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Selective Immobilization of Peptides Exclusively via N-Terminus Cysteines by Water-Driven Reactions on Surfaces

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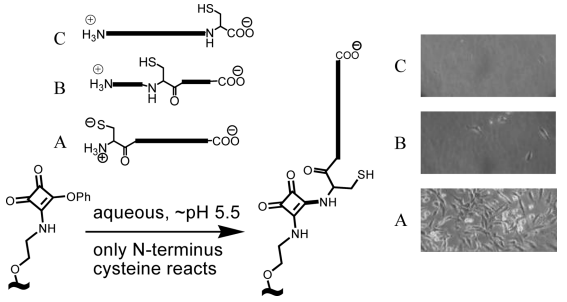

Immobilizing peptides or proteins on bioinert surfaces enables the elucidation of ligand−receptor interaction in complex biological systems. Here, we report a highly chemoselective surface reaction that immobilizes peptides exclusively via N-terminus cysteine residue in a peptide. At pH 5.5, only N-terminus cysteines of peptides couple covalently with phenoxy amino squarate moieties presented on self-assembled monolayers (SAMs) of alkanethiols on gold films. The selectivity of this surface reaction can tolerate the presence of internal cysteines in close proximity to basic residues such as histidines. We demonstrated this selective surface reaction by mammalian cell adhesion and by SAMDI mass spectroscopy of the SAMs.

Immobilization of peptides or proteins on surfaces is broadly useful for deciphering complex biological problems such as identifying new ligand−receptor binding[1−5] and for studying adhesion biology.[6−8] The ideal immobilization reaction should be chemoselective toward the designed functional group in a peptide to control the orientation of the immobilized ligands for optimal binding between the ligands and the targeted receptors.[2,9−14] Many aqueous-compatible chemoselective reactions are potentially suitable for immobilization, including native chemical ligation,[15] aldehyde-based coupling,[16] Staudinger ligation,[17] Diels−Alder reaction,[18] "Click" chemistry,[19,20] maleimide−thiol coupling,[21] and multicomponent reactions.[22,23] Among these reactions, maleimide chemistry is commonly used for peptide immobilization.[24] Because maleimide−thiol coupling does not distinguish the subtle difference in reactivity between cysteines at different positions in a peptide, this method is limited to immobilizing peptides not containing internal cysteines, and requires adding a terminus cysteine into the peptide for immobilization purposes. In a recent study, we discovered a class of chemoselective reactions between cysteine and amino squarate derivatives that is driven optimally by the hydrogen bonds from the solvent−water.[25] Structural study suggests that this reaction may also be facilitated by the more nucleophilic cysteine residues at neutral pH.[25] Here, we describe the use of this reaction to facilitate the immobilization of unprotected peptides exclusively via N-terminus cysteines while tolerating the presence of cysteines at other positions (Figure 1).

We used self-assembled monolayers (SAMs) of alkanethiols on gold films to elucidate this immobilization chemistry. This class of monolayers allows the control of both surface chemistry and the polycrystalline structure of the supporting gold films.[26] The monolayer consists of mixed alkanethiols, one terminated with an amino phenoxy squarate

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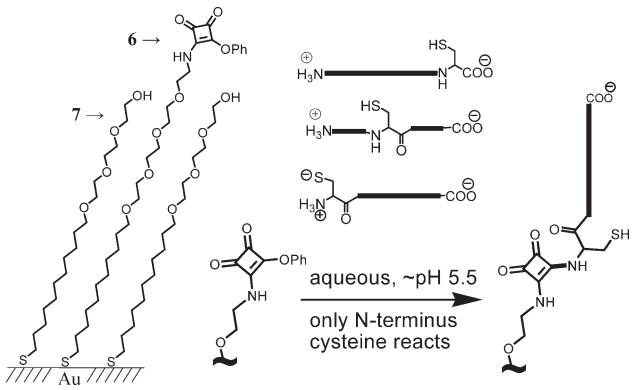
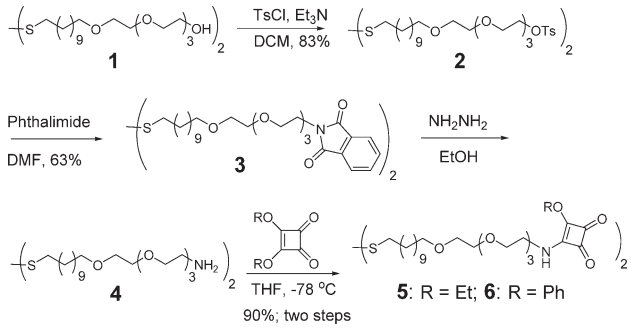


FIGURE 1. Selective immobilization of peptide containing N-terminus cysteine on phenoxy-squarate terminated SAMs.

