DEVELOPMENT OF THE ENGINEERED BIOLOGICAL MODEL SYSTEMS AND CHEMOSELECTIVE REDOX RESPONSIVE LIGATION (CRRL)

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A dissertation submitted to the faculty of the University of North Carolina at Chapel Hill in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Chemistry.

Chapel Hill
2010

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ABSTRACT

SUNGJIN PARK: DEVELOPMENT OF THE ENGINEERED BIOLOGICAL MODEL SYSTEMS AND CHEMOSELECTIVE REDOX RESPONSIVE LIGATION (CRRL)

(Under the direction of Dr. Muhammad N. Yousaf)

Biological model substrates were developed and engineered for cell adhesion and migration studies. The substrates are based on hydroquinone-presenting (HQ) self-assembled monolayers on gold surfaces that can be electrochemically activated for ligand immobilization. Combined with tetra(ethylene glycol)-terminated alkanethiol, the substrates secure biospecific interactions between cell adhesion receptors and ligands, as well as provide the ability to characterize the amount of immobilized ligand. In chapter 2, these model surfaces were used to study the biological role of decoupled PHSRN, found in the 9th type III domain of fibronectin when it is presented to Swiss 3T3 albino mouse fibroblasts. We found that PHSRN-integrin interactions enhanced lamellipodia protrusions by increasing Rac1 activity, which is associated with cross-talk between adhesion receptors and soluble factors. These novel biological model systems were further extended toward the development of a new platform based on reacting soluble ketone- or aldehyde-tethered ligands to surface-bound oxyamine-containing alkanethiols to generate a covalent oxime linkage to the surface in chapter 3. By photoprotecting the oxyamine group with nitroveratryloxycarbonyl chloride and then selectively deprotecting through microfiche film, bioligands and fibroblast cells could be immobilized in patterns and gradients. Expanding on the HQ platform in chapters 1 and 2, a general methodology to immobilize and release oxyamine ligands on an
electroactive quinone monolayer by electrochemical reduction was reported in chapter 4. The
HQ surface is catalytic, can perform several rounds of immobilization and release of ligands, and also converts the oxyamine functional group to a hydroxyl group by a mild
electrochemical potential. In chapter 5, a general chemoselective redox responsive ligation
and release strategy for molecular conjugation and cleavage was introduced. The HQ is
converted to benzoquinone to form stable quinone oxime with oxyamine-tethered ligands
that can be further cleaved by reduction, yielding aminophenol and hydroxyl-terminated
ligand. The conjugation and cleavage reactions are controlled by mild chemical or
electrochemical redox signals and can be performed at physiological conditions (pH 7.4, 37
°C) without the use of a catalyst.
ACKNOWLEDGEMENT

I would like to thank my advisor Muhammad Yousaf for guiding me through my Ph.D. course. His training and encouragement broadened my perspective over many diverse areas of science. I also would like to thank my committee members for their support and advice.

I will never forget the beginning of graduate school and time spent with Dr. Diana Hoover and Dr. Devin Barrett. We arrived at UNC and were together for a long voyage in the same small boat. I would like to extend my appreciation toward Dr. Eun-joo Lee for becoming a good friend to my family and me and also thank Abby and Nathan for being cake officers and good friends in need.

I am grateful to Professor Daewon Sohn in the chemistry department at Hanyang University for his continued support and advice. Thank you PPCL members for supporting me and providing exceptional humor. Also, thank you Hanyang Lions for being my spiritual home.

My five years in Chapel Hill became more colorful with the love and friendship of many good people I have met here along my journey. I would like to thank Soyeon, Sungkyu, Chansun, Jinwoo, Jaeho and Doohyun for the wonderful memories experienced in Chapel Hill. I also thank my dear friends, Taekwon, Yongdae and Woohee and departed friend, Juhyun for extending comfort and inspiration to me for a long time. I always feel loved by such good people, and they made my life in Chapel Hill even more memorable.
I would also like to show appreciation to my family in Korea and Japan for their support and love. I especially thank my parents for their teaching to live meaningfully and for the joy of being loved. I would like to thank my family in Japan as well for making me feel cared about all the time.

I truly appreciate my beloved wife, Mizumi Morohashi and daughter, Lena A. Park for being the greatest support for me during the most troublesome time. Meeting those two ladies were the two most amazing experiences in my life. The smiles and laughter in my family strengthen me and encourage me to progress and moved forward.
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Chapter I

INTRODUCTION TO THE CELL ADHESION AND NOVEL BIOLOGICAL MODEL SYSTEM USING SELF-ASSEMBLED MONOLAYERS (SAMS)
1.1 CELL ADHESION TO EXTRA-CELLULAR MATRIX

Most mammalian cells bind adhesion molecules to survive, migrate, proliferate, and differentiate\textsuperscript{1-3}. The cellular environment is mixture of many kinds of biomolecules that are secreted and deposited by various types of cells; collectively named the extracellular matrix (ECM). Cell adhesion onto the surface is mediated by cellular receptors such as integrins and syndecans engaging cell binding domains of ECM molecules. The interactions between the integrins and cell-binding molecules initiate co-localization of integrins, leading to recruitment of intracellular proteins at its cytoplasmic domain\textsuperscript{4}. The adhesion structures then transmit the adhesion mediated signals into the cells through various kinases and GTPases which are important for various biological consequences\textsuperscript{5,6}. The adhesion-mediated signals have high impact on various biological process that span survival, morphogenesis, differentiation, migration, proliferation and tissue formation. The extracellular matrix around the cell is not static; instead, cells actively change the composition and activity of adhesion molecules by degrading, secreting and stretching it\textsuperscript{6}.

Current model systems (Petri dishes covered with proteins) have been successful in providing biologists tremendous amount of important information about these adhesion mediated biological consequences. However, in order to fully understand adhesion mediated biological processes in vivo, it is essential to have a more sophisticated model system which can closely mimic the cell adhesion environment. For example, current model systems are not optimized to mimic dynamic and non-homogeneous nature of the ECM. Furthermore, most of biological consequences are result of converged stimulations from adhesion and other soluble factor receptors. Well-defined control over cell adhesion will become a facile
tool to solve such intricately linked processes by quantifying contributions from each pathway as well as study the mechanism of cross-talk between them. Therefore, we developed a novel biological model system with facile, real-time and well-defined control over cell adhesion conditions, which can expand our knowledge of biology.

1.1.1 Integrins

Integrins are major family of cell-surface-adhesion receptors, which are composed of two subunits (α and β) forming non-covalently associated heterodimers. Both subunits are type I transmembrane glycoprotein with relatively large extracellular domain and small intracellular domain. Until now, 18 α and 8 β subunit genes have been reported forming 24 different integrins (Figure 1.1)\textsuperscript{7,8}. The heterodimers bind to overlapping range of extracellular matrix molecules such as fibronectin, laminin, collagen, vitronectin or fibrinogen and cell surface counter-receptors such as cadherin, the Ig-superfamily proteins intercellular adhesion molecule–1 (ICAM-1) or vascular cell adhesion molecule–1 (VCAM-1) providing mechanical support for adhesion\textsuperscript{9-11}.

1.1.2 Adhesion Complex

Four kinds of adhesion structures have been discovered in mammalian cells including focal complexes, focal adhesions, fibrillar adhesions and 3D-matrix adhesions. Initially, cells make transient small contact structure at the leading edge or periphery of cell migration as a focal complex\textsuperscript{12}. If the focal complex is stabilized and matured, it progressively becomes a focal adhesion then fibrillar adhesion.
The cell-matrix adhesion complexes are dynamic bi-directional signal transmitting nexii as well as mechanical conduits. The bi-directional controls span all the steps of formation and disassembly of the adhesion structures; activation of integrins, engagement of activated integrin and ECM molecules, co-localization of engaged integrins, and disengagement of integrins. The spatially and temporally regulated assembly and disassembly of adhesion structures composed of multiple proteins are formed at the cytoplasmic tail of integrins (Figure 1.2).

**Figure 1.1** Some of the 24 mammalian integrins for different adhesion molecules are depicted. Each of them has specific and non-redundant functions. Not only do the integrins have specific target for interactions, the knock out experiments showed distinct biological phenomena supports their unique roles⁷,⁸.
In more detailed explanation, talin, one cytoplasmic component, binds to the integrin tail and increases affinity of the receptor to specific ECM ligands by reorganizing the ectodomain. Currently it is believed, talin interacts with cytoplasmic tail of beta integrin to disrupt the self-inhibitory interaction between α and β integrins. Talin mediates the initial connection between the ECM and cytoskeleton, which help transfer mechanical signals to stabilize and recruit other protein components. When the mechanical forces generated from ECM or internal actomyosin contraction is applied on the ECM-integrin-cytoskeleton complex, a focal complex matures into a focal adhesion connected to bundles of actin fibers called stress fibers\textsuperscript{13,14}. Then, the mature focal adhesion complex further recruits cytoplasmic components related to downstream signaling propagation. These signaling events are surface ligand dependent.
Figure 1.2 The major signal pathways and components are depicted for focal adhesion complexes. Many biological consequences on cell behaviors mediated by cell adhesion are often in concert with G protein coupled or kinase receptors for soluble factors. The link between signaling and receptors happen at the sub-membranous adhesion complex beneath the clustered complex.

1.1.2.1. Focal Adhesion Kinase and Src-family Kinases

Focal adhesion kinase (FAK) acts as a scaffold for phosphorylation mediated signaling generated at the adhesion complex. FAK has several domains for interaction with specific partners; 1. FERM domain for cytoplasmic tail of integrin and growth factor receptors, 2. Focal adhesion targeting (FAT) domain for Paxillin and 3. others region for SH2 and SH3 domain. FAK is non-receptor tyrosine kinase, which responds to the clustering of integrins by autophosphorylation. Once phosphorylated, the domain is now available to the kinases containing SH2 domains, like Src kinases, which then become activated and phosphorylate FAK to increase its kinase activity. The activated FAK (FAK-Src complex) is then able to interact with more proteins having SH2 and SH3 domains. These proteins are important in subsequent kinase and GTPase signaling for cytoskeletal organization, proliferation, survival, conversion of adhesion structure, motility, bi-directional mechanotransduction and gene expression.\(^{14}\)

1.1.2.2 Integrin-Linked Kinases (ILKs)

Integrin-Linked Kinases, a serine-threonine kinase, are signaling and scaffolding proteins, which are found in adhesion structures\(^ {15}\). The ILK, associated with β integrin cytoplasmic domain, regulates cell proliferation, survival, migration and Phosphoinoditide 3-kinase (PI3 kinase) -dependent signal transduction\(^ {16}\). It has been shown that the interaction
between ILKs and PINCH, paxillin, and parvin is important in regulation of cell adhesion and migration\(^1\). The ILK contains a N-terminal domain (ankyrin-repeat) for PINCH proteins and C-terminal domain (kinase domain) for parvin proteins, paxillin and β integrin tail\(^1\). The heterotrimeric complex of ILK, PINCH and parvin (paxillin binding protein) acts as a hub for integrin signaling network, similar to FAK.

### 1.1.2.3 Paxillin

Paxillin, with no kinase activity, is also signal scaffold protein for signal transduction. It assembles into the adhesion complex at early stage and recruits more components. Paxillin has numerous phosphorylation target sites for extra control for protein-protein interactions. It also has many protein-protein interaction modules such as leucin-rich repeat, proline-rich region and LIM domain for further interactions with kinases (FAK, ILK and Src), phosphatases (PTP-PEST), structural proteins (vinculin and parvins) and regulator and effector of Rho GTPases (CrkII-DOCK180-ELMO complex)\(^1\).

### 1.1.2.4 Vinculin

The vinculin is a structural protein, which connects cytoskeleton and the adhesion complex. It interacts with talin, F-actin, paxillin, VASP and Arp2/3, which regulate actin polymerization and the structure of adhesion complex\(^1\).

### 1.2 SENSING BIOLOGICAL CONTEXT: INTEGRIN-MEDIATED SIGNALING PATHWAYS
The engagement of integrins to ECM molecules and subsequent co-localization is related to various signal transduction associated with other receptors (e.g., G protein-coupled receptor and receptor kinases) controlling proliferation, apoptosis/survival, shape, polarity, motility, differentiation and transcription. It has been well known that cells have anchorage dependent response to soluble growth factors such as EGF, PDGF, LPA and thrombin, etc. which are essential for survival and proliferation. These integrin-mediated growth factor signals are well characterized for normal cells; blocking apoptosis via PI3-kinase and Akt and stimulate cell cycle progression via ERK and cyclin D1\textsuperscript{19,20}. For this, FAK may act as a signal converging point since the kinase can be activated by soluble growth factors and integrin co-localization\textsuperscript{20}. In addition, integrin-dependently activated FAK can recruit several adaptor proteins, which activate downstream signaling mediators previously implicated in growth control, including Src, Ras, and Raf\textsuperscript{21}.

1.2.1 Cross-talk With Receptor Tyrosine Kinase at the Adhesion Complex.

FAK may act as a mediator for cross-talk between integrin and receptor tyrosine kinase. Several experiments showed the adhesion complexes include not only integrins but also growth hormone receptors. In more detail, Plopper et al. used microbeads which were modified by minimum cell binding ligand of fibronectin, Arg-Gly-Asp (RGD), to induce integrin activation and formation of adhesion complex which included receptors for basic fibroblast growth factor (bFGF)\textsuperscript{22,23}. Similarly, Miyamoto et al. showed that receptors for platelet-derived growth factor (PDGF) and epidermal growth factor (EGF) are also incorporated in the adhesion complex\textsuperscript{24}. The recruitment of growth factor receptors results in
the activation of the growth factor receptors and accumulation of downstream signal components and eventually increment of mitogen response.

1.2.2 Cross-talk at the level of mitogen-activated protein kinases (MAPKs) cascade pathways: Parallel and independent controls by integrin and growth factor receptors.

Mitogen-activated protein kinases (MAPKs) are serine/threonine-specific protein kinases which respond to external signals, such as mitogens, osmotic stress and heat shock to regulate various cellular activities; gene expression, mitosis, differentiation, proliferation and survival/apoptosis.

The MAPKs are regulated by phosphorylation cascade, which consists of 3 kinases. External signal activates MAP kinase kinase kinase (MAPKKK, MAP3K), which activates MAP kinase kinase (MAPKK, MAP2K) and MAP kinase (MAPK). This MAPK cascade not only conveys an upper signal to downstream effectors but also amplifies and coordinates with other signals. The cascade diversifies the response pattern, which make it an important downstream cross-talk mediator for cell surface receptors.

Both integrins and growth factor receptors are involved in control of MAPK cascade, sometimes, by separate and different mechanisms. It is also known that the activation of MAPK by both receptors synergistically act on the activation of MAPK pathway together with FAK. However, the contributions of the two activation mechanisms onto the signaling pathways are very difficult because of intimate association of signaling by integrin and mitogen. Considering this, more facile and sophisticated control for the cell adhesion model surface must be used to resolve the multivariate equation.
1.2.3 Cross-Talk at Rho GTPases level

Rho GTPases, such as RhoA, Rac1 and Cdc42, are subfamily of Ras superfamily and act as a small biological switch molecule (~20kDa). They are essential upstream regulators in control of various cellular behaviors, such as cell adhesion, migration, gene expression, actin polymerization and cell polarization. The activated GTPase (GTP bound form) can interact with various signaling components and convey signal to downstream effectors. When the bound GTP is hydrolyzed to GDP, the GTPases are inactivated. The guanine nucleotide exchange factors (GEFs) move equilibrium to GTP bound status of the GTPases (‘on’ status) by facilitating the exchange of hydrolyzed GDP with GTP; while GTPase activating factor (GAP) facilitate the hydrolysis of GTP to GDP turning off the molecular switch.

1.2.3.1 Cdc42

Cdc42 conveys external signals from integrin, growth hormone reactor and G-coupled protein receptors (GPCR) to various effectors. Cdc42 is inhibited in the complex with RhoGDI until it is released from the Cdc42-RhoGDI complex by Cdc42-GDI kinase. Cdc42 can then be localized to the membrane and interact with SCAR and WASP regulating cytoskeletal organization and actin polymerization, MAPKs regulating cell growth, survival, apoptosis and differentiation, or PAK1/MLCK/Myosin to activate myosin.

Cdc42 also has role in regulation of cell behaviors like polarization by affecting the microtubule assembly and gene transcription. It had been shown that inhibition of Cdc42 in macrophage blocks its chemotactic ability, while maintaining its ability to migrate.
1.2.3.2 Rac1

Rac1 is most well studied among the Rac proteins, Rho GTPases subfamily (Rac1-4). Rac1 also can convey signals from integrin, growth factor receptors and GPCRs to the downstream effector molecules. Similar to Cdc42, Rac1 needs to be released from Rac1-GDI complex by Rac1-GDI kinase and localized to membrane by isoprenylation\textsuperscript{34-36}. It has been shown that Rac1 regulates the cell protrusions. Miroinjection experiments showed that constitutively active Rac1 increased membrane ruffling and lamellipodia extentions. Among three other Rho GTPases, Rac1 is the primary Rho-GTPase responsible for regulating the cytoskeletal remodeling involved with lamellipodia dynamics\textsuperscript{37-39}. There are two distinct pathways to activate Rac1 from external stimui; PI 3-kinase and PI(3,4,5)P\textsuperscript{3}\textsuperscript{40} and p130Cas/CrkII/DOCK180 pathways\textsuperscript{41,42}.

\textit{PI 3-kinase and PI(3,4,5)P3}. PI 3-kinase pathway has been shown to activate Rac1. The increased intracellular concentration of Phosphatidylinositol (3,4,5)-trisphosphate (PIP3) by microinjecting constitutently activated PIP2 kinase lead to vigorous lamellipodia protrusions and membrane ruffles which mediated by up-regulation of GTP bound Rac1\textsuperscript{40}. This is thought to be localization and translocation effect at the membrane for the GEFs having plecktrin domain (PH domain). All Dbl family of GEFs have PH domain, which can interact to PIP3 lipid at the membranes.

\textit{p130Cas/CrkII/DOCK180 pathway}. DOCK180 is a GEF, which can act as a complex with two other proteins (p130CAS, CrkII) to catalyze the exchange of the GDP to GTP for Rac proteins. It was shown that the complex can activate Rac and promotion of cell migration\textsuperscript{41,}}
1.2.3.3 RhoA

RhoA is a member of the Ras homology family of small GTPases. RhoA has been well characterized as important biological switch in regulation for the polymerization and rearrangement of actin cytoskeleton. However, further research has revealed more functions such as myosin chain elongation, gene expression, cell morphology decision, and proliferation\(^{43,44}\). Even though there are many stimuli to activate RhoA, such as growth factors, cytokines, adhesion molecules, and hormones, G-Protein coupled receptors (GPCR) are a major activator for RhoA. For example, when ligands are bound to IGF1/2 and IGF1R (insulin-like growth factor I receptor), a complex is formed with Leukemia-associated RhoGEF-12 (LARG), which then activate RhoA\(^{45-47}\).

