Electrochemically Controllable Conjugation of Proteins on Surfaces

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The rational design of surfaces for immobilization of proteins is essential to a variety of biological and medical applications ranging from molecular diagnostics to advanced platforms for fundamental studies of molecular and cell biology. We have developed an advanced electrochemically based approach for site-selective and reaction-controlled immobilization of proteins on surfaces. When a molecular monolayer of 4-nitrothiophenol on gold electrode surfaces is reduced electrochemically in a selective fashion at its nitro groups, to afford amino groups by potentiometric scans, the amine can be employed to orchestrate the immobilization of proteins to the surface. This protein immobilization strategy could allow one to fabricate intricate protein structures on surfaces for addressing fundamental and applied problems in biology and medicine.

INTRODUCTION

The controlled immobilization of proteins on surfaces is of paramount importance for a wide variety of applications, ranging from the development of model substrates for mechanistic studies of cell behavior to high-throughput protein assays for drug discovery, clinical diagnostics, and proteomics (1–7). In particular, studies by the use of DNA arrays in genomics (1), substantial scientific and commercial interest has been generated in the use of protein arrays as tools for proteomics (1, 2). However, proteins are much more chemically and structurally diverse than nucleic acids, making them intrinsically more complicated to be immobilized on surfaces (1, 2). Other difficulties result from the intrinsic fragility of proteins, which have modest conformational stability and can be damaged upon surface immobilization (1, 2). Moreover, proteins can adhere and adsorb to most surfaces through a variety of mechanisms (electrostatic interaction, hydrogen bonding, hydrophobic interactions, and/or a combination of these), resulting in nonspecific protein binding (8).

Therefore, a primary enabling technology is the ability of controlling the immobilization of proteins on surfaces while avoiding nonspecific protein binding and retaining protein native biological features and properties (1–3, 5–7). Molecular surface science has greatly contributed to the advancement of this technology by providing ideal platforms for bioengineering surfaces on a molecular level (9–15). For instance, self-assembled monolayers (SAMs), which form spontaneously by the adsorption of an active surfactant onto a solid surface, possess important properties of self-organization and adaptability to a number of technologically relevant surface substrates. The properties of a SAM (thickness, structure, surface energy, stability) can be easily controlled, and specific functionalities can also be introduced into the building blocks. For instance, SAMs of thiol on gold and triethoxysilanes on silicon dioxide (SiO2) have been exploited (6, 8, 16–18) to provide the surfaces not only with active groups that interact with specific moieties of the protein to be immobilized, but also with protein-resistant groups such as poly(ethylene glycol) (PEG).

Proteins have been immobilized onto these surfaces by either adsorption of the proteins (8), molecular recognition between the proteins and immobilized ligands (19), or covalent coupling to the SAM surface (16, 17). The most common conjugation methods rely on the covalent attachment of proteins to “static” (20) surfaces chemically modified with different protein-reactive groups, including activated esters (16), aldehydes (21), or maleimides (22–24). On the other hand, strategies for “turning-on” protein reactive groups at SAM surfaces by an “on demand” surface reaction using an external stimulus have great potential as versatile platforms for protein immobilization. Similar strategies have been employed to immobilize urease onto a polymer film (25), as well as other biomolecules, such as DNA (26) and peptides (27, 28) on SAM surfaces, yet protein immobilization onto a SAM using this “on demand” surface reaction approach has not been previously demonstrated.

Here, we report an approach to control protein immobilization on surfaces based on SAMs that can switch from bio-inert to bioactive states in response to an applied electrical potential. The approach we have developed is based on an electrochemical reaction in which aromatic nitro (NO2) groups, self-assembled on gold surfaces, can be chemically modified by a redox process to amino (NH2) groups, which can then be used to orchestrate the immobilization of the proteins. Our strategy involves (Figure 1) five steps: (1) functionalization of the electrically addressable gold surfaces with NO2-terminated SAMs; (2) spatially selective electrochemical reduction (26, 29–31) of the NO2 groups.

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to primary NH\textsubscript{2} groups; (3) selective reaction of the resulting NH\textsubscript{2}-terminated monolayers with a homobifunctional linker; (4) covalent immobilization of primary antibodies; (5) visualization of these bound primary antibodies using fluorescently labeled secondary antibodies. Fluorescence microscopy and surface plasmon resonance (SPR) spectroscopy were employed to characterize the immobilization of antibodies and their selective assembly to the modified areas of the SAM.