SCHEME 1



group, **6**, the other with a tri(ethylene glycol) group, **7**.[24] The tri(ethylene glycol)-terminated monolayer provides a bioinert surface that resists nonspecific protein adsorption and cell adhesion,[26] which is used in this work to assess the chemoselectivity of the surface reaction.

Alkanethiol, **6**, was synthesized in four steps in good yields from literature reported disulfide **1** (Scheme 1).[27] Briefly, tosylation of the hydroxyl group of **1** followed by nucleophilic substitution with potassium phthalimide afforded a diphthalimide derivative **3**. Reduction of the phthalimide with hydrazine by the Ing−Manske method[28,29] afforded an amine **4**. Substitution of phenoxy or ethoxy squarate with the amine **4** gave the desired amino ethoxy or phenoxy squarate-terminated disulfides, **5** or **6**, respectively. It is important to note that, unlike other reagents for thiol coupling, the amino phenoxy squarate moiety is stable in the presence of alkanethiols in organic solvents or at neutral conditions in aqueous solutions. Such unique reactivity is advantageous for making SAMs with good control of the surface density of alkanethiols.

To evaluate the chemoselectivity of this reaction, we employed a mammalian cell adhesion assay that depended on the successful coupling between the surface-bound squarate groups and the peptides in the solution. Mammalian cells adhere to a surface via specific ligand−receptor recognition. One of the well-known adhesion bindings is the recognition between membrane protein integrin and a tripeptide Arg-Gly-Asp (RGD) ligand on the proteins in the extracellular matrix.[30−32] In this work, we used peptides containing a RGD tripeptide and a cysteine residue at different positions in the peptide sequence to examine the chemoselectivity between the cysteines for immobilization. As the tri(ethylene glycol) background resists nonspecific cell adhesion and the pH-dependent chemoselective reaction governs the surface conjugation, any observed cell adhesion suggests successful immobilization of the RGD peptides on the surfaces.

Three peptides with cysteine residues located at the N-terminus (N′-**C**AGRGDS-C′), at the C-terminus (N′-AGRGDS**C**-C′), and at the internal position (N′-AG**C**SAGRGDS-C′) were used. Two N-acetylated peptides having N-terminus and internal cysteine residues (Ac-N′-**C**PHAAAARGDS-C′ and Ac-N′-GG**C**PHAAAARGDS-C′) were also included. Whereas the first three peptides are designed to test the reactivity of cysteine at N-, C-terminus and internal positions, acetylation of N-terminus nitrogen evaluates the effect of removing a basic moiety on the reactivity of cysteines. The sequence of the acetylated peptides was chosen such that the thiol group in the cysteine residue may exhibit a low pK_a.[33,34] The presence of a histidine residue in close vicinity of cysteine was also reported to potentially lower the pK_a of the thiol group.[35]

Mixed SAMs presenting low surface density of amino phenoxy squarate were prepared by soaking gold films in DMSO solution consisting of mixed molecules **6** and **7** (mole ratio 3/97). The surface reactions were conducted by soaking the SAMs in phosphate buffered saline (PBS) containing 2 mM of each of the five peptides at pH 7.52, 6.56, and 5.54 for 4 h at ambient temperature. The SAMs were then rinsed with distilled water and dried under a stream of nitrogen, and used for cell culture without delay. For cell culture, we incubated all of the SAMs in the same culture flask containing ~50 000 Swiss 3T3 albino fibroblasts and assessed the cell adhesion by optical micrographs of the SAMs. Direct examination of cell adhesion after 96 h of cell culture showed that cells adhered and proliferated on all of the SAMs treated with peptide at pH 7.52, with more cells on surfaces treated with N- and C-terminus cysteines than on those treated with internal cysteines (Figure 2). For surface reaction conducted at pH 6.56 and 5.54, the cell adhesion and proliferation were only observed on SAMs treated with peptide containing N-terminus cysteines. These results suggest that amino phenoxy squarate on SAMs selectively reacts with N-terminus cysteine in a peptide at slightly acidic conditions, optimized around pH 5.5. At higher pH, all cysteines exhibited reactivities while the cysteine residues at termini are more reactive than the internal ones.

To support the chemoselectivity of the surface reactions inferred from the cell adhesion experiments, we used matrix-assisted laser desorption/ionization and time-of-flight mass spectrometry on self-assembled monolayers (SAMDI-MS)[36,37] to directly characterize the mass of the molecules on SAMs.