1.3 GTPASES AND CELL BEHAVIORS: MIGRATION

In section 1-2, 3 representative levels of cross-talk between adhesion and soluble factors, which are essential for basic biological activities of cells, were shown: the adhesion complex, MAPK cascade, and GTPases. In this section, what effectors exist under GTPases and how the GTPases regulate biological phenomenon, especially migration and protrusions, will be described. Those behaviors associated with GTPases activities were observed for the cells on our engineered cell adhesion model surfaces.
Figure 1.3 The coordinated series of steps for migration are depicted. Initially, Cdc42 regulates the direction of migration. Then, Rac takes part in actin polymerization and formation of adhesion structure at the front of cell leading edge. Finally, Rho regulates actomyosin contraction in cell body to promote movement, turnover of adhesion complex and rear retraction.

1.3.1 Lamellipodia Extensions

Lamellipodia are cellular feet-like protrusions essential for cell migration. They seek a new adhesion site to help propel the cell forward. The requirement of Rac proteins for lamellipodia extensions and cell migration had been well supported by research. They demonstrated that cell could not extend lamellipodia and migrate responding to adhesion to ECM molecules, growth factors and cytokines when the Rac is mutated<sup>48-50</sup>.

It was shown that the formation of lamellipodia accompanies formation of branching out of actin filament flank using actin-nucleating activity of Arp2/3 complex<sup>51</sup> (Figure 1.3).
First, Rac interacts with IRSp53, which has SH3 domain for further interaction with WAVE, which then activates Arp2/3 complex. The IRSp53 has separate domains to interact with Cdc42 and Dia1, a target protein of Rho, enabling Cdc42 and Rho induced Rac-dependent lamellipodia extensions.

Rac can also stimulate actin polymerization by removing cap protein at the (+) side of barbed end on actin fiber. Rac is also involved in the stability of actin fibers by LIM kinase mediated inhibition of coflin. The Rac/Cdc42 target protein PAK activates LIM kinase, which then inactivates coflin, a protein that promotes actin depolymerization. These studies all indicate that Rac is a key regulator of migration because of its ability to stimulate lamellipodium extension.

### 1.3.2 Turnover of Adhesion Complex

Rac is needed for the formation of small adhesion structures (focal contacts) at the periphery of lamellipodia of migrating cells that are important for creating new attachment during migration. This focal contact is transient and Rho should act to mature the structure. Therefore, an increase in Rho activity leads to stronger adhesion decreasing the migration rate. Rac induces the disassembly of adhesion structures directly through PAK pathway or indirectly through antagonizing effect on Rho. PAK interacts with component of focal complex/adhesion, like paxillin, to be co-localized into the adhesion structure, and then induces disassembly by phosphorylating its effectors. Since the disassembly by Rac-mediated PAK is dependent on integrin interactions, it is thought that certain critical amount of integrin engagement may trigger PAK induced disassembly of the adhesion complex. Aside from Rac mediated disassembly, FAK and Src can signal for disassembly of the
structures. Furthermore, it was shown that microtubules can target the adhesion structures and transport proteins for disassembly.

### 1.3.3 Cell Body Contractions

Cell body contraction is required for cell migration to drag the cell body toward a newly formed cellular grip while facilitating detachment from previous adhesion sites. This cellular contraction is driven by contraction of actomyosin, a complex between actin and myosin and has been shown that Rho is important in inducing the actomyosin contraction. Rho regulates the phosphorylation of myosin light chain (MLC) enabling contraction by activating ROCK (Rho-kinases), directly phosphorylating MLC and inactivating MLC phosphatase. Similar to PAK, as described previous section, ROCK also activates LIMK to inactivate cofilin, leading to the accumulation of actin fibers. Rho is also related to activation of Dia, which interacts directly with Src kinases for its effect on stress fibers. As previously mentioned, the Dia proteins can interact with IRPsp53 and interact with WAVE to induce actin polymerization.

### 1.3.4 Filopodia Extension

Filopodia are actin-rich protrusions from plasma membrane, which form new adhesions for migration as well as antennae for cells to probe environment around them. The functions for filopodia are important in cell migration, neurite outgrowth and wound healing. Cdc42 initiates the actin polymerization by interacting with WASP, which activates Arp2/3 complex (Figure 1.3). RIF (Rho in Filopodia) can induce filopodia protrusions by activating Dia2 (diaphanous-related formin-2). Dia2 promotes nucleation and elongation of
linear F-actin to make longer filopodia than Cdc42 induced filopodia\textsuperscript{79}.

**Figure 1.4** A schematic view of actin polymerization, rearrangement, and upstream regulator GTPases are depicted: Rac controls lamellipodia and focal complex formation; Rho controls stress finer bundles and focal adhesion development; Cdc42 controls filopodia and focal complex formation. The lamellipodia formation is dependent on branching of new actin fibers by Arp2/3 complex. Rac activates WASP or WAVEs then they activate Arp2/3 complex. Cdc42 induces actin polymerization by either direct interaction with WASP or IRSp53 Tyr kinase – Arp2/3 pathway. Cdc42 and Rac also induce actin polymerization by activating PAK and LIMK, which phosphorylates cofilin inhibiting depolymerization of actin fibers. Rho activates MLCK which phosphorylates the light chain of myosin enabling actomyosin contraction and stress fiber formation. This mechanical stress imposed on the adhesion complex stimulates maturation to a focal adhesion.
1.4 FIBRONECTIN

Fibronectin (FN) usually exist as a dimer of identical subunits (~250kD) with a disulfide bond at near C terminus. Each FN homodimer consists of 3 different types of repeating units (FN I, FN II and FN III); 12 FN I, 2 FN II and 15~17 FN III repeating units.

Twenty different types of FN are generated from a single gene by alternative splicing\textsuperscript{80, 81}. This ability to generate multiple variants of FN is important for producing tissue specific FNs having specific cell-adhesive, ligand binding, and solubility properties. For example, 2 different classes of FNs exist based on solubility; cellular FN which (cFN) is less soluble and plasma FN (pFN), a more soluble form. The isoforms of FN are important mechanism in tailoring the ECM environment around cells in spatio-temporal manner by modulating which isoforms are present.

FN, not only interacts with many ECM molecules, but it interacts with at least 12 integrins, acting as main linker between the ECM and intracellular cytoskeleton. The minimum cell binding domain is located at 10\textsuperscript{th} FN III as 3 amino acid sequence (RGD)\textsuperscript{81}. PHSRN at 9\textsuperscript{th} FN III was also characterized as a synergistic cell binding site for $\alpha_5\beta_1$\textsuperscript{81}. The $\alpha_5\beta_1$ integrin can also interact with N-terminus of FN conveying distinct signals from RGD-integrin interactions. Integrins other than $\alpha_5\beta_1$ were reported to interact with FN. The $\alpha_4\beta_1$ integrins are reported to interact with REDV, LDV, IDAPS, KLDAPT and EDGIHEL sequence, $\alpha_4\beta_7$ can interact with REDV and LDV, and $\alpha_9\beta_1$ integrins can interact with EDGIHEL\textsuperscript{82}. FN also interacts with other ECM molecules. FN has two heparan sulfate proteoglycan interacting domains near C- and N- terminus, two domains for collagen...
interaction in FN I 6–9 and FN II 1–2, and two fibrin interacting domains (Fibrin I and Fibrin II)\textsuperscript{83}.

1.5 Dynamic Cell Environment

Cells actively modify their environment by secreting enzymes, cell-binding molecules, as well as controlling bi-directional mechanical tension across the adhesion complex. For example, lysyl oxidase is a cell-derived enzyme, which cross-links elastin and collagen associated with fibroblasts.\textsuperscript{84,85} The cross-linking is essential for forming an insoluble collagen, and elastin fibers control matrix porosity and normal mammalian development.

The adhesion complex is bi-directional molecular clutch, which modulates mechanical tension across the cell membrane.\textsuperscript{86} For example, the actomyosin driven cell migration transfers endogenous tension caused by actomyosin contraction to the ECM engaged adhesion complex. This stretching process increases exogenous tension of cell binding molecules such as fibronectin, and reveals hidden cross-linking sites where other ECM molecules can associate.\textsuperscript{87,88} Furthermore, cells actively remodel the ECM environment by secreting matrix metalloproteinases (MMPs) responding to stimuli like injury. The activity of MMPs is tightly controlled under normal conditions, which is important for development, morphogenesis, tissue repair, and remodelling.\textsuperscript{89} Many kinds of MMPs including Collagenases, Gelatinases, Stromelysins and Matrilysin exist. Among them, collagenase uncoils the triple helix of collagen fibril, so that susceptible proteolysis
sites are exposed increasing porosity of ECM meshworks.\textsuperscript{90} However, MMPs activity increases in the case of several diseases, which rapidly remodels the local ECM.

\section*{1.6 SAMs on Gold as a Platform for Cell Biology}

Self-assembled monolayers (SAMs) are monolayer films of molecules that form spontaneously on solid surfaces. This spontaneous organization is driven by thermodynamically favorable interactions between the molecules, surface, as well as inter-molecular forces.

Among many kinds of SAMs, alkanethiolates on gold have been studied extensively because of several inherent advantages. The gold-thiol bond forms quickly and gold resists oxidation well compared to the other noble metal based SAMs. Thiols are synthetically flexible to allow for the creation of a variety of surfaces. Specifically, SAMs of oligo (ethylene glycol) are inert to non-specific adsorption of proteins and the bond is stable for weeks when in contact with cell culture media. Also, many characterization methods are available for SAMs on gold, such TEM and SPR.\textsuperscript{91,92} These advantages of SAMs on gold allow for the control of the interfacial physical properties to study fundamental aspects of SAMs\textsuperscript{93,94}. Many efforts to characterize the properties of alkanethiolate SAMs on gold prove the monolayer is well ordered with well-oriented terminal groups and the kinetics of film formation follow the Langmuir isotherm.

\subsection*{1.6.1 Chemisorption of Alkanethiols onto the Gold Surface}
Several X-ray photoelectron spectroscopy (XPS) studies showed that alkanethiolates are formed after adsorption onto the gold surface\textsuperscript{95, 96}. IR and Raman spectroscopy could not observe the S-H stretching vibration either\textsuperscript{97, 99}. Laser desorption Fourier transform mass spectroscopy also found out that significant amount of thiolate negative ions was desorbed from the surface\textsuperscript{100, 101}.

SAM formation can be considered formally as oxidative addition of the S-H bond to the gold surface, then followed by a reductive elimination of the hydrogen:

$$\text{RSH + Au}(0)n \rightarrow \text{RS}^-\text{Au}^+\cdot\text{Au}(0)n-1 + 1/2 \text{H}_2$$

Bond energies for each component of equations are RS-H (87 kcal mol\(^{-1}\)), H-H (104 kcal mol\(^{-1}\)), and RS-Au (40 kcal mol\(^{-1}\)). The net energy for adsorption of alkanethiolates on gold is ~5 kcal mol\(^{-1}\). Therefore, the reductive removal of hydrogen atoms at the surface yielding H\(_2\) molecule is the most important exothermic step in the overall reaction\textsuperscript{102}.

1.6.2 SAM Formation on the Gold Surface Follows Langmuir Isotherm

The kinetics of SAM formation on the gold surface were extensively characterized by ellipsometry, XPS, surface acoustic waves, second harmonic generation and low-energy electron diffraction (LEED) to show it follows Langmuir adsorption model. Two kinetic steps are involved in formation of SAMs; the quick adsorption of molecules onto the surface, followed by the slow crystallization process through motion and extra adsorption of molecules. Typically, a well-ordered SAMs can take days to form and its surface density is ~4.5 \times 10^{14} \text{molecules/cm}^2\textsuperscript{103}. 
1.6.3 Structure of Monolayer on the Gold Surface (111)

Electron diffraction experiments as well as scanning tunneling microscopy (STM) showed the monolayers of alkanethiols on a gold lattice (111) form \( (\sqrt{3} \times \sqrt{3})R30^\circ \) overlayer structure\(^{104-106}\) (Figure 1.5). The distance between thiolate groups is 4.99 Å while the closest distance between alkyl chains induced by van der Waals is 4.24 Å. The tilting of alkyl chain maximizes the van der Waals interactions between two chains\(^{107}\).

![Diagram of monolayer on gold surface]

**Figure 1.5** A hexagonal coverage of alkyl thios on Au(111) surface. The alkyl chains are close-packed by Van der Waals interactions. The difference between thiol-thiol distance and Van der Waals distance tilt the chain approximately 30° from the normal to the surface to maximize the interaction.

1.6.4 SAMs on a Gold Surface as a Novel Biological Models System

With unique advantages as a biological model system, SAMs have been applied to study various aspects of cell adhesion and migration\(^{108,109}\). The unprecedented control at the molecular level was harnessed to mimic the dynamic ECM, where the presentation,
composition and distribution of cell binding ligands are constantly changing.

Liu et al. showed that RGD, a cell-binding peptide, presented over non-fouling SAMs could be hidden by illuminating light on to an azobenzene SAM\textsuperscript{110}. The azobenzene adjacent to RGD peptide switched between its diastereomeric E and Z isomers; 450-490 nm for E configuration and 340-380 nm for Z configuration. The Z configuration of the azobenzene hid the RGD peptide into the inert layer of poly(ethylene glycol) (PEG), losing cellular accessibility\textsuperscript{110}. This system allowed them to switch cellular adhesion on the surface by illuminating light of different wavelengths. Yeo et al. introduced similar dynamic surface where cell adhesion is switched by releasing the cell-binding molecule, RGD\textsuperscript{111}. They harnessed electroactivity of hydroquinone and its instant formation of cyclized lactone upon electrochemical reduction, releasing previously propionic acylated peptide tethered to the quinone group. Other approaches using various molecular controls have been used to create a dynamic surface\textsuperscript{112-114}.

The most common surface patterning technique, micro-contact printing (µCP), allowed the patterning of alkanethiolates on gold. To perform the procedure, elastomeric stamps inked with a hydrophobic alkanethiol were gently pressed onto the gold surface to generate a hydrophobic domains, which were later backfilled by non-fouling molecules (TEG terminated alkanethiol). Then, the hydrophobic domains were selectively modified to allow cell adhesion in pattern\textsuperscript{115}.

Maduram et al. applied this method to observe cellular responses to different geometric cues in 2D tissue\textsuperscript{116}. Similarly, Xia et al. introduced nanopatterns of Fn to show corresponding cellular responses of intracellular signaling (Rac) and focal adhesions formation\textsuperscript{117}. Luo et al. showed the effect of geometry and cell culture size on the rate of
stem cell differentiation\textsuperscript{118}. Hoover et al. created nano arrays of RGD peptides with dip-pen nanolithography (DPN) to show the correlation of ligand binding affinity and asymmetric distribution for cellular orientation\textsuperscript{119, 120}.

There have been many efforts to create surface ligand gradients on SAMs to study haptotaxis. Chan et al. introduced gradient of RGD peptide by photodeprotection through a gradient mask to show cellular preference of ligand density as well as directed migration along the axis to the higher density of RGD. Lamb et al. further introduced larger scale gradients by \( \mu \)CP\textsuperscript{121, 122}. This method harnessed percolation of alkanethiols from PDMS microfluidic channels followed by subsequent stamping onto the gold substrate.

### 1.7 BIO-ORTHOGONAL CONJUGATION

Countless biological molecules and ions exist in the cytoplasm, interacting for a number of different functions; activating or deactivating counterparts, becoming a complex to converge and diverge signals, and manipulating different biopolymers. Methodologies for transport characterization, localization, and biological changes for certain components \textit{in vivo} are important since they often act differently from \textit{in vitro}. In other words, target biomolecules need to be tagged with a probe, which can be monitored non-invasively in a living cell.

Without a doubt, genetic tagging with a fluorescence probe (GFP and its variants) to a target protein has been most the widely used methodology\textsuperscript{123-126}. However, genetic tagging has its limitations. It cannot be applied to other kinds of biomolecules, such as carbohydrates,
nucleic acids and lipids. In order to overcome these problems, bio-orthogonal chemistry has emerged as general tagging method for non-protein biomolecules. Bio-orthogonal reactions must be invisible to biological functional groups (bio-orthogonality), mild and occur close to physiological conditions for cell survival (aqueous, pH7.4, 37°C), robust, fast, and high yielding at low concentrations, non-cytotoxic, and products must be stable in the cell. A few reactions satisfy these tough criteria, and the representative reactions will be described below.

1.7.1 Staudinger Reaction

Hermann Staudinger reported in 1919 that azides react with triphenylphosphine under mild conditions to produce an aza-ylide intermediate. In the presence of water, the intermediate is subsequently hydrolyzed to yield two products: an amine and corresponding phosphine oxide\(^{127, 128}\). Noting the bio-orthogonality of the azide and phosphine group, and the mild reaction conditions, the Bertozzi group utilized this reaction as a ligation strategy\(^{129}\). They installed an intramolecular electrophile trap next to phosphine group, so the result of the reaction is amide and phosphine oxide formation, known as a Straudinger ligation (Figure 1.6). Even though the oxidation of phosphine by air or metabolic enzymes was a potential problem, this ligation methodology was successful in fulfilling several needs for bio-imaging\(^{130-132}\). Soon after this report, several efforts to improve the ligation methodology by remove the auxiliary phosphine from final ligation products were undertaken. In this traceless ligation, the phosphine group is linked to nucleophile, which is then acylated by the carboxyl group of molecule A. If the azide-containing molecule B is introduced, the aza-ylide intermediate attacks acyl group and hydrolyzes to produce amide and phosphine oxide.
without side products\textsuperscript{133-135}. Although the traceless Staudinger ligation has not been used in living cells, it has been applied to immobilization of molecules onto the surfaces\textsuperscript{136}.