**EXPERIMENTAL PROCEDURES**

**SAM Preparation.** Substrates with 30 nm thick Au employing a 10 nm thick Ti adhesion layer on an n-type Si wafer were fabricated by electron-beam evaporation. The Au substrates were cleaned with piranha solution (3:1, H\textsubscript{2}SO\textsubscript{4}/30\% H\textsubscript{2}O\textsubscript{2}), rinsed with ultra high purity (UHP) H\textsubscript{2}O (resistivity = 18 M\textOmega \text{cm}) and then HPLC-grade EtOH thoroughly for 1 min. (Caution: Piranha solution reacts violently with all organic compounds and should be handled with care). Subsequently, the substrates were immersed in a 0.1 mM ethanolic solution of 4-nitrothiophenol (Sigma-Aldrich) for 12 h to form the SAMs on the Au surfaces. The substrates were rinsed with EtOH and UHP H\textsubscript{2}O.

**Electrochemical Conversion.** The cyclic voltammograms (CVs) of the NO\textsubscript{2}-terminated SAMs and their conversion on the Au electrodes were performed using a Princeton Applied Research VMP multichannel potentiostat with a custom-designed Teflon cell, equipped with the functionalized Au substrate as the working electrode, a Pt wire as the counter electrode, and the standard calomel electrode (SCE) as the reference electrode. The planar gold working electrode exposes a circular geometric area of 50 mm\textsuperscript{2} to the electrolyte solution. Cyclic voltammetry was carried out at a scan rate of 200 mV/s.

**Protein Immobilization.** The pristine 4-nitrothiophenol and converted 4-nitrothiophenol SAMs were incubated with a 5 mg/mL solution of bis(sulfosuccinimidyl)suberate (BS\textsuperscript{3}; Pierce) in phosphate buffered saline pH 7.4 (PBS) for 20 min at room temperature. Samples were rinsed with PBS (∼1 min) and incubated with a primary mouse anti-human vascular endothelial growth factor (VEGF) antibody (Biosource) with a concentration of 25 µg/mL in PBS containing 0.1% Triton X-100 (PBS–Triton) for 20 min. The samples were rinsed with PBS buffer solution for 10 min and incubated in the dark for 20 min in a 20 µg/mL PBS–Triton solution of Alexa Fluor 568 goat anti-mouse IgG antibody (Molecular Probes). The substrates were rinsed with PBS for 10 min and mounted for fluorescence microscopy. For the control samples, the pristine 4-nitrothiophenol and converted 4-nitrothiophenol SAMs were stained as described above, without first immobilizing the BS\textsuperscript{3} homobifunctional linker.

**X-ray Photoelectron Spectroscopy (XPS).** XPS experiments were performed in an Omicron XPS/UPS system using a monochromatic Al K\textsubscript{α} X-ray source (1486.6 eV) with a pass energy of 50 eV. Fitting of XPS peaks was performed using the Avantage v2.2 processing software. All spectra were fitted using 30\% Lorentzian/70\% Gaussian peaks.
Figure 2. CV (blue curve) of a SAM of 4-nitrothiophenol—the reduction of NO₂ to NH₂ groups appears as a pronounced peak at around −0.85 V. CV (red curve) of the SAM of 4-nitrothiophenol after the reductive scan.

**Fluorescence Microscopy.** Fluorescence images were collected on a Zeiss Axiostar 200 fluorescence microscope, equipped with an AxioCam MRm monochrome camera. Pictures were acquired using AxioVision LE 4.1 with identical exposure parameters and analyzed using Image J 1.33u (NIH). No postexposure image processing was performed.

**Surface Plasmon Resonance.** SPR spectroscopy experiments were performed with a Biacore X at 25 °C at a flow rate of 2 µL/min. Two different monolayers—pristine 4-nitrothiophenol and converted 4-nitrothiophenol—were prepared on Biacore gold sensor chips. Prior to protein binding studies, 40 µL of a 5 mg/mL solution of the BS³ homobifunctional linker in PBS, was injected into the gold sensor, followed by a PBS–Triton washing for 10 min. The functionalized gold surfaces (either pristine or converted) were subsequently exposed to a primary antibody by injection of 40 µL of anti-streptavidin produced in rabbit (Sigma) at a concentration of 1.4 mg/mL in PBS–Triton. The protein solution was replaced with the PBS–Triton solution for 25 min to remove any unbound primary antibody. Subsequently, a 40 µL sample of the secondary antibody, Alexa Fluor 488 goat anti-rabbit IgG (Molecular Probes), with a concentration of 20 µg/mL in PBS–Triton was injected, followed by a 10 min PBS–Triton washing.