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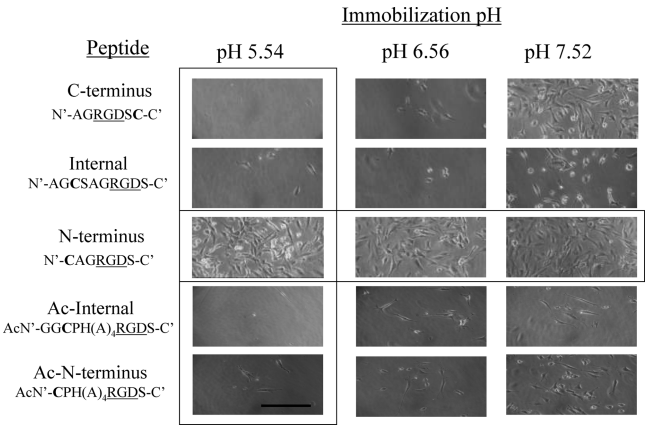

FIGURE 2. Optical micrographs of Swiss 3T3 albino fibroblasts (96 h in culture) on mixed SAMs of **6** and **7**, treated with different peptides at pH 5.54, 6.56, and 7.52 for 4 h at ambient temperature. The pH of the immobilization reaction is listed above the micrograph and the peptide sequence is shown to the left. Scale bar = 38 μm.

The substrate was prepared via identical procedures as for the cell adhesion experiments except that the surface density of squarate-terminated alkanethiols was higher (prepared from 50/50 of **6** and **7** in DMSO) to facilitate mass ionization. After rinsing and drying subsequent to the surface reaction at different pH values, SAMs were treated with matrix 2,4,6-trihydroxyacetophenone (THAP, ~10 mg/mL in acetonitrile) and analyzed by SAMDI, using a reflectron mode and positive polarity to obtain a mass spectrum for each SAM.

Figure 3 shows selected SAMDI mass spectra of the products from the peptide immobilization on the mixed SAMs prepared from alkanethiols **6** and **7** (50/50) in DMSO. For reactions with peptide having N-terminus cysteine at both pH 5.54 and 7.52, we observed the sodium adduct (m/z 1145.40) and the hydrogen adduct (m/z 1456.44) corresponding to the peptide−alkanethiols conjugate on a mixed disulfide of **6** and **7**. For reaction with peptide having C-terminus cysteine at pH 5.54, we only observed the disulfide of unreacted **6** and **7** ($[M+H]^+$ m/z 908.41 and $[M+Na]^+$ m/z 930.37). For reaction conducted at pH 7.52, the masses for both the peptide−alkanethiol conjugate and the corresponding mixed disulfide were observed: $[M + H]^+$ m/z 1122.17, $[M + Na]^+$ m/z 1144.12 (conjugated alkanethiol); $[M+H]^+$ m/z 1456.33, $[M+Na]^+$ 1478.27 (mixed disulfide). We note that SAMs presenting amino *ethoxy* squarate groups, **5**, do not afford any detectable conjugation with any peptides based on both cell adhesion assays and SAMDI experiments.

These masses suggest a pH-dependent chemoselectivity for the immobilization reactions that is consistent with cell adhesion experiments. We attribute this chemoselectivity of the water-driven reaction on surfaces to the high acidity (low pK_a), and thus high nucleophilicity, of the thiol groups of N′-terminus cysteine because the thiol group, in close proximity to the basic amino group, likely forms a zwitterion complex. Both the internal cysteine and acetylated N′-terminus cysteine are not reactive at these slightly acidic conditions. Another important observation is that the presence of basic amino acid residues (histidine) in the peptide does not readily activate the reactivity of internal cysteines, suggesting that sterics may also contribute to the inactivity for internal cysteines.

This reaction appears to be unique because while the thiolate is a better leaving group than the phenoxy group, the thiol addition