\textbf{1.7.2 1,3-Dipolar Cycloaddition}

Huisgen first reported the scope of reactions between activated alkyne and azide as a \textit{1,3 dipole}\textsuperscript{137}. The activation was achieved by adding an electron-withdrawing group, like an ester, to the alkyne resulting in $\alpha,\beta$-unsaturated carbonyl structure. If there was no activation of alkyne, the reaction mixture needed to be boiled to obtain the conjugated product. The Sharpless group reported that with the use of the catalyst, Cu(I), the rate of cycloaddition increases $\sim 10^6$ fold\textsuperscript{138,139} (Figure 1.7A). This Cu(I) catalyzed 1,3-dipolar cycloaddition reaction, also known as ‘click’ chemistry, happens at physiological conditions in the presence of cell lysate. In spite of the impressive thermodynamic stability of the conjugated product (30-35 kcal/mol), and fast reaction rate in physiological conditions, these reactions could not be used in cells because it lacks bio-orthogonality (activated alkyne can be a Michael receptor for various biological functional group) and the cytotoxicity of metal catalyst.
The Bertozzi group developed catalyst free 1,3-dipolar cycloaddition by introducing ring strain to the alkyne, which is now bio-orthogonal but activated enough for conjugation in physiological conditions without catalyst\textsuperscript{140} (Figure 1.7B). The alkyne located in the 8 membered ring has \(~18\) kcal/mol strain which can be released when it forms intermediate of the [2+4] cycloaddition. Further activation of the alkyne by adding electron-withdrawing group (Fluorine), increased the second order reaction rate from \(1.2 \times 10^{-3} \text{M}^{-1}\text{s}^{-1}\) to \(7.6 \times 10^{-2} \text{M}^{-1}\text{s}^{-1}\) \textsuperscript{141}. These strategies were successfully applied to various in vivo imaging.
Figure 1.7 (A) 1,3-Dipolar cycloaddition (B) Copper-free click chemistry

1.7.3 Bioorthogonal Peptide Sequence

Tsien and coworkers have developed short amino acid sequence which act as a bio-orthogonal tagging target for special chemical moiety\textsuperscript{142, 143}. They introduced 6 amino acid sequence containing 4 cysteins (CCXXCC, where XX can be any amino acid but proline and glycine yields optimal results) that react with biarsenicals (Figure 1.8). The peptide reporter is expressed by genetic modification and a cell-permeable chemical reporter is introduced later to form covalent bond under physiological conditions. The ethanedithiol substituents
protect the arsenic group from reacting with single thiols in vivo. This methodology has been applied as a supplement to GFP strategies, because GFP labeling sometimes perturbs proteins due to its size\(^{144,145}\). This methodology was further applied to protein tracking using FlAsH and ReAsH, staining for electron microscopy\(^{146}\), attaching small molecule to protein\(^{147-150}\), and labeling with small-molecule binding peptide\(^{151-153}\).

**Figure 1.8** Bio-orthogonal peptide sequence for chemoselective conjugation
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FUNCTIONALLY DECOUPLED RGD AND PHSRN ON ENGINEERED SUBSTRATES GENERATE DIFFERENT ENVIRONMENTAL CONTEXT IN CONVEYING SOLUBLE FACTORS SIGNALS
2.1 INTRODUCTION

Many cell biological consequences, such as survival, proliferation, differentiation, polarization, migration, apoptosis, and gene expressions require stimuli from upstream adhesion receptors like integrins and growth hormone receptors\(^1,2\). When integrins engage ligands, they co-localize and eventually form adhesion complexes at the cytoplasmic tail that act as a bidirectional signal-mediating centers\(^3,4\). Since the adhesion structure often involves growth factor receptors, the complex is an important signal convergence point and the adhesion receptor is thought to provide environmental context for the cells translating other extracellular signals\(^5,6,7,8\). The focal adhesion complex, MAPK cascade and GTPases often contribute to converging signaling levels through independent mechanisms, but it is not trivial to assign their contributions without a proper biological model system.

The interaction of cells with extra-cellular matrix (ECM) proteins such as fibronectin (FN) play a crucial role in various biological processes, including cell survival, proliferation, migration, differentiation, and apoptosis\(^9-11\). Many cell types bind to specific central cell binding domains of FN spanning from the 8\(^{th}\) to the 10\(^{th}\) type III domain (FIII\(_{8-10}\)). Pierschbacher and Ruoslahti reported that the short peptide sequence Gly-Arg-Gly-Asp-Ser (GRGDS) in the FIII\(_{10}\) domain is a minimum cell adhesion site\(^12\). However, FIII\(_9\) acts as a synergy site for interaction with integrins \(\alpha_5\beta_1\) and \(\alpha_{IIb}\beta_3\) to mediate cell adhesion and is necessary for obtaining maximal cell-binding activity. Later, PHSRN was isolated as the minimum synergistic sequence enhancing cell adhesion, although it was believed to be incapable of supporting attachment alone\(^13,14\).

However, recent work has shown that PHSRN may have more functions than initially believed. Garcia \textit{et al.} have suggested a model of \(\alpha_5\beta_1\)-mediated adhesion of K562
erythroleukemia cell\textsuperscript{15}. The integrins first bind to RGD, which modulates the integrin orientation, conformation, and activates them. Once activated, the integrins then bind PHSRN. Direct measurement of the binding force showed substantial mechanical force between $\alpha_5\beta_1$ integrins and PHSRN. Furthermore, Feng and Mrksich have reported that PHSRN can act as a weak cell binding ligand using a self-assembled monolayer (SAM)\textsuperscript{16} model system. The authors showed that several cell lines bind biospecifically to the PHSRN ligand via $\alpha_5\beta_1$ integrins. Livant \textit{et al.} have shown that acetylated and amidated soluble PHSRN has a surprisingly high activity in changing keratinocytes into their invasive phenotype\textsuperscript{17}. These results strongly suggested that PHSRN may interact with cellular receptors and have its own biological effects outside of synergistic RGD mediated cell adhesion.

In this paper, we compared cellular phenotypes mediated by functionally decoupled RGD and PHSRN using our novel model system. We engineered our model system for cell adhesion using self-assembled monolayers (SAMs) composed of 1:99 hydroquinone tethered alkanethiolate (HQ) / tetra(ethyleneglycol) alkanethiol (TEG) SAMs on gold surfaces (HQ/TEG system). With facile ligand immobilization to the chemically defined surfaces and the biospecific interactions between cell and ligands, HQ/TEG system has been successfully applied as simplified biological model system for cell adhesion, migration, intracellular nanoarchitecture formation, cell polarization, and differentiation\textsuperscript{18-22}.

Similar to the previous reports of Feng and Mrksich\textsuperscript{16}, PHSRN demonstrated its potency as a cell binding ligand. We further compared cell phenotypes between two different ligand presenting surfaces, studying cell migration rate, membrane ruffling, and cytoskeleton arrangement. These results show PHSRN may induce different downstream signaling from
that of RGD, which was proved by a GTPase pull-down assay with or without serum stimulus. Furthermore, we could modulate such cellular phenotypes of 3T3 fibroblasts by controlling the ratio of two ligands on the surface. These results demonstrate the HQ/TEG system can be a valuable addition to the tool set of cell biology research, especially in studying cross-talk between adhesion and growth factor signals.

2.2 EXPERIMENTAL

2.2.1 Substrate Preparation and Cell Culture Condition

Substrate preparation. Gold substrates were prepared by electron-beam deposition of titanium (3 nm) and then gold (12 nm) on glass microscope slides using a vacuum evaporator system with a 3kW electron beam gun (Model VE-100, Thermionics Laboratory, Inc., Port Townsend, WA). In order to form a SAM, newly coated substrates were first washed with absolute ethanol and then immersed into a 1mM ethanolic solution of alkanethiolates (1:99 HQ/TEG) for at least 12 hours. The molecules are synthesized as previously described\textsuperscript{51}. The hydroquinone-containing surfaces were oxidized by applying a potential of 750mV (vs. Ag/AgCl) for 15 seconds in a 1 M solution of HClO\textsubscript{4} using a BAS 100B/W Electrochemical Analyzer (Bioanalytical Systems, Inc., West Lafayette, IN). Ligands were immobilized by immersion into the corresponding oxyamine-peptide solution (10 mM in PBS) for 4 hours.
Cell culture. 3T3 Swiss Albino (SA) mouse fibroblasts were cultured in Dulbecco's modified Eagle's medium (Sigma) with 10 % bovine calf serum and 1 % penicillin/streptomycin (37 °C in a humidified 5% CO₂ atmosphere). In order to plate cells onto the SAM substrates, cells were removed from the culture flask with a solution of 0.05% trypsin/0.53 mM EDTA, re-suspended in serum-containing or serum-free culture medium (5,000~10,000 cells/mL), and seeded for 2 hours. The cell suspended solution was then removed and the substrates were further cultured in fresh serum-containing or serum-free media.

2.2.2 Characterization for Cell Behaviors

Fluorescence imaging and cell area measurement. For imaging and data processing, cells were fixed with 3.2 % formaldehyde in PBS and then permeated with PBS containing 0.1 % Triton X-100. A fluorescent dye mixture containing DAPI (4',6-diamidino-2-phenylindole dihydrochloride, nucleus; Sigma), phalloidin-TRITC (tetramethylrhodamine B isothiocyanate, actin; Sigma), and anti-paxillin (BD Biosciences, San Jose, CA) with a secondary fluorescently labeled antibody (Cy-2 conjugated goat anti-mouse IgG, Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). In conducting cell area measurements, images were taken at 10 different random spots of each substrates and further analyzed by cell scoring mode from Metamorph software. A Nikon Eclipse TE2000-E inverted microscope (Nikon USA, Inc., Melville, NY) was used to obtain fluorescence images.
**Migration rate determination.** 3T3 SA fibroblasts were seeded onto prepared substrates at low concentration (1,000 ~ 5,000 cell/mL) to minimize the effects of cell-cell contacts. After incubation for 2 hours in cell culturing conditions, movies were taken for 20 hours (brightfield mode). Cell displacements at each hour were tracked by Metamorph program.

**Scanning electron microscopy.** Cells were cultured 10 hours and dehydrated in a 50, 70, 95% and 100% EtOH/H₂O series followed by critical point drying. A Pd/Au alloy (Cressington 108 auto sputter coater, Cressington Scientific Instruments Ltd.) was deposited (~1 nm) onto the samples and the surfaces were imaged using a Hitachi model 2-4700 scanning electron microscope.

**GTPases activity assay.** Cells were incubated in 0.5% delipidated BSA (Sigma) or 1% serum containing media for 16 hours. After trypsinizing from the culture flask, the suspended cells were pelleted by centrifugation (1000rpm, 5min) and followed by re-suspension in corresponding cell culture media. The cells were plated onto SAMs substrates and incubated for 2 hours. The Rac1 pull-down assay was performed using assay kit purchased from Cell Biolab inc. (San Diego, CA) and carried out according to the supplier’s instruction.

### 2.3 RESULTS AND DISCUSSIONS
Recently, a novel biological model system that utilizes SAMs of HQ/TEG on a conductive surface for cell biology studies was introduced\textsuperscript{18-22}. The \( p \)-hydroquinone functional group was electrochemically converted to the corresponding quinone form, allowing immobilization of soluble aminooxy-terminated (-ONH\(_2\)) ligands to the monolayer. The quinone oxime product is also redox active and has its own unique diagnostic cyclovoltammetric (CV) peaks like hydroquinone. This control provides real-time monitoring of the ligand immobilization reaction to the surface, as well as quantification for the ligand density. It also opens up spatial control by creative patterning methods. In combination with the anti-fouling molecule TEG, known to resist nonspecific protein adsorption, this platform enables the study of biospecific interactions between ligands and cells with homogeneous activity of the ligands\textsuperscript{23-26}. For our studies, we have chosen to use SAMs composed of HQ mixed with TEG (1 : 99 = HQ : TEG) to compare biological effects of different integrin-ligand engagement (RGD and PHSRN) to cellular phenotypes. The oxyamine tethered RGD and PHSRN ligands are immobilized to the surface through the general immobilization scheme in figure 1 (as well as figure S1 in supporting material) in order to generate defined bio-specific interactions between cell adhesion receptors and the ligands.
2.3.1 Biological Activity of Ligands: Mixed RGD and PHSRN Loses Synergistic Effect.
Figure 2.2 Cell areas on different ligand presenting surfaces are measured at different time points after the cell seeding (R: RGD, N-G₆-R: GPHSRN-G6-RGDS, N+R: mixed PHSRN and RGD(1:1), N: PHSRN). Error bars are standard deviation. At least 200 measurements were performed for each data. Cells on N-R shows highest activity for cell spreading while mixed ligands (N+R) could not mimic such activity, confirms that decoupled RGD and PHSRN looses synergistic effect. PHSRN can act as a cell binding ligand but, still, its activity is lower than that of RGD.

A series of experiments were performed in order to compare biological activity of RGD, PHSRN, mixed RGD and PHSRN (RGD+PHSRN), and glycine hexamer linked RGD and PHSRN (PHSRN-G₆-RGD) by assessing cell spreading on each ligand presenting surfaces. 3T3 swiss albino fibroblasts were cultured on substrates presenting each ligand and cell area was measured at time points ranging from 10 to 65 min (Fig. 2). The glycine hexamer of PHSRN-G₆-RGD was based on the natural distance between PHSRN, which allowed higher cell adhesion activity than RGD alone²⁷. It was found that PHSRN-G₆-RGD-
presenting substrates induced the most cell-spreading. The mixed RGD and PHSRN cannot reproduce the cell spreading activity of PHSRN-G<sub>6</sub>-RGD. These results emphasize the importance of orientation and distance between the RGD and PHSRN sequences to have a synergistic effect in cell spreading. As reported by Feng and Mrksich, cells adhered and spread on the PHSRN-presenting SAM substrate<sup>16</sup>. However, cells spread slowest on the PHSRN presenting SAM, implying decoupled PHSRN is less active for inducing cell spreading compared to RGD.

2.3.2 Different Morphology and Behaviors for Cells on PHSRN- and RGD- Presenting Surfaces

*Cytoskeleton and Focal Adhesion.* In order to compare adhesion structures and actin fiber organization on two functionally decoupled ligands, cells were stained for vinculin (anti-paxillin with Cy-2, green), nucleus (DAPI, blue) and F-actin (phalloidin, red) after 10 hours of culture on RGD- and PHSRN- presenting surfaces (Figure 3). Cells adhered to RGD-presenting surface had a rigid appearance with well-developed stress fiber bundles that filled the entire cytoplasmic region of the cell body. The focal adhesions at the end and periphery of stress fibers were observed as a dot or oval like shapes. On the other hand, cells on PHSRN formed less stress fiber bundles and had less adhesion structures.
Figure 2.3  Focal adhesion and actin fiber stain images for cells after 10 hours of culture on PHSRN (left) and RGD (right) presenting SAM substrate. Cells on the PHSRN show rounder shape with less stress fibers while cells on RGD has well developed stress fibers and rigid shape. Correspondingly, cells on the RGD has many adhesion complexes while cells on PHSRN have less number of definite adhesion structures (Green: paxillin, Red: actin, Blue: Nucleus; bar indicates 20µm).

Migration Rate. In order to compare migration rate of cells on both surfaces, live-cell images were taken to evaluate cellular migration rates on RGD- and PHSRN- surfaces. The 15h movies were started 2 hours after cell seeding onto each substrate in the presence of serum (10 % bovine calf serum/DMEM). The tracking of cellular migration by Metamorph software was used to calculate the cellular migration rate on both substrates; 15.1 µm/h for cells on PHSRN and 6.7 µm/h for cells on RGD surface (Figure. 4B). The images taken every 20 min showed the different migration rates resulted from different protrusion dynamics for lamellipodia and filopodia. Figure 4A shows that cells on RGD spread well and produced fewer protrusions, resulting in a rigid shape and relatively slow migration rate.
However, cells on PHSRN showed high protrusion dynamics, extending lamellipodia and filopodia 0.5 ~ 1 times of cell body length. These results showed the increased migration rate is related to the cellular lamellipodia protrusion dynamics on PHSRN.

**Figure 2.4** Protrusions of cells on the surface presenting PHSRN (A, up) or RGD (A, down) were recorded by live cell imaging (Images were taken every 20 minutes). Cells on PHSRN surface showed highly dynamic protrusions. (B) Cell migration rate is almost 3 times higher for cells on the PHSRN than cells on the RGD (N=43 per each substrate). (C) Soluble PHSRN enhance also enhances cell migration rate and protrusions on RGD and cyclic RGD (cRGD) presenting SAM substrates (N=40 per each measurements). This establishes that the high migration rate and dynamicity of protrusions on the PHSRN-presenting surface are not the sole consequence of weak binding affinity of PHSRN but the interaction between PHSRN and cellular receptors.

**Soluble PHSRN Increase Migration Rate.** In order to confirm that the high migration rate on PHSRN-presenting surface is not only because of weak affinity of PHSRN, soluble PHSRN
(Ac-PHSRN-NH$_2$) was added to the culture medium (10 µM in 10 % bovine calf serum / DMEM) and cell migration rates were measured (Figure. 4C). Increased protrusion and migration rates were observed for cells cultured on all ligands (a mixture of RGD and PHSRN (PHSRN+RGD), RGD, and cyclic RGD (cRGD)) except PHSRN-only surfaces.

<table>
<thead>
<tr>
<th>PHSRN Surface</th>
<th>RGD Surface</th>
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<tr>
<td><img src="image1" alt="SEM image of PHSRN surface" /></td>
<td><img src="image2" alt="SEM image of RGD surface" /></td>
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**Figure 2.5** SEM images were taken after critical temperature dried cells on PHSRN (left) or RGD (right). Cells were cultured for 10 hours on each substrate before proceeding to critical temperature drying. Peripheral and dorsal ruffling is observed from cells cultured on PHSRN-presenting surface while cells on RGD have smooth dorsal surface and trim periphery.

cRGD is known to have a higher cell binding activity than the linear RGD by 1,000-fold due to its structural similarity to the natural RGD loop located in FIII$_{10}$ of FN$^{28,29}$. Furthermore, the live-cell imaging showed that soluble PHSRN changed rigid and spread cells to rounder forms, similar to the phenotype observed for cells on PHSRN-presenting surfaces. The treatment with soluble PHSRN increased long positive protrusions and lamellipodia stretching. However, when soluble NRSHP was treated to the cell culture media (10 µM), migration rate and protrusions did not noticeably change, which emphasized the sequence specificity. Since soluble PHSRN can increase protrusion and migration rates on cRGD as
well as linear RGD in sequence specific manner, this suggests our observations of cells on PHSRN are caused by ligand effects rather than the weaker cell binding affinity of PHSRN relative to RGD. Prior reports that Ac-PHSRN-NH₂ significantly increases invasion to extracellular matrices of sea urchin embryos of keratinocytes, fibroblasts and corneal epithelial cells seem to support our data¹⁷,³⁰.

