hidden and revealed peptides. Cells were able to differentiate between hidden and revealed RGDS peptides more effectively at lower surface density.

molecule to a surface through an electroactive oxime linkage and a Huisgen cycloaddition reaction. By application of a mild, noninvasive electrochemical potential, we show that a surface presenting cell-adhesive RGD peptides can change their affinity (from linear to cyclic RGD) for cell integrin receptors, which modulates cell spreading and cell migration behavior. We further show how this method can be used to make materials adhesive and nonadhesive to cells by a hide-and-reveal ligand approach. The methodology is efficient and may be applicable to eliciting molecular transformations for a wide range of biological small molecules. Furthermore, it may be useful for physical/mechanical studies that require switchable surfaces. By synthesizing bifunctional molecules, a variety of dynamic ECM mimics may be possible for stimulating diverse cell functions, especially tissue remodeling to direct organism development.[32] Future experiments will apply this strategy to nanoparticles for the redox-state-dependent delivery and release of therapeutics and imaging probes *in vitro* and *in vivo*.

■ ASSOCIATED CONTENT

Supporting Information. Synthesis of **9** and spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

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