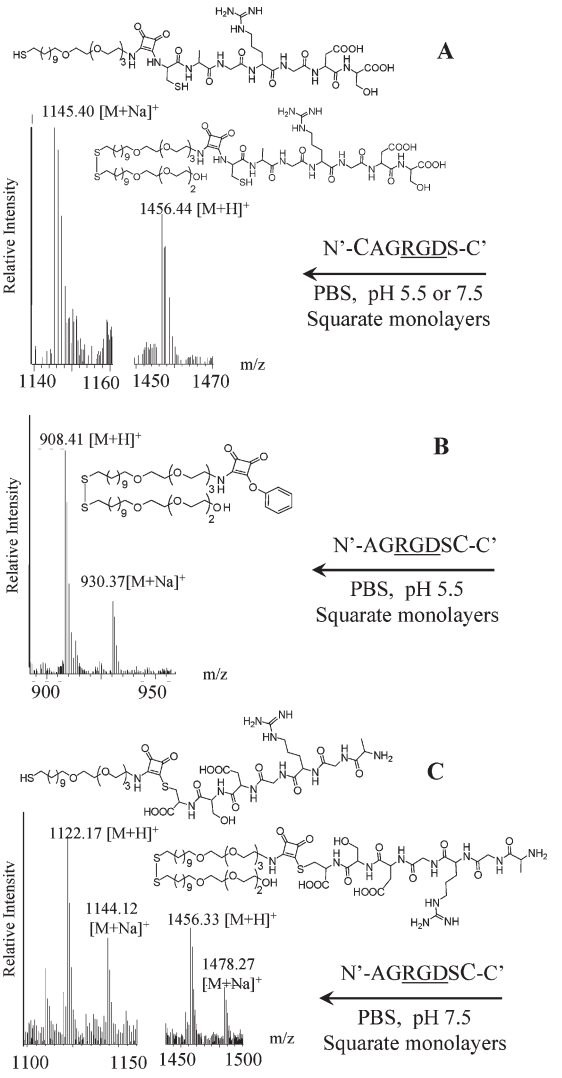

FIGURE 3. SAMDI-MS of monolayers presenting a phenoxy-squarate group treated with (A) peptides with N′-terminus cysteine at pH 5.5 and 7.5, (B) peptides with C′-terminus cysteine at pH 5.5, and (C) peptides with C′-terminus cysteine at pH 7.5.

and phenoxy elimination still proceeds in water. We believe that phenoxylate anions are readily protonated in water at neutral conditions to generate poor nucleophiles, phenols, making the elimination irreversible. A possible mechanism that avoids displacing the phenoxy group by the thiolates violates the Baldwin rule (see the Supporting Information).[38] Because the selectivity of the surface reaction is optimized at pH 5.5, we believe that tri(ethylene glycol)-terminated SAMs may provide an interfacial environment that lowers the pK_a of thiol groups of peptides. We note that free phenoxy squarate molecules in solution also coupled with peptides containing N′-terminus cysteines faster than C′-terminus cysteines (see the Supporting Information).

Many proteins contain functionally important internal cysteines such as proteases,[39] phosphatases,[40] cysteine-rich

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secretory proteins (Crisp),[41,42] G-protein coupled receptors, and potentially many others yet to be discovered.[43] For example, protein tyrosine phosphatases are inhibited by covalent modification of a cysteine at the active site.[44] The thiol activities can also be masked by the existence of disulfide forms in the protein. For instance, the activity of some disulfide-containing proteases is observed only when reducing agent is added.[45,46] Thus, this squarate moiety also provides a class of candidates for developing specific covalent cysteine inhibitors.[44]

To conclude, we demonstrated a highly selective surface reaction that utilizes the unique reactivity of the cysteine residue at the N′-terminus of a peptide. By adjusting the pH of the buffer, the reactivity of the internal cysteines is turned off, leaving only the N′-terminus cysteine reactive for conjugation. This control of selectivity allows the immobilization of peptides or proteins without tampering with existing internal cysteines for biological studies. Different interfaces, such as monolayers presenting nonpolar or aprotic polar groups, may also have an impact on this class of reactions in aqueous solution due to, for example, the difference in acidity of the thiol groups at the interface. As such, this immobilization method is useful for studying interfacial reaction mechanisms.[47]

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Experimental Section

Disulfide of 11-Mercaptoundecyltri(ethylene glycol)ethylamino-4-phenoxycyclobut-3-ene-1,2-dione (6). To a solution of crude amine **4** (120 mg, 0.15 mmol) in anhydrous tetrahydrofuran (5 mL) at −78 °C was added 4 equiv of 1,2-diphenoxycyclobutene-3,4-dione (160 mg, 0.60 mmol). The reaction mixture was then allowed to stir for 1 h at −78 °C and slowly warmed to room temperature. The reaction mixture was stirred for an additional hour at room temperature. The solvent was removed under reduced pressure to obtain a colorless residue. Purification by column chromatography (silica gel), using a gradient solvent system (50% EtOAc in hexane → 100% EtOAc → 10% MeOH in CH_2Cl_2), afforded compound **6** in 90% yield. ^{1}H NMR ($CDCl_3$, 300 MHz) δ 7.39−7.14 (m, 5 H), 4.02−4.01 (br s, 1 H), 3.86−3.59 (m, 16 H), 3.54−3.47 (m, 2 H), 2.86−2.79 (t, 2 H, J = 8.6 Hz), 1.83−1.68 (m, 4 H), 1.52−1.3 (m, 16 H); ^{13}C NMR ($CDCl_3$, 300 MHz) δ 129.7, 129.6, 125.4, 118.0, 71.5, 70.4, 69.8, 69.6, 44.3, 39.1, 29.5, 29.4, 29.4, 29.1, 28.4, 26.0; HRMS m/z calcd for $C_{58}H_{88}N_2O_{14}S_2$ $[M + 23]^+$ 1123.5569, found 1123.5558.

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Supporting Information Available: General experimental procedures and spectroscopic data for the compounds and copies of ^{1}H NMR, ^{13}C NMR, and MALDI-TOF MS spectra. This material is available free of charge via the Internet at http://pubs.acs.org.