*Rufflings are Observed on PHSRN-Presenting Surface.* Cell morphology was further investigated by scanning electron microscopy (SEM) after performing critical point drying. As shown, cells on the RGD surface (Figure 5) have smooth and flat dorsal surface. The bundles of stress fibers are visible as the denser contrast areas. However, cells on PHSRN showed a rough and bulged dorsal surface with a clear distinction between cell body and lamellipodia, accompanying a significant amount of ruffling at the periphery as well as dorsal ruffling. This extensive peripheral and dorsal ruffling is a characteristic phenotype of cells with constitutively activated Rac GTPase. GTPases in eukaryote cells behave like molecular switches to control various biological processes such as adhesion, migration, polarization, proliferation, differentiation, and cell survival. In fibroblasts, Rho activation increases cell contractility, which leads to the formation of focal adhesions and actin stress fibers³¹,³². Whereas, Cdc42 and Rac activation leads to the formation of filopodia, lamellipodia, and membrane ruffles as well as focal contacts or complexes³³-³⁵.

2.3.3 Upstream Regulator Rac1 and RhoA GTPases Are Responsible for the Different Observations
To confirm the observed difference in cellular behaviors on RGD and PHSRN surfaces were related to the activities of GTPases, pull-down assays were performed for cells subjected to

![Image of pull-down assay results]

**Figure 2.6** Rac1 and RhoA pull-down assays are performed for the cells on different ligand presenting substrate. Cells were seeded and cultured 2 hours on the ligands presenting surface before proceeding to the pull-down assay. Cells (serum starved for 16 hours in 0.5% delipidated BSA/DMEM solution) were cultured in the presence (1% serum) or without serum (BSA) for 2 hours then proceeded for the assay. When serum stimulus exists, activity of Rac1 is higher for the cells on PHSRN presenting surface while activity of RhoA is higher for the cells on RGD presenting surfaces. These results are well matched to the previous results.

Each cell binding ligand with or without serum stimulus. Cells were incubated for 2 hours before proceeding with the activity assays to study sustained behaviors rather than an instant reaction to surface adhesion. Figure 6A shows the distinguished Rac1 activity changes with the presence of serum stimulation for the cells on RGD- and PHSRN- presenting surfaces.
The activity of Rac1 became higher for PHSRN surfaces and lower for RGD surfaces. Interestingly, Rac1 activity was more upregulated by adhesion to the RGD surface (without serum stimulus) compared to PHSRN surface, which was reversed when serum stimulation was added. These results suggest that the adhesion engagements to two different ligands provided diverse environmental contexts for serum-stimulated signaling, which converged at the GTPase level.

2.3.4 A New Question

Many researchers reported that the precise spatial positioning between RGD and PHSRN (30-40Å) is a critical factor in the synergistic effect in cell adhesion and focal adhesion kinase (FAK) phosphorylation\textsuperscript{36}. However, the two domains are not always ideally aligned for integrin receptor because of the flexible linker connecting them. The substratum FN binds influences its conformation. Its conformation affects many biological phenotypes such as focal adhesion assembly, cell adhesion, cell differentiation, gene expression, migration, and apoptosis\textsuperscript{37-46}. The FN adhered to different surfaces varies cellular integrin specificity, which leads to these distinct biological consequences\textsuperscript{37,46}. Even though it is unclear how exact changes in FN affect integrin specificities, Garcia et al showed that changes in orientation and distance of the flexible linkage between 9\textsuperscript{th} and 10\textsuperscript{th} type III domain of FN central cell binding domain may be the key factor effecting integrin specificities.
Furthermore, cells actively modify FN into the fibril form to obtain the full biological activity of FN. In the assembly of the FN fibril, cells contract to stretch FN and expose buried sites\textsuperscript{48}. In addition to revealing binding sites, stretching induced changes in the relative distance between them and straightened their hydrophilic loops\textsuperscript{49}. By using steered molecular dynamics simulation, Krammer et al. predicted the stretching of FN unravels the connection between the synergy site and the RGD loop, which increases the distance and relative orientation between them by 20 Å and 50°\textsuperscript{50}. Such functional decoupling of RGD and its synergy site may reduce α\textsubscript{5}β\textsubscript{1} affinity because of the strict structural requirements\textsuperscript{36}. Together with previous reports and the existence of invasin, a bacterial adhesion protein having 25-100 times more binding affinity than FN with no flexible linker within the integrin binding site\textsuperscript{51}, these results prompt a new question. Is the transient decoupling of the two domains containing RGD and PHSRN a relevant environmental cue in cellular adhesion and migration?

2.3.5 Modulation of the Observed Cellular Behaviors by Changing Compositions of Mixed Ligands on the Surface

We further showed that cellular phenotypes could be modulated by the mixed presentation of PHSRN and RGD. It was found that the migration rate
Figure 2.7 Cell migration on the surface presenting RGD and PHSRN with different ratios on 1% HQ SAM (RGD+PHSRN) are evaluated. The migration rate increases, as the ratio of PHSRN increases on the mixed ligand presenting surface (filled square). Migration rate of cell could be modulated by controlling ratio of the presented ligands using HQ/TEG model system in easy and well-defined way. The corresponding change of surface concentration of RGD didn’t show any significant change for the cell migration rate (reversed triangle); implying that the change is caused by PHSRN, not by concentration change of major cell binding ligand RGD.

increased with a higher ratio of PHSRN presented in mixed peptides (Fig. 7, filled square). Notably, migration rates were influenced by the mixed ligand effects of RGD and PHSRN rather than by the concentration change of RGD. The RGD concentration corresponding to its mixed ligand counterpart was mimicked by controlling HQ concentration of HQ/TEG yielding no noticeable changes in migration rate (Fig 7, reversed triangle). In detail, this mimcking of RGD ligand concentration on the mixed ligand surface was achieved by immersing gold substrates into the solution of HQ molecule (0.2, 0.4, 0.6, 0.8%) and TEG
**Figure 2.8** Focal adhesion and actin fiber stain images for cells cultured 10 hours on SAM substrate presenting mixed RGD and PHSRN (Green : Paxillin, Red : actin, Blue : Nucleus; bar indicates 20\(\mu\)m). When PHSRN is dominant (right up), cells show rounder shape with cortical actin while cells show rigid shape with stress fibers on RGD dominant surface (right down). These results are well matches with cell migration rate evaluation data on mixed ligand presenting surface.

molecule (99.8%, 99.6%, 99.4% and 99.2%) to prepare SAMs, followed by oxyamine tethered RGD immobilization as shown in figure 1. Correspondingly, figure 8 showed that cell morphology could be also modulated by the relative amount of presented ligands. When PHSRN was dominant (RGD:PHSRN = 2:8 and 4:6), more cells formed rounded shapes with a hollow center. On the other hand, when RGD was dominant (RGD:PHSRN = 6:4 and 8:2),
cells formed well-defined stress fiber bundles and dot like focal adhesions similar to a RGD-presenting surface.

2.4 CONCLUSION

These results show how the facile control of ligand immobilization can be applied to design a flexible and an unprecedented experimental setup. Combined with the bio-specificity of ligand presentations, this system will be valuable in deciding how the nature of adhesion (engagement of integrins to bio-ligands) will affect intracellular signal convergence, between cell adhesion and other extracellular signals like growth hormones. For example, the HQ/TEG system has already been applied to generate geometries, nano scale patterning, and ligand gradients, which are important characteristics of the ECM. These properties are not easy to mimic with current biological model systems (petri dish with protein adsorbed). The effect of adhesion conditions will add valuable pieces to the big puzzle of all adhesion-mediated signaling pathways, such as MAPK cascade, focal adhesion complexes, and GTPases. It will be easier to solve this multivariable equation with ability to control each factor independently.

In this report, we applied novel biological model substrate using the HQ/TEG system to present two functionally related ligands of FN; RGD and PHSRN. We showed that PHSRN, as previously reported\textsuperscript{16}, can act as a weak cell binding ligand for 3T3 Fibroblasts. We further showed that the interaction of cells with PHSRN promoted cell migration, through increasing activity of Rac1, an upstream regulator for lamellipodia protrusion and
migration rate. We also showed evidence the adhesion to different ligands on SAMs provided specific context to cells, when they are stimulated by other extracellular signals (Figure 9). The functional and structural coupling between two ligands in FN prompted a new question

![Diagram showing the interaction between RGD and PHSRN](image)

**Figure 2.9** Decoupled RGD and PHSRN interact with cellular receptors to generate different phenotypes of cellular behaviors. Those consequences are caused, at least partially, from cellular interpretations for soluble factors under different adhesion context. The close functional and structural coupling of the two ligands liked by flexible linker prompts new question; if the transient decoupling for two domains are relevant biological que for cellular decisions in vivo.

concerning the transient decoupling of two domains of FN and subsequent PHSRN-receptor interaction. This process occurs in vivo owing to the flexible linker between two domains, and is a relevant biological que for cellular decision pertaining adhesion and migration. This facile and tight control of the adhesion environment is promising for studies for many other difficult questions; the quantitative assessment of the contributions from various extracellular stimuli, cross-talk mechanism between adhesion and growth factor receptors, as well as the
reasons for retaining seemingly independent and redundant signalling pathways through evolution. We believe that HQ/TEG system will be a valuable addition to bolster the current biological model systems and provide a platform for further cell studies.
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AN INTERFACIAL OXIME REACTION TO IMMOBILIZE LIGANDS AND CELLS
IN PATTERNS AND GRADIENTS TO PHOTOACTIVE SURFACES
3.1 INTRODUCTION

In nature, cells exist in a highly evolving complex environment where numerous spatial input cues, such as gradients or patterns of ligands, are dynamically presented, which can then directly modulate a range of cellular behavior.\textsuperscript{1,2} In order to generate new in vitro model systems to study complex cell behavior, there has been tremendous interest in interfacing material science with cell biology. For fundamental cell biological investigations, self-assembled monolayers (SAMs) on gold have been the model system of choice for a variety of cell adhesion,\textsuperscript{3} migration,\textsuperscript{4} coculture,\textsuperscript{5} dynamics of intracellular protein activation,\textsuperscript{6} subcellular nanoarchitecture formation,\textsuperscript{7,8} cell polarization,\textsuperscript{9} and cell patterning studies.\textsuperscript{10} There are several advantages of using SAMs on gold as a platform for preparing model systems to study cell phenomena over other materials: (1) SAMs are synthetically flexible (routine organic synthesis to generate almost any alkanethiol tethered molecule); (2) many surface spectroscopies to characterize interfacial reactions or associations are available (for example, surface plasmon resonance spectroscopy, atomic force microscopy, scanning tunneling microscopy); (3) mono-layers are electroactive (can modulate and/or characterize surface properties by electrochemistry); (4) for biointerfacial studies, SAMs can become inert to nonspecific protein adsorption (incorporation of ethylene glycol-terminated alkanethiols renders the surface inert); and (5) the method is compatible with cell culture conditions (pH 7, 37 °C) and live-cell high resolution fluorescence microscopy.\textsuperscript{6} Taken together, these features allow for the generation of well-defined surfaces for biospecific interactions between ligands presented on the SAM and cell surface receptors.

To generate model surfaces to investigate complex biospecific cell phenomena, a synthetically simple method for immobilizing ligands in spatially controlled patterns and
gradients would lead to the design of more sophisticated model surfaces for interrogating cell behavior. Although several methodologies for biomolecule conjugation to SAMs on gold have been developed, including acid-base chemistry,\textsuperscript{11–13} Staudinger ligation,\textsuperscript{14–16} Click chemistry,\textsuperscript{17,18} Diels-Alder reaction,\textsuperscript{19} Michael addition,\textsuperscript{20} disulfide-thiol exchange reaction,\textsuperscript{21} as well as other methods,\textsuperscript{22} only a few are ideal as an immobilization method to generate patterns, multiple ligand patterns, and gradients on SAMs for cell biological applications. To access biospecific dynamic cell behavior, the model surface must be able to change its properties by either releasing ligands or immobilizing ligands rapidly and in the presence of attached cells.

Herein, we report an interfacial oxime reaction as a conjugation strategy to immobilize ligands and cells in patterns and gradients to a surface. We characterize the reaction by electrochemistry and demonstrate the utility of this method by patterning multiple ligands and show it is amenable to generating surfaces for biospecific studies of cell adhesion and migration. We show that the reaction between surface-bound oxyamine groups and soluble carbonyl tethered groups is kinetically well behaved, fast, and generated in high yield under physiological conditions.\textsuperscript{23,24} Also, the reaction is chemoselective in the presence of cell lysates,\textsuperscript{23,24} and the resulting oxime is chemically stable for a range of pH’s and temperatures on a gold surface. From a synthetic perspective, the ketone moiety can also be easily incorporated into peptide ligands using solid-phase peptide synthesis.\textsuperscript{26} We further demonstrate that photochemical lithography can be applied to produce patterns and gradients at the microscale on the surface.

Nitroveratryloxycarbonyl (NVOC)-oxyamine surfaces are deprotected by UV illumination through a photolithographic mask to reveal oxyamine functional groups in
spatially controlled patterns. Ketone-GRGDS peptide is chemoselectively immobilized and shown to support biospecific ligand mediated cell adhesion.

3.2 EXPERIMENTAL

All amino acids were purchased from Anaspec, Inc. (La Jolla, CA). Rink amide MBHA resin was purchased from NovaBiochem. All reagents and solvents were purchased from Aldrich and used as received. THF was distilled from sodium benzophenone under nitrogen before use. All reagents used in cell culture were obtained from Gibco BRL.

3.2.1 Synthesis

2-(Undec-10-enyloxy)isoindoline-1,3-dione (1). A solution of N-hydroxyphthalimide (9.9 g, 60.7 mmol) and sodium bicarbonate (4.95 g, 59 mmol) in DMF (100 mL) was heated to 80 °C. After the mixture turned dark brown, bromoundecene (5.26 mL, 23 mmol) was added and the reaction mixture stirred for 10 h. The reaction mixture was extracted with water and ethyl acetate. The organic phase was concentrated and purified by column chroma- tography with 1:3 ethyl acetate/hexane to afford 7.1 g (97%) of product as a colorless oil: 1H NMR (CDCl3) δ 7.82 (m, 2H), 7.72 (m, 2H), 5.80 (m, 1H), 4.95 (m, 2 H), 4.17 (t, 2 H, J = 6.8), 2.03 (m, 2H), 1.77 (m, 2 H), 1.32 (m, 2 H), 1.19-1.27 (br s., 10 H).
Figure 3.1 Synthesis of NVOC-protected oxyamine terminated alkanedisulfide (7)

Figure 3.2 Synthesis of ketone functionalized fluorescent dyes (9, 10)
**S-11-(1,3-Dioxoisindolin-2-yloxy)undecyl Ethanethioate (2).** A solution of 1 (2.0 g, 6.4 mmol) and a catalytic amount of azobis(isobutynitrile) in dry THF was heated under reflux. Upon observing N₂ bubbles, thioacetic acid (0.91 mL, 12.7 mmol) was added to the reaction mixture and the mixture was kept stirring under reflux for 6 h. The reaction mixture was concentrated and separated by column chromatography with 1:3 ethyl acetate/hexane to afford 2.1 g (84%) of product as a white solid: \(^1^H\) NMR (CDCl₃) δ 7.82 (m, 2H), 7.72 (m, 2H), 4.17 (t, 2H, J = 6.8), 2.84 (t, 2H, J = 6.8), 2.30 (s, 3H), 1.54 (brs, 4H), 1.34 (m, 2H) 1.28 (brs, 12 H).

**S-11-(Aminooxy)undecyl Ethanethioate (3).** To a solution of 2 (2.8g, 7.1mmol) in DCM (30 mL) was added hydrazine (23 mL of 1.0 M solution in THF, 23 mmol) and the mixture was stirred at room temperature for 3 h. The reaction mixture was filtered and the filtrate was concentrated and then separated by column chromatography with 1:3 ethyl acetate/hexane to afford 1.7 g (91%) of product as colorless oil: \(^1^H\) NMR (CDCl₃) δ 3.65 (t, 2H, J = 6.8), 2.84 (t, 2H, J = 6.8), 2.30 (s, 3H), 1.54 (brs, 4H), 1.28 (brs, 14 H).

**11-(Aminooxy)undecane-1-thiol (4).** To a solution of 3 (1.5 g, 5.7 mmol) in EtOH (22 mL) was added 12 N HCl (36 mmol, 3 mL) and the mixture was heated under reflux for 3 h. The reaction mixture was concentrated and dissolved in ethyl acetate. The organic phase was washed with a saturated aqueous solution of NaHCO₃, water, brine, and sodium sulfate. The organic phase was concentrated to afford 1.0 g (79%) of a yellowish oil (4.5 mmol): \(^1^H\) NMR (CDCl₃) δ 3.65 (t, 2H, J = 6.8), 2.51 (q, 2H, J = 6.8), 1.54 (m, 4H), 1.28 (br s, 14H).
2-(Decyl-disulfanyl)pyridine (5). A reaction mixture of decanethiol (1.0 g, 5.7 mmol), dipyridyl disulfide (1.9 g, 8.6 mmol), and dimethylaminopyridine (1.1 g, 8.7 mmol) in methylene chloride (30 mL) was stirred at room temperature for 8 h. The reaction mixture was concentrated and separated by column chromatography with 1:2 ethyl acetate/hexane to afford 1.5 g (92%) of product as a colorless oil (5.3 mmol): 1H NMR (CDCl3) δ 8.44 (d, 1 H, J = 3.8 Hz), 7.72 (d, 1 H, J = 3.8 Hz), 7.61 (t, 1 H, J = 2 Hz), 2.78 (t, 2 H, J = 7.4 Hz), 1.64 (m, 2 H), 1.34 (m, 2 H), 1.22 (m, 12 H), 0.85 (t, 3 H, J = 6.8 Hz).

O-(11-(Decyl-disulfanyl)undecyl)hydroxylamine (6). A reaction mixture of 4 (465 mg, 2.12 mmol) and 5 (723 mg, 2.55 mmol) in 15 mL of chloroform was heated under reflux overnight. The reaction mixture was concentrated and separated by column chromatography with 1:3 ethyl acetate/hexane to afford 450 mg (55%) of white solid: 1H NMR (CDCl3) δ 3.65 (t, 2 H, J = 6.8 Hz), 2.65 (t, 4 H, J = 7.2 Hz), 1.64 (m, 4 H), 1.52 (m, 6 H), 1.28 (brs, 24 H), 0.86 (t, 3 H, J = 6.6 Hz).