**RESULTS**

Cyclic voltammetry (CV) was used to investigate the characteristics of the SAMs and for the chemical conversion (NO₂ → NH₂) on the gold surfaces. The CV study of the NO₂-terminated SAMs was carried out in three subsequent CV scans. A 0.1 M KCl solution (9:1, EtOH/UHP H₂O) (26) was employed as the electrolyte to check the characteristic CV peak that the NO₂ group exhibits (blue curve in Figure 2) (29). Next, a second scan was carried out in a 0.1 M KCl aqueous solution to ensure (29) an adequate supply of Na⁺ ions for the complete electrochemical reduction of the NO₂ to NH₂ groups. Finally, the disappearance of the NO₂ group-related peak was observed by running a third scan (red curve in Figure 2) in a 0.1 M KCl solution (9:1, EtOH/UHP H₂O). The potential was swept from 0 to −1.0 V with respect to the SCE. The reductive peak of the NO₂ group was observed at −0.85 V (blue curve in Figure 2). By integrating the area under the reductive peak, the SAMs density was found to be 2.6 × 10¹⁴ molecule/cm². After the reduction of the NO₂ groups during the second scan, no redox activity was observed (red curve in Figure 2), implying complete conversion of the NO₂-terminated SAMs.

High-resolution XPS spectra were acquired on the gold electrodes before and after surface electrochemical modification. XPS measurements confirmed the conversion of the NO₂ groups (N(1s) binding energy = 405.6 eV; blue curve in Figure 3) to the NH₂ counterparts (N(1s) binding energy = 399.6 eV; red curve in Figure 3). XPS of the pristine 4-nitrothiophenol surfaces also indicated the presence of primary amines. This behavior can be attributed (32, 33) to the gradual reduction of the NO₂ groups to NH₂ groups (Figure 3; blue curve) during X-ray irradiation initiated by the photoelectrons and secondary electrons emitted from the surface. However, in the electrochemically converted NO₂-terminated SAM, the XPS of the N(1s) binding energy region indicated only the presence of the NH₂ group with a strong peak at 399.6 eV.

Following the XPS confirmation of the efficient electrochemical reduction, the viability of this strategy for the site-selective assembly of proteins on gold surfaces was demonstrated first by fluorescence microscopy. After performing electrochemical conversion on large-scale electrodes (converted area: 50 mm² on a 1 cm² gold surface area), the electrochemically generated NH₂ groups were activated with the BS³ homobifunctional linkers to expose amine-reactive N-hydroxysulfosuccinimide (Sulfo-NHS) groups. Subsequently, the primary mouse anti-human VEGF antibody was allowed to react with the modified gold surface, followed by incubation with a red fluorescent secondary antibody. Detection of the binding of the primary antibodies to the electrochemically modified surfaces was evaluated by fluorescence microscopy. Figure 4a,b illustrates the fluorescence microscope images collected from the NO₂- and NH₂-terminated regions of the surface, respectively. The fluorescence images of the regions terminated by the amino groups (Figure 4b) clearly revealed high fluorescence intensity, while the unmodified areas (NO₂ groups) showed minimal fluorescence. The signal-to-noise ratio (specific binding on NH₂-terminated regions/nonspecific binding on NO₂-terminated regions) was determined to be 26, a ratio which demonstrates the high degree of biological specificity to the NH₂ surface over the NO₂ surface.

Control experiments were also established to verify that the selective immobilization of proteins on the NH₂-terminated regions relied on the formation of amide bonds first between
tion was observed for the NO$_2$-terminated monolayers.

Specific adsorbed on the gold surface and thus are likely surface. These data establish that the proteins are not nonspecifically no protein desorbed from the converted monolayer buffer was passed through the system for approximately 30 min, converted NO$_2$-terminated monolayers, particularly after wash.

results contrasted with the negligible nonspecific protein binding compared to 8 ng/cm$^2$ on the pristine NO$_2$ monolayer regions.

LITERATURE CITED


CONCLUDING REMARKS

The NO$_2$-terminated groups in the SAMs of 4-nitrothiophenol on gold surfaces can be reduced electrochemically and selec- tively to NH$_2$ groups by applying a negative voltage between the addressed electrode and its counter electrode in the presence of an electrolyte. By employing the homobifunctional activated ester linker, proteins can then be attached specifically to the NH$_2$ monolayer region. We have demonstrated that immobilized antibodies exhibit high affinity and selectivity for these NH$_2$ regions, after activation, as monitored by fluorescence microscopy and surface plasmon resonance spectroscopy. This strategy can potentially be extended to immobilize sequentially a series of different proteins onto selected gold nanoelectrodes by carrying out repeated cycles with the entirely aqueous solution-based, and hence bio compatible, electrochemical and protein immobilization processes. Efforts in this direction are underway.

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