4,5-Dimethoxy-2-nitrobenzyl 11-(Decyl-disulfanyl)undecyloxycarbamate (7). A reaction mixture of 7 (150 mg, 0.38 mmol), dimethylaminopyridine (61 mg, 0.5 mmol), and 4,5-dimethoxy-2-nitrobenzyl chloroformate (140 mg, 0.5 mmol) in 10 mL of dry THF was stirred at room temperature for 12 h. The reaction mixture was then concentrated and separated by column chromatography with 1:2 ethyl acetate/hexane to afford 120 mg (87%) of product as white solid: 1H NMR (CDCl3) δ 7.70 (s, 1 H), 7.39 (s, 1 H), 7.01 (s, 1 H), 5.57 (s, 2 H), 3.96 (s, 3 H), 3.94 (s, 3 H), 3.88 (t, 2 H, J = 6.8 Hz), 2.65 (t, 4 H, J = 7.2 Hz), 1.64 (m, 6 H), 1.24 (28 H), 0.86 (t, 3 H, J = 6.6 Hz); ESI mass in CH2Cl2 calcd 630.3, found 630.3.
Synthesis of Ketone-Terminated Fluorescent Dyes (9, 10). To a mixture of rhodamine sulfonyl chloride and triethylamine (1:3 mol equiv) in DMF (just enough to dissolve the two molecules), 1,4-dioxa-8-azaspiro[4.5]decane (1.2 mol equiv) was added and stirred at room temperature for 4 h. The product is then precipitated by ethyl ether. The dark purple precipitate is treated with HCl solution (15%) and stirred for 3 h at room temperature. Triethylamine was added until the solution reached pH 7. The reaction mixture was then concentrated to afford a dark purple powder (9): ESI mass in H2O calcd 639.2, found 639.1. The same procedure was used to synthesize a fluorescein-ketone dye molecule (10): ESI mass in H2O calcd 488.1, found 488.2.

Molecule 11 was prepared as previously described.28,29

Solid-Phase Peptide Synthesis of GRGDS-ketone (12). Ketone-functionalized GRGDS peptide was synthesized using a peptide synthesizer (CS Bio) at 0.1 mmol scale. 4-Acetylbutylic acid was used without protection of the ketone group. The peptide was obtained from the resin after treating with 10 mL of TFA containing 5% water and 5% methylene chloride for 2 h under stirring and filtration. The filtrate was mixed with ethyl ether (40 mL) and the mixture was centrifuged at 3000 rpm for 15 min to obtain a white precipitate (2×). The precipitate was lyophilized overnight to obtain a white solid. ESI mass H2O calcd 714.4, found 714.3.
Figure 3.3 Synthesis of an ethylene(glycol) incorporated oxyamine terminated alkanedisulfide (13)

1-Bromo-7,10,13,16,19-pentaoxatriacont-29-ene (16). A solution of 15 (2.4 g, 6.9 mmol) in 40 mL of dry THF was stirred in a ice bath for 30 min. NaH (1.34 g, 56 mmol) was then added to the solution and stirred at room temperature for 30 min. After further stirring in room temperature for 1 h, 1,6-dibromohexane (5.6 g, 23 mmol) in 5 mL of dry THF was added. The reaction mixture was stirred at room temperature overnight and the reaction mixture was concentrated in vacuo and separated by column chromatography with EA to afford 1.6 g (45%) of product that was a transparent oil: 1H NMR (CDCl3) δ 5.75 (m, 1 H), 4.95 (m, 2 H), 3.5-3.7 (m, 18 H), 3.32-3.45 (m, 4 H), 2.0 (m, 2 H), δ 1.80-1.85 (m, 2 H), δ 1.5-1.59 (m, 4 H), δ 1.2-1.45 (br s., 16 H).
2-(7,10,13,16,19-Pentaoxatriacont-29-enyloxy)isoindoline-1,3-dione (17). A solution of N-hydroxyphthalimide (1.08 g, 9.4 mmol) and sodium bicarbonate (789 mg, 9.5 mmol) in DMF (70 mL) was heated to 80 °C. After the mixture turned dark brown, 16 (1.6 g, 3.1 mmol) was added and the reaction mixture stirred for 10 h. The reaction mixture was extracted with water and ethyl acetate. The organic phase was concentrated and purified by column chromatography with 1:1:1 ethyl acetate/ethyl ether/hexane to afford 1.3 g (45%) of product as a colorless oil: 1H NMR (CDCl3) δ (CDCl3) 7.82 (m,2H), 7.72 (m, 2H), 5.75 (m, 1H), 4.95 (m, 2H), 4.18 (t, 2 H, J = 6.8 Hz), 3.5-3.7 (m, 16 H), 3.32-3.45 (m, 4 H), 2.0 (m, 2 H), 1.80-1.85 (m, 2 H), 1.5-1.59 (m, 4 H), 1.2-1.45 (br, 16 H).

S-11-(1,3-Dioxoisoidolin-2-yloxy)undecyl Ethanethioate (18). A solution of 17 (1.3 g, 2.2 mmol) and a catalytic amount of azobis(isobutylnitrile) in dry THF was heated to reflux. After observing N2 bubbles, thioacetic acid (0.33 mL, 4.4 mmol) was added to the reaction mixture and the mixture was stirred under reflux for 6 h. The reaction mixture was concentrated and separated by column chromatography with 1:0.7:0.3 ethyl acetate/hexane/ethyl ether to afford 400 mg (71%) of product as a pale yellow oil: 1H NMR(CDCl3) δ7.82 (m, 2H), 7.72 (m, 2H), 4.37 (t, 2H, J = 6.8 Hz), 3.84 (t, 2H, J = 2 Hz), 3.63 (t, 2H, J = 2Hz), 3.50-3.62 (m, 14 H), 3.41 (t, 2 H, J = 2 Hz), 2.83 (t, 2 H, J = 6 Hz), 2.30 (s, 3 H), 1.80-1.85 (m, 2 H), 1.5-1.59 (br, 6 H), 1.2-1.45 (br, 18 H).

2-(29-Mercapo-7,10,13,16,19-pentaoxanonacosyloxy)isoindoline- 1,3-dione (19). To a solution of 18 (400 mg, 0.61 mmol) in EtOH (10 mL) was added 12 N HCl (12 mmol, 1 mL)
and the mixture was heated under reflux for 3 h. The reaction mixture was concentrated and dissolved in ethyl acetate. The organic phase was washed with a saturated aqueous solution of NaHCO₃, water, brine, and sodium sulfate. The organic phase was concentrated to afford 370 mg of product (~100%) as a transparent oil: ¹H NMR (CDCl₃) δ 7.82 (m, 2 H), 7.72 (m, 2 H), 4.17 (t, 2 H, J = 6.8 Hz), 3.67 (t, 2 H, J = 2 Hz), 3.50-3.62 (m, 16 H), 3.41 (t, 2 H, J = 2 Hz), 2.5 (q, 2 H, J = 6 Hz), 1.80-1.85 (m, 2 H), 1.5-1.59 (br, 6 H), 1.2-1.45 (br, 18 H).

2-(60-Hydroxy-7,10,13,16,19,42,45,48,51,54-decaoxa-30,31-dithi-ahexacontyloxy)isoindoline-1,3-dione (21). To a solution of 19 (370 mg, 0.60 mmol) in methylene chloride (20 mL) was added 20 (280 mg, 0.50 mmol). The reaction was stirred under reflux for 3 h. The reaction mixture was concentrated and separated by column chromatography with 1:1 ethyl acetate/ethyl ether (5% MeOH) to afford 134 mg of product (25%) as a transparent oil: ¹H NMR (CDCl₃) δ 7.82 (m, 2 H), 7.72 (m, 2 H), 4.17 (t, 2 H, J = 6.8 Hz), 3.67 (t, 2 H, J = 2 Hz), 3.50-3.65 (m, 32 H), 3.41 (t, 4 H, J = 2 Hz), 2.65 (t, 4 H, J = 6 Hz), 1.80-1.85 (m, 2 H), 1.5-1.59 (m, 10 H), 1.2-1.45 (brs., 32 H).

60-(Aminoxy)-7,10,13,16,19,42,45,48,51,54-decaoxa-30,31-dithi-ahexacontan-1-ol (13). To a solution of 21 (134 mg, 0.12 mmol) in DCM (10 mL) was added hydrazine (0.4 mL of a 1.0 M solution in THF, 0.4 mmol) and the mixture stirred at room temperature for 3 h. The reaction mixture was filtered and the filtrate was concentrated followed by washing with a saturated NH₄Cl aqueous solution. The reaction mixture was concentrated to afford 102 mg (90%) of product as a colorless oil: ¹H NMR (CDCl₃) 3.67 (t, 2 H, J = 2 Hz), 3.50-3.65 (m, 34 H), 3.41 (t, 4 H, J = 2 Hz), 2.65 (t, 4 H, J = 6 Hz), 1.80-1.85 (m, 2 H), 1.5-1.59 (m, 10 H), 1.2-1.45.
(br s., 32 H); ESI mass in CH2Cl2 calcd 873.6, found 873.6.

### 3.2.2 Other Methods

*Preparation of Monolayers.* The glass substrate (75 mm × 25 mm) from Fisher was immersed into a piranha solution (1:1 volume ratio of H2SO4 and 31.6% hydrogen peroxide) for 4 h and cleaned thoroughly with deionized water and absolute ethanol. Electron-beam deposition of Titanium (10 nm for electrochemical measurements and 3 nm for cell adhesion studies) and gold (100 nm for electrochemical measurements and 12 nm for cell patterning studies) on the glass substrate was performed consecutively. The prepared gold substrate was cut into 1.25 × 1.5 cm2 and washed with absolute ethanol. All substrates were treated with a solution of the corresponding alkanethiols in absolute ethanol (1 mM) for 12 h and then rinsed with ethanol.

*Electrochemical Measurements.* A Bioanalytical Systems CV-100W potentiostat was used for all electrochemical measurements. HClO4 solution (1 M) was used as an electrolyte solution. A platinum wire was used as the counter electrode and Ag/AgCl was used as a reference electrode. All cyclic voltammograms were scanned at 100 mV/s. The substrates of SAM molecule 6 were immersed in the FcCHO solution (150 mM) and then transferred to 1 M HClO4 solution in order to measure every 4 min. A SAM of 7 was immersed in a FcCHO solution (150 mM) after UV deprotection for 0 and 60 min.

*Photodeprotection of NVOC-oxyamine (7) Monolayers.* The photodeprotection was
performed in a saturated semicarbazide solution to quench the aldehyde containing photobyproduct. The saturated solution was purged by N2 for 30 min before reaction.

Cell Culture. Swiss Albino 3T3 cells (ATCC) were cultured in Dulbecco’s Modified Eagle Medium (Gibco) supplemented with 10% calf bovine serum and penicillin/streptomycin. The cells on the flask bottom were detached by treating with a solution of 0.05% trypsin/0.53 mM EDTA for 2 min in the incubator (37 °C in a humidified 5% CO2 atmosphere). Then serum-free media was added and cells were precipitated by centrifugation at 1000 rpm for 10 min. The precipitate was resuspended in serum-free culture medium (~50,000 cells/mL) and added onto the substrates for 2 h. The substrate was then transferred to serum-containing media.

3.3 RESULTS and DISCUSSIONS

3.3.1 Interfacial Oxime Ligation after Photodeprotection: General Scheme and Characterizations

The general scheme to photochemically unveil a reactive oxyamine surface in patterns and gradients and subsequent ketone tethered ligand immobilization is shown in Figure 3.5. A Photoprotected oxyamine alkanethiol (7) was synthesized and assembled on a gold surface (Figure 3.1). The photochemical protecting group NVOC effectively hides the oxyamine group from reacting with carbonyl-functionalized ligands. Upon UV illumination through a
microfiche mask, the NVOC group is effectively removed to reveal the oxyamine group, which can subsequently chemoselectively react with carbonyl-functionalized ligands to generate an immobilized oxime linkage to the surface.

We first showed an oxyamine alkanethiol surface can react with carbonyl-bearing
ligands (Figure 3.6A) rapidly and in high yield. To characterize the real-time kinetics of the interfacial reaction, we immobilized a redox active ferrocenecarboxaldehyde [FcCHO (8)] to a surface containing a 1:1 mixture of oxyamine alkanethiol (6) and decanethiol. The extent

![Image of Scheme](here)

**Figure 3.5** Scheme describing a chemoselective interfacial oxime reaction to immobilize ligands to a photo-active surface. Illumination with ultraviolet light (365 nm) onto a NVOC-oxyamine terminated SAM yields an oxyamine group which subsequently reacts with a ketone functionalized ligand forming a stable interfacial oxime linkage.

and kinetics of the reaction were characterized by cyclic voltammetry. The surface initially has no redox peaks, but after the addition of FeCHO (150 mM, in ethanol), a peak at 348 mV steadily increased with reaction time, indicating that ferrocene is immobilized to the surface. Analysis of the rate of increase in peak currents of the ferrocene monolayer followed pseudo-first-order kinetics, because the FeCHO was present in large excess relative to the immobilized oxyamine. The data was fit to an exponential decay (equation 3.1) to give a pseudo-first-order rate constant \( k' \) of 0.13 min\(^{-1}\), where \( I_t \) is the peak current at time \( t \) and \( I_0 \) is the initial peak current.
\[ It = I_0 (1 - \exp^{-k't}) \text{ (equation 3.1)} \]

This result shows that the oxyamine monolayer reacts chemoselectively with carbonyl containing ligands to give the corresponding oxime on the surface. To show the reaction went to completion, we compared the theoretical and calculated amount of ferrocene immobilized to a surface containing 50% oxyamine (a surface presenting molecule \( 6 \) should have 1:1 ratio of oxyamine to decanethiol, since it is a disulfide). After 60 min of reaction time, the redox peak for the ferrocene does not increase, indicating complete conversion of the surface from the oxyamine to the oxime conjugate. To determine the amount of ferrocene-oxime conjugate on the surface, we used the equation \( Q = nFA\Gamma \), where \( Q \) represents the charge and was calculated to be 6.38 \( \mu \)C, \( n \) represents the number of moles of electrons and is 1 for the redox interconversion of ferrocene and ferrocenium, \( A \) is the area of working electrode and was determined to be 1.3 \( \text{cm}^2 \), and \( F \) is the Faraday constant and is 96485 \( \text{C/mol} \). The surface coverage (\( \Gamma \)) was determined to be \( 4.7 \times 10^{13} \) molecules/cm\(^2\) and is experimentally close to the theoretical coverage of a 50% surface of \( 5.0 \times 10^{13} \) molecules/cm\(^2\). This result shows that the interfacial reaction is fast, kinetically well-behaved, and can potentially tailor surfaces with a variety of carbonyl containing ligands. As controls, the redox peaks characterizing ferrocene are not observed when the substrate is first treated with a 10% aqueous acetone solution for 3 h (acetone will react with oxyamines to generate an oxime, effectively eliminating the oxyamine group from the surface) before the addition of the FcCHO solution.
Figure 3.6 Cyclic voltammetry characterization of the kinetics of the interfacial oxime reaction and the photo-deprotection of NVOC-oxyamine SAMs. All cyclic voltammetry was performed in 1M HClO₄ solution against Ag/AgCl reference electrode with scan rate of 100mVsec⁻¹. (A) A SAM of molecule 6 was immersed in a FcCHO (8) solution (150 mM) to determine the rate of interfacial oxime reaction. (inset) The plot of time versus normalized peak current (Ip) at 348mV was fit to an exponential decay and gave a pseudo-first order rate constant of 0.13 min⁻¹. (B) A SAM of NVOC-oxamine (7) was immersed into a FcCHO solution (150 mM) after UV deprotection (365 nm) for 0 min and 60 min. After 60 minutes of illumination the FcCHO immobilized to the surface shows a cyclic voltammogram similar to a control substrate composed of molecule 6 showing no desorption or damage to the surface due to uv exposure. The photo-deprotection was performed in a saturated semicarbazide solution to quench the aldehyde functional group of the photo-byproduct.

To generate molecularly controlled complex patterns and gradients on the surface, we synthesized a photoprotected oxyamine alkanethiol (7). By illuminating the surface with ultraviolet (UV) light of 365 nm through a patterned microfiche, we demonstrate spatial unveiling of the oxyamine group and subsequent immobilization of oxime ligands. We chose NVOC as the photoprotecting group because of the high efficiency of deprotection and UV
compatibility with SAM surfaces. However, upon UV illumination the photobypoduct of the NVOC- oxyamine bears an aldehyde functionality that immediately reacts with the oxyamine surface to essentially quench its subsequent reactivity. In order to eliminate the competing aldehyde photo- tobyproduct, the photodeprotection was performed in a saturated solution of semicarbazide (14) (in H₂O and N₂ purged for 30 min before usage), which acts as an efficient carbonyl scavenger. Under these conditions, UV irradiation of a SAM composed of molecule 7 revealed the oxyamine group for subsequent reactivity. The substrate was then immersed in FcCHO solution to show ligand immobilization. For complete photodeprotection, the surface was illuminated with UV light for 60 min and revealed approximately 95% of the oxyamine functional groups relative to a control substrate made of oxyamine disulfide (6) (Figure 3.6B). These results show the oxyamine can be revealed and immobilized with ligands with no deterioration in the surface or the oxyamine molecule. No immobilization was detected by cyclic voltammetry when the substrate was treated with a 10% aqueous acetone solution for 1 h before introducing the FcCHO solution.

3.3.2 Ligand Immobilization in Pattern and Gradient

To demonstrate that the methodology can immobilize ligands in a spatially controlled manner, we illuminated the photoprotected oxyamine surface with UV light through a patterned microfiche mask and added ketone-functionalized fluorescent dyes. Figure 3.7 shows the general scheme of photopatterning and ligand immobilization. To introduce the ketone group to the fluorescent dyes (fluorescein isothiocyanate and sulforhodamine B acid chloride), we modified the structures by reacting with 1,4-dioxa- 8-azaspiro[4.5]decane,
followed by treatment with 15% HCl solution (Figure 3.2). To generate complex surface

Figure 3.7 Scheme describing the spatial unveiling of oxyamine groups for ligand immobilization. A mixed monolayer of NVOC-protected oxyamine terminated SAMs is illuminated through a patterned microfiche mask to reveal the oxyamine group for subsequent chemoselective immobilization of ketone-tethered ligands.

patterns and multiple ligand immobilizations, we illuminated the surface through a microfiche patterned mask consecutively to pattern two different fluorescent dyes. The ketone-functionalized dyes 9 and 10 are immobilized after each round of photodeprotection through a microfiche mask of pentagon and circle patterns, respectively. The fluorescence image shows that the dyes were immobilized onto only selected regions conforming to the photolithographic patterns (Figure 3.8) after two separate rounds of photodeprotection and immobilization. To create molecularly defined gradients on a surface that can immobilize a
variety of ligands, we extended the photodeprotection and capture strategy.

**Figure 3.8** Fluorescent micrographs demonstrating multiple ligand immobilization in patterns to a surface. Two consecutive rounds of photodeprotection with different photolithographic patterned masks followed by the immobilization of ketone tethered rhodamine (red (9)) (A) and fluorescein (green (10)) (B) fluorescent dyes, respectively. (C) A combined image of (A) and (B).

Figure 3.9A shows the fidelity of the microfiche gradient mask and the corresponding immobilization of fluorescent dye 9. Analysis of the fluorescent intensity profile along the axis of the dumbbell shape gradient surface shows that the relative fluorescence intensity closely corresponds with the gradient slope of the microfiche mask and confirms that the ligand is immobilized in a gradient on the surface.

### 3.3.3 Cell Culture in Pattern

To demonstrate that this strategy is capable of immobilizing biologically relevant molecules and is compatible with cell culture conditions, we selectively immobilized the GRGDS peptide as a cell-binding ligand to an otherwise inert surface. The GRGDS peptide motif is found in the extracellular matrix protein fibronectin and is known to bind to cell surface integrin receptors to promote cell attachment, migration, and proliferation. A ketone functional group was introduced to the GRGDS peptide as a 4-acetylbutylic acid without any
protecting groups during routine solid-phase peptide synthesis (12). To show a biospecific interaction between the cells and the surface, SAMs consisting of a 2:98 ratio of molecules 13 and 11 were generated and exposed to the ketone-peptide (12) for immobilization (10 mM, in PBS, 2 h) (Figure 3.8). The major component of the surface consists of the ethylene glycol group due to its ability to resist nonspecific protein adsorption and cell attachment, a critical requirement for biospecific studies of mechanistic cell adhesion and migration.

Fibroblasts were then added, and after adhering to the surface, they migrated and proliferated to eventually become a lawn of cells that are contact inhibited. To show the interaction was due only to the peptide, addition of soluble GRGD peptide (without the ketone group, 10 µM, 1 h) caused the cells to detach from the surface, indicating that the cells were adhered only via a ligand-receptor interaction. Furthermore, surfaces that were not exposed to the peptide, and therefore only present the oxyamine group and ethylene glycol groups, showed no cell attachment.

Figure 3.9 Fluorescent micrographs and line profiles of molecularly defined gradients on the SAM surface. (A) Microfiche film with patterned dumb-bell gradient (above) and the corresponding fluorescent image of the photo-deprotected and oxime immobilized ketone-rhodamine (8) dye. (B) Intensity profile along the axis of the patterned surface showing a gradient fluorescent pattern.
Figure 3.10 A phase contrast image of patterned attached cells on a SAM photo-deprotected and immobilized with ketone-GRGDS. The patterns are triangle, trapezoid and right triangle from the top row. The scale bar indicates 100µm. The micrograph was taken 3 days after cell seeding.

To attach cells in patterns biospecifically, we prepared a mixed SAM of molecule 7 and tetra(ethylene glycol)-terminated alkanethiol (11) 2:98 and immobilized ketone-GRGDS (12) (10 mM in PBS, 2 h). Ultraviolet light illumination through a patterned microfiche mask followed by peptide immobilization and cell seeding shows cells patterned on the surface (Figure 3.10). To demonstrate that the cells were attached biospecifically via their integrin receptors to RGD-presenting surfaces, we showed that the addition of soluble RGD peptides (10 µM, 1 h) detached the cells from the surface. Furthermore, when a scrambled GRD-ketone peptide was immobilized, no cells attached to the surface.
3.4 CONCLUSION

We have developed an interfacial oxime reaction that chemoselectively immobilizes ligands to photo-active surfaces in patterns and gradients. We showed the sequential immobilization of two fluorescent dyes in patterns and also the immobilization of ligands in gradients. The interfacial oxime reaction is fast, kinetically well-behaved, high yielding and can be characterized by electrochemistry. Furthermore, we extend the strategy to immobilize peptide ligands (ketone-GRGDS) for biospecific cell attachment in patterns. The strategy presented here can be used to immobilize a variety of ligands containing the ketone group and may be used for spatial and temporal control of ligands to generate dynamic surfaces to study cell behavior. This methodology is compatible with attached cell culture and provides molecular level control of patterned ligands and gradients on surfaces. This strategy can be extended to generate high-throughput arrays and in combination with high-resolution live-cell microscopy can be used to investigate a variety of cell adhesion, cell signaling and cell migration phenomena.
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Chapter IV

AN ELECTROACTIVE CATALYTIC DYNAMIC SUBSTRATE THAT IMMOBILIZES AND RELEASES PATTERNED LIGANDS, PROTEINS, AND CELLS
4.1 INTRODUCTION

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\(^1\text{–}^5\). Various approaches have been developed to promote or inhibit cell attachment by altering the macroscopic properties of the materials in situ, including mechanical stretching,\(^[6\text{–}8]\) photochemical illumination,\(^[9\text{–}11]\) electrochemical modulation,\(^[12,13]\) and thermal activation.\(^[14\text{–}16]\) 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.\(^[17\text{–}23]\) 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 photo-chemical approach, we demonstrate the immobilization and release of peptide ligands that mediate cell attachment in defined gradient patterns on inert surfaces.

4.2 RESULTS and DISCUSSIONS

4.2.1 Scheme of Electroactive Dynamic Surface

![Image](image.png)

**Figure 4.1** Interfacial reaction between soluble oxyamine and quinone-terminated SAMs. Electrochemical oxidation [O] of the mixed mono-layers 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-ONH₂) to give the redox-active oxime conjugate on the surface. The oxime is chemically stable and undergoes reversible redox coupling in HClO₄ (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.

Our approach is based on a redox-active hydroquinone-terminated SAM that can be electrochemically oxidized to the corresponding quinone (Figure 4.1). The resulting quinone 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 HClO4, pH 0) with the ligand still covalently bound. However, electrochemical reduction of the monolayer at a higher pH value (phosphate-buffered saline (PBS), pH7) 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.

Figure 4.2 Surface groups used in this study.
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.\textsuperscript{24,25} Second, the electroactive quinone and oxime monolayers permit quantitative characterization of the extent of ligand immobilization and release by cyclic voltammetry (CV).\textsuperscript{26,27} 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.\textsuperscript{28} 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.

4.2.2 Characterization of Ligand Release

We first prepared monolayer surfaces presenting both hydroquinone (1) and tetraethylene glycol (2) groups (Figure 4.1), and characterized the interfacial immobilization and subsequent release of aminooxyacetic acid by CV. Figure 4.3 A shows the cyclic voltammograms of a mixed monolayer presenting both the hydroquinone and tetraethylene
Figure 4.3 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 mVs$^{-1}$; the intersection of the crosshairs represents zero current.
glycol groups (1:1) in 1m HClO₄ and PBS. The hydro-quinone 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 H₂O, 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 1m HClO₄ correspond to the oxidation and reduction of the oxime monolayer (Figure 4.3 B). The oxime is stable in 1 M HClO₄ solution and can be cycled between the oxidized and reduced forms at least 50 times with no change in the voltammograms.

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 4.3 C). 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 1m HClO₄, and confirmed the oxime conjugate on the surface (Figure 4.3 D).

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 ¹H 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 (NH₃) is also released during the electrochemical reduction, but have not yet been able to identify this species.

Figure 4.4 Normalized peak currents (Ip/Io) at 425 mV versus time for the consecutive cyclic voltammograms shown in Figure 4.3 (C). Inset: Plot of pseudo-first-order rate constants versus pH, which shows the relative rates of oxime degradation in various buffer solutions.

To further characterize the kinetics of oxime degradation in PBS, we examined the cyclic voltammograms obtained for the interfacial reaction. Figure 4.4 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 4.3 C. The data were fitted to an exponential decay (equation 4.1) to give a pseudo-first-order rate constant of 0.01 s⁻¹ in PBS, where I₀ is the peak current at time t, I₀ is the initial peak current, and Iᵣ is the residual non-faradaic current.
\[ I_t = I_f + (I_0 - I_f) \exp(-k't) \] (equation 4.1)

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 4.4 (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. We prepared a mixed monolayer presenting 1% hydroquinone group and 99% tetra(ethylene glycol) groups. A high percentage of background tetra(ethylene glycol) density ensures that the monolayer surface completely resists nonspecific protein adsorption. 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, 4h). Association of FLAG antibody to the peptide-immobilized monolayer substrate was characterized by surface plasmon resonance (SPR) spectroscopy.
Figure 4.5 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.

Figure 4.5 (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 50 mV for 5 min in PBS causing release of peptide, a SPR sensorogram showed that the anti-FLAG did not bind to the surface (Figure 4.5, bottom). This result confirmed that electrochemical reduction of the monolayer released the immobilized FLAG peptide ligands from the surface through oxime degradation.

4.2.3 Dynamic Co-culture Platform
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 nitroveratryloxy carbonyl (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.\textsuperscript{31,32}

Figure 4.6 shows the photopatterning of attached cell culture on surface-immobilized RGD gradients, and subsequent release of the patterned cells by an electrochemical potential.\textsuperscript{33} To demonstrate that electrochemical treatment of the monolayer is noncytotoxic to the attached cell culture, we used microcontact printing (mCp) to pattern hexadecane thiols that promote adhesion of another subset of cells through hydrophobic interactions on the same substrate.\textsuperscript{34}

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\% tetra(ethylene 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 electro-chemically oxidized and then treated with soluble RGD oxyamine (0.1m, 4h) 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
Figure 4.6. 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 photo-mask 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.

photoactivated gradient region of the monolayer (presenting adhesive RGD peptides; Figure 4.6 H).

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 4.6 I 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.

4.3 CONCLUSIONS AND DISCUSSIONS

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.35-37
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A GENERAL CHEMOSELECTIVE REDOX RESPONSIVE LIGATION (CRRL) AND RELEASE STRATEGY
5.1 INTRODUCTION

The ability to chemoselectively ligate a range of biomolecules, polymers, nanoparticles and nanomaterials is fundamental to current chemistry research and is crucial for a wide range of applications from protein conjugation, pharmaceutical therapies, molecular electronics, polymer synthesis and tissue engineering.\textsuperscript{1-4} The most used methods rely on ‘click chemistry’ type reactions that span Diels-Alder, Michael additions, thiol-ene reactions, Staudinger reactions, nucleophilic ring opening reactions, 1,3-dipolar cycloaddition, oxime/hydrozone formation reactions and native chemical ligations.\textsuperscript{5-18}

Although several conjugation methods exist and have been used for many applications the use of these methods for in \textit{vivo} biological studies and applications has been severely limited due to the special nature of the biological environment that limits the scope of reactions that may be employed.\textsuperscript{19-32} This sub-set of conjugation reactions for in \textit{vivo} studies and applications ideally, at physiological conditions, require kinetically well-behaved reactions that are fast, bioorthogonal, stable (covalent bonds), modular to conjugate a diverse range of molecules and non-cytotoxic. Moreover, a conjugation strategy that can be: 1. Monitored and characterized with simple non-intrusive analytical methods, 2. Turned on or off for conjugation and 3. Release molecules and be inexpensive would greatly complement and expand the scope of existing conjugation/release methods for a range of new applications.

It is well known that oxyamine groups (R-ONH\textsubscript{2}) selectively react with carbonyl groups to form stable oxime products, which have been used as a popular bioconjugation methodology for the preparation of ligand microarrays and cell arrays\textsuperscript{33,34}, therapeutics\textsuperscript{35,36},
fluorescent labeling\textsuperscript{37}, combinatorial libraries\textsuperscript{38}, artificial proteins\textsuperscript{39}, glycoprotein mimetics\textsuperscript{40}, chemically tunable polymer\textsuperscript{41}, and biosensors.\textsuperscript{42} Oxime formation is chemoselective, fast, and stable in physiological condition.\textsuperscript{43,44} There are many types of oximes reported and characterized and each have unique characteristics including the quinone oxime; having an oxime moiety (R\textsubscript{1}R\textsubscript{2}C=N-O-R\textsubscript{3}) directly adjacent to the aromatic ring.\textsuperscript{45} Among them, the quinone oxime ether (QO) and ester have been studied in the field of stereo-electronic control in organic chemistry\textsuperscript{46}, and applied to cytotoxic molecules\textsuperscript{47}, therapeutics\textsuperscript{48}, and photo-induced iminyl radical generation.\textsuperscript{49} We have extensively studied and employed the quinone oxime ether (QO) as a quantitative immobilization strategy on gold and indium tin oxide (ITO) surfaces for a range of biotechnological and cell behavior studies.\textsuperscript{50-62} However, until now, the QO with its interesting aromatic structure and redox properties, has not been explored as a solution based conjugation strategy.

The ability to cleave specific chemical linkages with mild stimuli is a powerful tool in designing small molecule drugs, in vivo biosensors, solid phase synthesis as well as protein-protein interactions.\textsuperscript{63-68} There are several popular classes of cleavable linkers for these applications: cleavage by reduction (sulphydryl and diazobenzene), oxidation (periodate), nucleophilic substitution, and base labile sulfones.\textsuperscript{69} Among them, reduction-induced cleavage is attractive due to its mild condition for cleavage at physiological conditions. For example, N-Succinimidyl 3-(2-pyridyldithio)propionate (SPDP) and Succinimidyl oxycarbonyl-\(\alpha\)-methyl-\(\alpha\)-(2-pyridyldithio)toluene (SMPT) are often used for the preparation of immunotoxin conjugates that contain a monoclonal antibody which is cross-linked to a protein toxin molecules; the antibody directed against tumor-associated antigens. Lambert et al. showed that a disulfide linkage between the antibody and toxin molecules
results in a potent toxicity when compared to non-cleavable linkages. Although the disulfide linkers have been successful in various fields where cleavable properties are required, there are always issues regarding disulfide exchange and premature cleavage at physiological conditions. A major limitation of this method is that it is incompatible with cysteines due to their intrinsic lack of bio-orthogonality with disulfide bond formation.

In this report, we develop a general strategy termed a chemoselective redox responsive ligation (CRRL) and release strategy for molecular conjugation and cleavage of a range of molecules in solution. This strategy is based on the reaction of oxyamine tethered ligands with a redox controlled benzoquionone group for subsequent oxime formation and release. This CRRL strategy is dynamic, kinetically well behaved, stable, bio-orthogonal, inexpensive, synthetically flexible and can be regenerated for subsequent rounds of conjugation and release at physiological conditions. Furthermore, the CRRL reactions can be monitored by simple UV-Vis spectroscopy methods and controlled by electrochemistry (either chemical redox reagents or applied potentials). To our knowledge, the CRRL strategy is the only single method that is able to chemoselectively ligate molecules and to then release the molecules under redox control with regeneration for multiple subsequent rounds of conjugation and release.

5.2 EXPERIMENTAL

5.2.1 Synthesis and Purification of Peptide
All CRRL peptides were synthesized using an automated solid phase peptide synthesizer (CS Bio) with standard Fmoc SPPS methods at a 0.1 mmol scale. All amino acids were purchased from Anaspec. To ensure reaction completion, a 4-fold excess of Fmoc-amino acids were used for each step of amino acid addition in the presence of HBTU and DIPEA in DMF. The SPPS resin (Fmoc-rink amide MBHA resin, Anaspec) was treated with a TFA solution containing 2.5 % water and 2.5 % triisopropylsilane for 1 h with bubbling nitrogen and filtered. The filtrate was mixed with cold ether to precipitate. The precipitate was washed with ether one additional time to completely remove TFA, and then extracted with water. The resulting CRRL peptides were purified by reverse-phase HPLC, using a Vydac C-18 semi-preparative column with a gradient of 0 - 100% organic solvent for over 50 min (4 mL/min). The aqueous solvent was 95:5 water:acetonitrile with 0.1 % TFA while the organic solvent was 95:5 acetonitrile:water with 0.1 % TFA. The products eluted at 14.5 min for inter-CRRL RGD (6) and at 16.5 min for intra-CRRL RGD (9). The collected eluate was frozen and lyophilized to a white powder.

5.2.2 Synthesis of HQAA (1)

1,4-bis((tetrahydro-2H-pyran-2-yl)oxy)benzene (3) To a solution of hydroquinone 2 (4.6 g, 42.2 mmol) in THF (40 mL), dihydropyran (16 mL, 206 mmol) and 3 drops of concentrated HCl were added. The reaction was stirred overnight at room temperature and then concentrated. The crude product was dissolved in a minimal amount of ethyl acetate and recrystallized in ethyl acetate/hexane affording 8.3g of white powder (71%). $^1$H NMR (400 MHz, CDCl$_3$): δ 1.54-1.65 (m, 6H), 1.80-1.84 (m, 4H), 1.96-1.97 (m, 2H), 3.56-3.59 (m, 2H), 3.88-3.94 (m, 2H), 5.28 (t, 2H), 6.96 (s, 4H).
2,2’-((2-(6-bromohexyl)-1,4-phenylene)bis(oxy))bis(tetrahydro-2H-pyran) (4) To a solution of 3 (1.9 g, 6.8 mmol) in dry THF (Acroseal, Acros) in an ice bath, tert-butyl lithium (4.8 mL of 1.6 M, 7.68 mmol) was added dropwise over 5 min. This mixture was stirred for 15 min, followed by the addition of an excess of 1,6-dibromohexane (3.19 mL, 20.7 mmol); the reaction was then placed at room temperature. A white precipitate initially formed, however, after stirring for 12 h, the solution became clear yellow. The reaction mixture was diluted with 40 mL of methylene chloride and washed (1 x 20 mL of NH₄Cl and 1 x 20mL of brine) and concentrated to a transparent oil. Flash chromatography was performed (8:1:1 hexane:ethyl acetate:methylene chloride), affording the product (1.94 g, 4.6 mmol, 68%). 

1H NMR (400 MHz, CDCl₃): δ 1.62-2.02 (m, 20H), 2.66 (t, 2H), 3.38 (t, 2H), 3.58-3.62 (m, 2H), 3.88-3.93 (m,2H), 5.27 (m, 2H), 6.82-6.84 (m, 2H), 6.97-6.70 (m, 1H).

2,2’-((2-(6-azidohexyl)-1,4-phenylene)bis(oxy))bis(tetrahydro-2H-pyran) (5) To a solution of 4 (2 g, 4.5 mmol) in DMF (30 mL), sodium azide (0.6 g, 9 mmol) was added. The reaction mixture was stirred for 2 h at 50 °C. The reaction was then extracted with water/methylene chloride and the organic phase was washed with water twice. The organic phase was concentrated to yield a clear oil with quantitative yield. 

1H NMR (400 MHz, CDCl₃): δ 1.32-2.02 (m, 20H), 2.56 (t, 2H), 3.21 (t, 2H), 3.54-3.2.59 (m, 2H), 3.90 (t, 2H), 5.26 (m, 2H), 6.80 (m, 2H), 6.94 (m, 1H).

HQAA (1) To a solution of 5 (1.4 g, 3.4 mmol) and propargylglycine (0.6 g, 1.8mmol) in 70 mL DMF, an aqueous solution (30 mL) of CuSO₄·5H₂O (750 mg, 3 mmol) and sodium
ascorbate (600 mg, 3 mmol) was added. The reaction mixture was stirred overnight at room temperature, concentrated in vacuo, and purified by flash chromatography (9:1 methylene chloride:methanol) to afford a clear oil (1.7 g, 2.3 mmol, 67%). $^1$H NMR (400MHz, CDCl$_3$): δ 1.19-1.92 (m, 22H), 2.44 (t, 2H), 3.24 (t, 2H), 3.54 (t, 2H), 3.91 (t, 2H), 4.16 (t, 1H), 4.28-4.47 (m, 3H), 5.27 (s, 2H), 6.71-6.96 (m, 3H), 7.28 (t, 2H), 7.37 (t, 2H), 7.58 (t, 2H), 7.69 (s, 1H), 7.76 (d, 2H).

5.2.3 Characterization

Spectroscopy. Spectra were obtained using an Agilent Technologies model 8453 diode-array spectrophotometer for both standard and kinetics measurements. All the measurements were performed using quartz spectrophotometer cells (Aldrich chemical, 10mm, rectangular) in Dulbecco’s PBS (used as received) at room temperature. All measurements were initiated as immediately after sample preparation.

Chromatography and Mass Spectroscopy. Analytical HPLC was performed on a Waters HPLC 1525 equipped with a 2998 diode array detector and an automated sampler. The samples were eluted through an Atlantis analytical column (C18, 5 µm, 6 x 150 mm) with a binary solvent system (Solvent A: optima grade water (Fisher) with 5% optima grade acetonitrile (Fisher) and 0.1% TFA; Solvent B: acetonitrile with 5% water and 0.1% TFA) with a gradient of 0–100% solvent B over 50 min (1 mL/min). For the LC-MS data for intra-CRRL RGD (6) and inter-CRRL RGD (9) peptides, samples were run on a Waters Acquity UPLC system coupled to a Waters Micromass ZQ Mass Spectrometer (Scanning
300-1600m/z) using a Waters Acquity UPLC column (BEH C18 1.7 μm, 2.1 x 150 mm, 30 °C). The elution occurred in a gradient of binary solvents (Solvent A: optima grade water (Fisher) with 0.2 % formic acid (Fluka); Solvent B: Optima grade acetonitrile with 0.2 % formic acid), where solvent B increased from 1 % to 40 % over 20 min.

5.3 RESULTS AND DISCUSSION

5.3.1 Scheme of CRRL Conjugation and Release

Figure 5.1 Cartoon scheme of the chemoselective redox responsive ligation (CRRL) and release strategy. A CRRL receptor tethered to a molecule 1 can be switched between the “off” state (full circle) and “on” state (incomplete circle) by mild chemical or electrochemical oxidation and reduction. Only when the receptor is turned “on”, the CRRL ligand (red arrow) can react chemoselectively to form a conjugated molecule pair. The covalent ligation product is stable but can be selectively released by a mild chemical or electrochemical reductant. The CRRL cleavage reaction regenerates the receptor group while the ligand is irreversibly altered. Conjugation between the receptor and the ligand, as well as the cleavage reaction, are fast, mild, clean (no need for catalyst), bio-orthogonal, and
chemoselective in physiological conditions (pH of 7.4 and 37 °C) and complex protein mixture (cell lysates), which are essential characteristics for biological applications.

Figure 5.1 shows a cartoon scheme of the dynamic CRRL strategy. A CRRL receptor can be switched between the ‘off’ state and ‘on’ state by mild chemical or electrochemical redox changes. Only when the CRRL receptor is turned ‘on’ can it react chemoselectively with a CRRL ligand to generate a stable covalent conjugated pair. The covalent ligation product is stable but can be selectively released by a mild chemical or electrochemical reductant. The CRRL cleavage reaction regenerates the receptor group while the released ligand is irreversibly altered at the ligation site. Subsequent rounds of conjugation and cleavage at physiological conditions (pH 7.4 and 37 °C) can be performed. The reaction is bio-orthogonal, chemoselective and may be done in complex protein mixtures and cell culture.  

Figure 5.2 shows the molecular structures of the solution based CRRL conjugation and release strategy. A hydroquinone (HQ) is the “off” CRRL receptor and can be converted to the benzoquinone (BQ) (“on” CRRL receptor) by mild chemical oxidants or electrochemical oxidation. This interconversion process is chemically and electrochemically reversible, which enables switching between the active and inactive forms for chemoselective conjugation. An oxyamine-tethered molecule (R-ONH₂; the CRRL ligand) can conjugate selectively to the BQ form generating a covalent and stable quinone oxime ether (QO) as a ligation product. The QO can then be reduced by mild chemical reductants or electrochemical reduction to yield the aminophenol (AP) with release of the ligand as a hydroxyl-terminated molecule (R-OH). The HQ can then be regenerated from aminophenol (AP) by a subsequent mild redox process in low pH with the liberation of ammonia.
Figure 5.2 The molecular cycle of the CRRL conjugation and release strategy. A hydroquinone (HQ) is the “off” CRRL receptor and can be oxidatively converted to the benzoquinone (“on” CRRL receptor) form by mild chemical reagents or electrochemical methods. This interconversion process is chemically and electrochemically reversible, which enables switching between the active and inactive forms for chemoselective conjugation. An oxyamine-tethered molecule (R-ONH₂; the CRRL ligand) can conjugate selectively to the BQ form generating a covalent and stable quinone oxime ether (QO) as a ligation product. The QO can then be reduced in mild conditions to yield the aminophenol (AP) with release of the ligand as a hydroxyl-terminated molecule (R-OH). The HQ can then be regenerated from AP by a subsequent mild redox process.

5.3.2 Characterization of CRRL Conjugation and Release by UV-Vis Spectroscopy

To monitor and characterize the kinetics of the stable intermediate products of the CRRL conjugation and cleavage strategy we used standard UV-Vis spectrometry. Upon oxidation of hydroquinone (HQ), benzoquinone (BQ) is formed, which can react with oxyamine groups (R-ONH₂) to form the quinone oxime ether (QO). The QO can then be
Figure 5.3 Monitoring the kinetics of CRRL conjugation between BQ (200 µM) and methoxyamine (100 mM) by UV-Vis spectrometry. (a) UV-Vis spectra showing the conversion from BQ (248 nm) to QO (320 nm) with a clear isosbestic point. (b) A plot of absorbance maxima versus time for BQ and QO. An exponential increase of QO and decay of BQ are fitted to pseudo first order kinetics to obtain k’ (4.2 x 10^{-3} s^{-1}). (c) A plot of k’ versus varying [CH_3-ONH_2] to determine the second order rate constant k for the reaction of BQ with CH_3-ONH_2 to generate QO. The rate constant for CRRL conjugation is 3.4 x 10^{-2} M^{-1}s^{-1}.
Figure 5.4 Determining the kinetics of CRRL cleavage of QO (200 μM) with dithiothreitol (DTT) (100 mM) by UV-Vis spectrometry. (a) UV-Vis spectra characterization for the cleavage reaction versus time (QO: 320 nm, AP: 292 nm). (b) A plot of QO (200 μM) absorbance (320 nm) versus time in the presence of the reductant DTT (100 mM) follows a pseudo 1$^{st}$ order exponential decay ($k' = 7.3 \times 10^{-2} \text{ s}^{-1}$). (c) A plot of $k'$ versus varying [DTT] to determine the second order rate constant $k$ for the cleavage of QO to generate AP. The CRRL cleavage rate constant $k = 6.5 \times 10^{-1} \text{ M}^{-1}\text{s}^{-1}$.
cleaved to generate the aminophenol (AP) with all species having diagnostic absorbances (HQ: 288 nm, ε = 2,520 M⁻¹cm⁻¹, BQ: 248 nm, ε = 20,300 M⁻¹cm⁻¹, QO: 320 nm, ε = 18,163 M⁻¹cm⁻¹, AP: 297 nm, ε = 2,150 M⁻¹cm⁻¹). Figure 5.3a shows the conjugation reaction between BQ and methoxyamine characterized by UV-Vis spectroscopy. To the solution of BQ (PBS, 200 μM), a solution of methoxyamine (PBS, 100 mM) was added immediately after the initial measurement (0 min). The visible isosbestic point demonstrates that BQ and QO are linearly related by stoichiometry, supporting that the reaction is quantitative. The absorbances at 248 nm and 320 nm were plotted against time in an exponential plot (Figure 5.3b). The exponential graph was fitted to pseudo first order reaction kinetics in order to obtain k’. After solving for a series of k’s from different reaction conditions with excess methoxyamine, the k’ values can be plotted against [methoxyamine] (Figure 5.3c) to obtain the second order rate constant for CRRL conjugation, k = 3.41 x 10⁻² M⁻¹s⁻¹ (PBS, room temperature).

The same experiments and calculations were performed for the CRRL cleavage reaction. To a solution of QO (PBS, 200 μM), dithiothreitol (DTT) (PBS, 100 mM) was added (Fig 5.4a). Although DTT masks the spectrum that is lower than 260 nm, the decrease of QO absorbance at 320 nm and the appearance of AP at 292 nm could be observed. The absorbance peak is shifted from 297 nm to 292 nm for AP because of the high concentration of DTT. For the case of reduction in low glutathione (GSH) concentration, AP has an absorbance peak at 297nm (Figure S2 in the Appendix A). Commercial AP (200 μM) in 100 mM DTT and PBS also shows the same absorbance shift. The isosbestic point was not observed for the conversion of QO to AP because of the absorbance peak overlap. The rate constant for the CRRL cleavage reaction was determined to be 6.5 x 10⁻¹ M⁻¹s⁻¹ (Fig 5.4c).
The reaction products from both CRRL conjugation (QO) and cleavage reactions (AP) were characterized by NMR and mass spectrometry (Figure S1 in the Appendix A). These UV characterizations clearly show that the CRRL conjugation and cleavage reactions are fast and

![Diagram of CRRL conjugation and cleavage reactions](image)

**Figure 5.5** CRRL conjugation and cleavage reactions monitored and controlled by an electrode. A self-assembled monolayer of a HQ-tethered alkanethiol on a gold electrode has a characteristic cyclic voltammogram (top). The HQ can be electrochemically oxidized to BQ for CRRL ligation with oxyamine tethered ligands (R-ONH₂), resulting in a shift of the diagnostic reduction peak associated with the redox species of QO (bottom cyclic voltammogram). The redox cycle of QO is stable at low pH (pH < 7) and does not cleave. When this redox cycle is performed at higher pH (pH > 7), the oxime (QO) is efficiently cleaved to generate AP and then upon subsequent redox cycles to hydroquinone (HQ). The regenerated HQ can then continue the cycle for subsequent rounds of conjugation and release of oxyamine tethered groups (R-ONH₂).

clean at pH 7.4. We found that the key CRRL conjugation product (QO) is stable at room temperature in PBS for several weeks and could be stored indefinitely at 0 °C (Figure S3 in the Appendix A)
5.3.3 Characterization of CRRL Conjugation and Release by Cyclovoltammetry

In order to demonstrate the CRRL conjugation and cleavage reactions could be monitored and controlled by electrochemical redox signals, we prepared self-assembled monolayers (SAMs) of HQ-tethered alkanethiols on a gold electrode. The HQ head group of the SAMs can be electrochemically oxidized and reduced (switched) between BQ and HQ repeatedly, without any loss of signal or side reactions. This HQ/BQ pair showed diagnostic potentials (Ox at 576 mV; Red at 230 mV) in the corresponding cyclic voltammograms (CV) (Figure 5.5, top). When an oxyamine-tethered ligand (R-ONH₂) was introduced to the oxidized BQ SAMs, QO formation was accompanied by a shift of the diagnostic reduction peak (470 mV), displayed in the CV (Figure 5.5, below). Note that the CV not only conveys ligand immobilization by the peak shift, but also the extent of ligand immobilization by calculating the area of redox peaks (Q = nFAT; Q = charge, n = number of electron, F = Faraday’s constant, A = surface coverage of the electroactive molecule). The QO formed after immobilization of oxyamine acetic acid was stable to an electrochemical redox cycle at pH 0 (1 M perchloric acid solution). However, after ligand conjugation, and upon increasing the pH the interfacial oxime ligand was rapidly released. After scanning the gold surfaces with CV, the original HQ/BQ redox signal was regenerated. These results clearly demonstrate that both mild chemical redox agents and electrochemistry (-200 mV to 800 mV) can control CRRL conjugation and cleavage from a surface, which is attractive in designing new biotechnological microarray platforms or as a bioengineering tool for studying cell behavior.
5.3.4 Characterization of CRRL Conjugation and Release by HPLC

Figure 5.6 shows HPLC chromatograms for the characterization of the molecules and reactions involved in the CRRL strategy. The CRRL reaction mixtures for conjugation and cleavage were stirred for 5 minutes in PBS and then characterized by HPLC. When HQ (200 µM, 5.9 min) was mixed with methoxyamine (100 mM, 2.4 min), there was no formation of QO (Fig 6a). However, the BQ (200 µM) formed QO instantly with methoxyamine, which resulted in a shift of the BQ peak (7.9 min) to the newly formed QO (16.7 min) (Fig 6c). Figure 5.6d shows that AP is exclusively eluted from a CRRL cleavage reaction; AP elutes earlier than HQ (4.5 min). The HPLC chromatograms at 214 nm for each reaction showed that CRRL conjugation and cleavage reactions are exceptionally clean and fast at pH 7.4 (PBS, room temperature). All of the peaks were separated and characterized by NMR and mass spectrometry (Figure S1 in the Appendix A). The ability to switch between HQ and BQ, as well as the orthogonality of the reaction between BQ and oxyamines, is crucial for designing smart molecules that contain both a CRRL receptor and a CRRL ligand. As long as the CRRL receptor is in the off state (HQ), it cannot react with the oxyamine moiety until the molecule encounters an oxidative environment (as shown for intramolecular and intermolecular CRRL peptides below), enabling temporal and/or spatial control of molecular conjugation and release. This dynamic behavior can also be applied to intermolecular CRRL conjugation, in that the ligation only occurs when oxidative conditions exist in the reaction mixture. It should be noted that the initiation cue for the CRRL strategy is not a catalyst, but an oxidative environment.
Figure 5.6 HPLC chromatograms for the molecules and reactions involved in the CRRL strategy. (a) Reaction between HQ and methoxyamine. There is no formation of the conjugate QO when the CRRL receptor (HQ) is in the “off” state. (b) HPLC chromatogram of BQ formed by the oxidation of HQ via chemical or electrochemical methods. (c) Reaction between BQ and methoxyamine quantitatively yields the QO product. (d) The CRRL cleavage reaction in reductive conditions converts the QO to AP quantitatively. All the reactions were in PBS (pH 7.4) at 25 °C.

5.3.5 Proof-of-Concept Peptide: Intra-CRRL RGD and Inter-CRRL RGD

To conjugate a range of biomolecules using the CRRL strategy, we used standard solid phase peptide synthesis (SPPS) to generate peptides containing the hydroquinone group
at various locations. We first synthesized a protected hydroquinone tethered Fmoc amino acid (HQAA; 1) that could easily be incorporated into a standard solid phase peptide synthesizer for site-specific CRRL chemistry (Figure 5.7a). A Tetrahydro-2H-pyran (THP)
protected HQ-tethered alkyl azide (5) was synthesized and conjugated to an Fmoc-protected propargyl glycine by 1,3-dipolar cycloaddition. The HQAA (1) can be incorporated into any position in a peptide sequence, enabling control of the conjugation site with routine Fmoc SPPS (Figure 5.7b). The THP group can then be deprotected yielding the HQ using the standard trifluoroacetic acid (TFA) cleavage step for peptide release from a resin. CRRL’s compatibility with facile SPPS is an important feature for generating a range of biomolecules including carbohydrates, polymers, nucleic acids, proteins and small molecule ligands and nanomaterials that contain the hydroquinone group for a broad range of applications in material science and biology.

In order to demonstrate the utility of the CRRL strategy we used the well-known cell adhesion peptides discovered by Pierschbacher and Ruoslahti as a model system. They reported the short peptide sequence Gly-Arg-Gly-Asp-Ser (GRGDS) found in the extracellular matrix protein fibronectin to be a minimum cell adhesion ligand for integrin receptors on cell surfaces. Although GRGDS alone can result in cell adhesion, several other peptide sequences including PHSRN (found in fibronectin within the FIII9 domain) have been shown to have a synergistic adhesion effect. Furthermore, it has been shown that cyclic-RGD has a higher activity towards cell binding than linear-RGD ($K_d$ of nM versus $\mu$M, respectively) due to the structural similarity between the cyclic form and the natural RGD loop in the FIII10 domain. We chose to focus on the RGD cell adhesive peptide to demonstrate the generation of a dynamic biomolecule using CRRL, which can modulate its biological activity by inducing structural changes upon sensing different redox environments. Two proof-of-concept peptides, intra-CRRL RGD (6) and inter-CRRL RGD (9), were synthesized to demonstrate the utility of the CRRL methodology.
The intra-CRRL RGD peptide (6) has the HQAA (1) at the C terminus and an oxyamine group at the N terminus (Figure 5.8a). An added feature of the CRRL strategy is the ease of incorporation of an oxyamine group into a peptide by using a commercially available boc-protected oxyamine acetic acid during standard solid phase peptide synthesis. In a reducing environment (1mM DTT in PBS), the intra-CRRL RGD (6) remains in the linear form for more than 4 days (PBS, 25 °C). When the molecule encounters oxidative conditions (10 equivalents of copper (II) sulfate pentahydrate in PBS), the HQ is immediately oxidized to the BQ form, which reacts rapidly with the oxyamine-functionalized N terminus to generate a cyclized RGD peptide (7) (Figure 5.8b). The cyclized intra-CRRL RGD peptide (7) is stable for weeks in PBS and room temperature (25 °C). To regenerate the linearized RGD peptide, the addition of a chemical (100 mM DTT in PBS) results in the cleavage of the oxime bond to generate the AP group and a hydroxyl-functionalized N terminus (8) (Figure 5.8c). As this intra CRRL peptide has both a CRRL receptor and a ligand, characterization of each state of the peptide by UV-Vis spectroscopy is possible because of the diagnostic signature absorbances of each electroactive group (HQ, BQ, QO, AP). This allows for facile monitoring of the chemical changes occurring during the CRRL cyclization and cleavage steps. As added evidence, mass spectrometry characterization of each reaction product confirms that each transformation is essentially quantitative and proves the existence of the HQ, oxyamine, QO, AP, and the corresponding hydroxyl terminated group in a single peptide during the course of the CRRL conjugation and release steps.

Similarly, inter-CRRL formation and cleavage between RGD and PHSRN was characterized by mass spectroscopy. The inter-CRRL RGD peptide (9) (Figure 5.9a) has a HQAA at the N-terminus for the conjugation of an oxyamine-tethered PREDRVPHSRN
Figure 5.8 Intramolecular CRRL. A peptide (intra-CRRL RGD, (6)) containing the HQAA (1) and a terminal oxyamine group was synthesized using standard solid phase peptide synthesis. The CRRL strategy was used to generate a cyclic peptide characterized by liquid chromatography and mass spectrometry. (a) Structure of the intra-CRRL RGD (6) peptide. (b) A mild oxidant Cu$^{2+}$ was added in order to oxidize the HQ to BQ followed by rapid intramolecular oxime formation to generate a cyclic peptide (7). (c) A mild reductant DTT was added to the solution of cyclized RGD peptide (7) to reduce and therefore cleave the oxime bond to generate the linear peptide (8). Mass spectrometry characterization of each product in the intramolecular CRRL strategy were shown. All the reactions were performed at physiological conditions (PBS, pH=7.4, 37.0 °C).
Figure 5.9 Intermolecular CRRL demonstrating the conjugation of two different peptides. One peptide (inter-CRRL RGD (9)) contains the HQ group while the other (PREDRVPHSRN) has an oxyamine group. Each are easily synthesized using standard solid phase peptide technology. (a) Inter-CRRL RGD (9) peptide in PBS before reaction. (b) A mild oxidant Cu$^{2+}$ and oxyamine-PREDRVPHSRN were added to the PBS solution of inter-CRRL RGD (9) peptide to enable rapid intermolecular CRRL conjugation of the two peptides (10) (PBS, 37.0 °C). (c) The CRRL product peptide (10) was then selectively cleaved upon treatment of the reductant DTT to yield the linear RGD peptide (11). (Bottom) Mass spectrometry characterization of each product in the intermolecular CRRL strategy. All the reactions were performed at physiological conditions (PBS, pH=7.4, 37.0 °C).
peptide. Oxidative conditions yielded the large hybrid peptide (10) having a molecular mass of 2479.25 (Figure 5.9b), which can then be dissociated by DTT; yielding the corresponding AP-RGD (11). We have a program to study cell adhesion, polarization and migration and future studies will aim to use these peptides in cell adhesion and motility studies. 77-79

Figure 5.10 The intra-CRRL RGD peptides were also characterized by selected ion chromatography. Structures with a cartoon representation of each of the intramolecular peptide CRRL species. Each peak represents a specific mass number that corresponds to each peptide species undergoing the CRRL and release. (a) Linear intra-CRRL RGD (6) peptide in PBS. (b) The RGD peptide (6) is cyclized (7) in an oxidative environment. The multiple peaks are related to isomers of QO conjugation. (c) Linear form of RGD (8) is generated by reduction.
As further characterization, a selected ion chromatogram was presented to demonstrate that each intra-CRRL peptide species behaves similarly to the corresponding parent molecule without the peptide (Figure 5.6), in terms of the elution durations from a C18 column (Figure 5.10). UV-Vis spectroscopy confirmed that the HQ-peptides (9) behave similarly to the parent molecules (Figure 5.11). The reduced form of the inter-CRRL RGD (HQ-peptide (9)) was dissolved in H₂O and oxidized by the addition of 10 equivalents of Cu²⁺ to generate the oxidized BQ-peptide. When methoxyamine (100 mM, ddH₂O) was added, oxime formation occurred instantly (QO-peptide). The QO-peptide was converted to the AP-peptide by treatment with DTT (100 mM). The HQ can be regenerated by a redox process on AP at low pH with release of ammonia. All of the UV spectra for the various CRRL peptides correspond to those of HQ, BQ, QO and AP (Figure 5.3, 5.4). Taken together, these results show the CRRL strategy can be used to cyclize molecules, conjugate to other molecules and release molecules responding to environmental redox stimuli with real-time characterization by a non-invasive straightforward UV-Vis absorption spectroscopy technique. This facile characterization is not only important for identifying species but for also determining actual amounts of the CRRL species and for monitoring the kinetics and therefore degree of conjugation.

The CRRL strategy is a new class of conjugation chemistry in that it allows chemoselective conjugation and cleavage that is responsive to specific redox stimuli. The protected HQ can be incorporated into routine solid phase peptide synthesis as a HQAA (1). This allows for precise control of the site for chemoselective conjugation to other molecules or for intramolecular cyclization. For the cleavage reaction, the released molecule undergoes a functional group transformation, converting the oxyamine moiety to a hydroxyl group.
CRRL can therefore also be used in protecting functionally important hydroxyl groups, which can be critical in the synthesis of carbohydrates or prodrugs.

Figure 5.11 The different stages of the CRRL and release strategy can be monitored by UV-Vis absorbance spectroscopy. For example, the inter-CRRL RGD (9) peptide (HQ-peptide) undergoing the CRRL process contains a distinct electroactive group with a corresponding diagnostic absorbance spectra. HQ-peptide was oxidized with Cu$^{2+}$ to yield BQ-peptide (ddH$_2$O, RT). When methoxyamine (100 mM) was added to the solution, a QO-peptide (10) formed, which can be cleaved by mild reduction to produce the AP-peptide. UV absorbance of each peptide species (HQ: 288 nm, BQ: 248 nm, QO: 320 nm, AP: 292 nm).

The chemical conversion of QO to AP is also important for the industrial production of AP, which is the major chemical intermediate of acetaminophen and other drugs and is also critical for the photography industry. The most popular synthesis for AP involves the metal catalyzed reduction of nitrophenol in harsh (high temperature and pressure) conditions.
However, this method produces excessive environmental pollution and wastewater, as well as residual catalyst. Since the CRRL cleavage occurs at mild physiological conditions with no waste other than an alcohol and with no use of a metal catalyst, it may be able to improve industrial production of AP and/or drug production.

Additionally, QO offers an alternative to disulfide bonds as a cleavable linker when cysteine groups are present on any of the two target biomolecules. We determined that CRRL cleavage takes 10 hr for reaction completion in the presence of 5 mM glutathione (GSH) in PBS (Figure S2 in Appendix A). The facile electrochemical CRRL reaction on conductive surface may also be applied to industry applications, such as affinity chromatography (protein concentration) or dynamic solid phase organic synthesis or renewable microarrays.

5.4 CONCLUSIONS

In summary, we introduce a new chemoselective redox responsive ligation and release strategy. This strategy is based on the ability of a hydroquinone to oxidize to a benzoquinone for subsequent reaction with oxyamine tethered ligands to generate a stable oxime product. The oxime can then undergo an irreversible cleavage upon a reductive environment to generate an aminophenol and a primary hydroxyl terminated ligand. The aminophenol is stable and can be brought back to the original hydroquinone with mild redox chemistry for subsequent rounds of conjugation and release. The CRRL strategy can be monitored in real-time by standard UV-Vis absorption spectroscopy and modulated by chemical or electrochemical methods. A non-natural amino acid containing the
hydroquinone group was synthesized and shown to incorporate at selective positions within a peptide by using standard solid phase peptide synthesis. As a demonstration, an intermolecular and intramolecular CRRL strategy was used to generate dynamic cyclic peptides and two tethered peptides that could be conjugated and cleaved. The CRRL methodology not only shares all the advantages of traditional oxime chemistry (fast, quantitative and chemoselective), but also has a dynamic attribute associated with conjugation and cleavability. CRRL conjugation and cleavage occurs fast and quantitatively in physiological conditions without the need for a catalyst. Furthermore, the CRRL strategy is synthetically flexible and can also be used with hydrazine type molecules instead of oxyamines to generate conjugated hydrazones.\textsuperscript{80} We believe that CRRL is a general methodology for conjugation and release and will be a useful tool for scientists and engineers in various fields aiming to design smart molecules and materials, which either alter their biological activities according to specific changes in their physiological environments or for material science applications.\textsuperscript{81,82} The CRRL strategy is inexpensive, bioorthogonal, dynamic, kinetically well behaved, synthetically modular and straightforward to monitor and control. Preliminary evidence shows that the QO may be cleaved with strong ultraviolet light for potential photo-induced cleavage applications. To our knowledge, the CRRL strategy is the only single method that is able to chemoselectively ligate molecules and to then release the molecules under redox control with regeneration for multiple subsequent rounds of conjugation and release. Future research will explore how the CRRL strategy may be used to release or conjugate molecules to generate dynamic polymers, carbohydrates and mixed CRRL bio-molecules (peptides, nucleic acid, lipids) \textit{in vivo}.\textsuperscript{83-87} We believe, the ease of the CRRL strategy should find wide use in a range of applications in biology, tissue engineering,
nanoscience and material science and will significantly add to the suite of current conjugation and release strategies.
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6.1 CONCLUSIONS AND FUTURE DIRECTIONS

This dissertation focused on the development of a novel engineered biological model system based on self-assembled monolayers. Bio-specific interactions between ligand and cell adhesion receptors as well as facile control over cell adhesion conditions were major advantages over current model systems. They helped provide deeper insight of adhesion mediated signaling as well as cross talk with other receptors. For example, HQ/TEG system was applied to study adhesion effects of two structurally and functionally coupled ligands, RGD and PHSRN, on 3T3 swiss albino fibroblasts in chapter 2. The observed difference in cellular behaviors was caused by corresponding differences in activities of Rac1 and RhoA. Furthermore, cross-talk was observed between integrins and growth factor receptors on PHSRN-presenting surface, but not RGD-presenting surfaces. In this chapter, cells behaved very differently on both ligands; migrating faster with dynamic lamellipodia protrusions on the PHSRN-presenting surface while stationary with well developed adhesion structures and stress fibers on the RGD-presenting surface.

Consideration of these dramatically different adhesion effects in the context of close coupling of these ligands prompted a new question; is the transient decoupling of two domains a relevant biological question \textit{in vivo}? The question is further supported by several reports of FN stretching by endogenous cellular tension inducing decoupling and adsorption onto the substratum. This process potentially changes the distance and orientation between the two domains containing RGD and PHSRN as well as their engaged receptors. The question only can be answered by developing non-invasive, real-time monitoring and control over the distance and orientation of 9th FNIII and 10th FNIII domains of FN. Since the distance between two domains (~40Å) is in the applicable range of fluorescence resonance
energy transfer (FRET) microscopy (10–100 Å), FRET donor and acceptor incorporation onto the two domains of FN seems to be promising approach. The real-time change of distance and orientation of FN by endogenous tension of cell will be correlated to the changes in cellular behaviors in terms of orientation, contraction, actin rearrangement, focal adhesion turn over, and lamellipodia protrusions.

Chapter 3 focused on the development of a novel methodology to immobilize ligands with spatial control by applying a photolithographic strategy. The NVOC-protected oxyamine was revealed only when UV light was impinged through photomask to selectively reveal an oxyamine group. The revealed oxyamine group formed oxime ligations with ketone-functionalized bioligands. The mixed SAMs with TEG enabled immobilization of multiple ligands in various shapes, enabling cell culture in specific geometries as well as gradients.

Chapter 4 introduced dynamic control over quinone oxime conjugation and release on a conductive surface in physiological conditions. First, the hydroquinone is oxidized to the benzoquinone form, which reveals a ketone for oxyamine conjugation. The resulting quinone oxime ether is stable for the electrochemical reduction in pH 0 (1M perchloric acid soln.). Interestingly, the quinone oxime conjugation is cleaved by reduction in higher pH buffer. The conjugation and cleavage was confirmed by cyclovoltammetric (CV) characterization, surface plasmon resonance experiment (SPR), and X-ray photoelectron spectroscopy (XPS). Immobilizing bio-specific ligands to activate cellular receptors, the potential application as a platform for dynamic co-culture was shown.

The methodologies developed will be collectively applied to develop a model system to study adhesion-mediated signaling. The cellular response to geometry and ligand gradients
in real time will open new insights of adhesion mediated signaling. Furthermore, the ability to control conjugation and cleavage in physiological conditions (pH 7.4, 37.5°C) will be valuable to investigate real-time cellular response to adhesion condition changes. For example, installing a hydroquinone and oxyamine in both ends of a RGD peptide will enable real-time control over the structural constraint of the peptide. Since the cyclic form of RGD peptide has ~1000 times stronger cell binding affinity than linear form due to the structural similarity to FN\textsuperscript{1}, the cellular response to adhesive changes may be monitored (Figure 6.1). The results from the dynamic model surfaces will provide new insights to adhesion mediated signaling by depicting connections between adhesion-mediated phenomenon.

![Diagram](image)

**Figure 6.1** The RGD peptide flanked by CRRL counterparts immobilized onto the surface and presented to cells. Since the cyclic forms of the RGD ligand have significantly higher integrin binding affinity than the linear form\textsuperscript{1}, this substrate can modulate the adhesiveness of the surface by electrochemical switching. The dynamic surface will enable real time monitoring of cellular response to the dynamic change of adhesion.

In chapter 5, the conjugation and release strategy on the conductive surface (chapter 4) was extended as a strategy to form a cleavable linker between two biomolecules in
aqueous solution. The quinone oxime ether in solution, not on the surface, could be cleaved by mild biological reductants such as DTT and GSH. Interestingly, the cleaved quinone oxime ether yielded alcohol and aminophenol, which is different from the products of surface cleavage. The electrochemical cleavage on the surface produced hydroquinone and alcohol, where the difference may be caused by the instant release of ammonia by subsequent electrochemical redox cycles for the aminophenol on the surface. The electrochemical conversion of aminophenol to hydroquinone has been well described in the literature²,³.

The clean synthesis without the use of catalyst in aqueous solvent to produce aminophenol may have a potential impact on the pharmaceutical industry where aminophenol is an important chemical intermediate for acetaminophen. Usually industrial scale synthesis of aminophenol includes metal catalyzed reduction of nitrophenol in harsh conditions (high temperature and pressure), which produces excessive environmental pollution and wastewater. More importantly, small amounts of residual catalyst may remain in the product batch. Future research will focus on improving applicability and cost efficiency of this reaction at the industrial scale.

The CRRL methodology has the unique ability to switch ‘on’ and ‘off’ with physiological que (mild oxidative or reductive environment) for chemoselective conjugation. This characteristic is important in designing a smart molecule having both CRRL counterparts in same molecule. The smart molecule (bio-molecule or chemical sensor) can change structural constraints to respond to an oxidative environment, which may cause measurable changes in biological activity or signal of the probes.

Chemical linker cleavability in a reducing environment has been important in the preparation of immunotoxins⁴. Immunotoxins, first reported 30 years ago, are a chimeric
targeting protein (either an antibody or growth factor) for specific cells and toxins\textsuperscript{5,6}. Lambert \textit{et al.} showed that a disulfide linkage between the antibody and toxin molecules resulted in a potent toxicity when compared to non-cleavable linkages\textsuperscript{7}. The disulfide bond, a natural reductively cleavable linker, has been applied successfully to design effective immunotoxins. However, there always have been concerns about the intrinsic lack of chemoselectivity and premature cleavage associated with the disulfide bond. For example, when several cysteins are exposed at the surface of target proteins, application of this methodology produces isomers, which should be avoided in pharmaceutical applications. The easy installation of hydroquinone and oxyamine groups, the chemoselective conjugation between them, as well as linear cleavage profile in 5mM GSH in PBS (pH 7.4) strongly suggests this methodology can be used as a cleavable linker for immunotoxins. Future efforts will directed towards obtaining actual cleavage profiles in the cytosol. Membrane permeable FRET pairs connected by quinone oxime ether will be introduced to the cell to monitor CRRL cleavage in real time. Several targeting moieties (antibodies, growth factors or peptides) for cancer cells tethered with FRET donor will be connected to FRET quencher through CRRL linker. The endocytosis, dissociation and subsequent fate will be monitored and characterized. Cytotoxicity and cellular metabolism of aminophenol, a CRRL byproduct, should also be characterized and an optimized strategy to incorporate a hydroquinone or oxyamine in the antibody or growth factor also needs to be developed. The CRRL strategy is unique and novel class of chemistry that allows bio-orthogonal and chemoselective conjugation followed by subsequent cleavage. Furthermore, CRRL conjugation and cleavage can be controlled by biologically relevant redox signals. The reactions are fast, quantitative and clean (no catalyst) in physiological conditions. Together
with our novel biological model system, we believe that this new chemistry will enable us to interrogate unprecedented areas of biology as well as adding valuable bio-organic tools for scientist in various fields.
REFERENCES


Appendix A

SUPPORTING INFORMATION FOR CHAPTER 5
Figure A1. NMR and MS data for quinone oxime (QO) and aminophenol (AP) separated
by HPLC from reactions of CRRL conjugation and cleavage (Figure 6c and 6d) (a) NMR data of QO (b) NMR data of AP (c) MS data of AP

Figure A2. UV spectrometry characterization of QO degradation in the 5 mM glutathion (GSH) which is cytoplasm concentration of GSH. (a) UV spectra characterization for the cleavage reaction versus time (QO: 320 nm, AP: 297 nm). Spectra are obtained in every 20
min. (b) A plot of QO (100 μM) absorbance (320 nm) versus time in the presence of the reductant GSH (5 mM).

Figure A3. UV spectrometry characterization of QO stability in the PBS solution. QO solution (100 μM, PBS) is stored in a glass bottle for two weeks under dark condition. The negligible change of the UV absorbance at 320 nm suggests that the QO is very stable in PBS.
Figure A4. UV absorbance spectra of CRRL species. The molecules were dissolved in PBS solution (100 μM). BQ (248 nm, 20,310 M⁻¹cm⁻¹); HQ (288 nm, 2,520 M⁻¹cm⁻¹); QO (320 nm, 18,163 M⁻¹cm⁻¹); AP (297 nm, 2,150 M⁻¹cm⁻¹)