Diels−Alder Reaction for the Selective Immobilization of Protein to Electroactive Self-Assembled Monolayers

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The immobilization of biologically active molecules is important for preparing substrates used in diagnostic assays, high-throughput drug discovery, and attached cell culture.[1] A wide variety of methods are available, and the choice for a particular application depends on the relative importance of many factors, including selectivity and efficiency of the coupling reaction, the stability of the resulting complex, and the suitability for preparing patterned arrays of ligands. No single method meets all of these criteria. The complex of streptavidin with biotin is overall the best available method, but it has the limitations that the complex is large and intrusive, and nonspecific adsorption of the protein can be problematic.[2] Many chemical methods, including the condensation of amines with activated carboxylic acids or with aldehydes, are convenient and applicable to most molecules but are limited by a lack of selectivity.[3a,b] Others, including the coordination of Ni(II) complexes with oligo(histidine) motifs, have excellent selectivity but often lack long-term stability.[3c] In this paper we use self-assembled monolayers (SAMs) that present a quinone group to demonstrate that the Diels−Alder (D−A) reaction of this group with cyclopentadiene (cp) is an excellent method for immobilization. This design also permits the reactivity of the quinone to be modulated either chemically or electrochemically, by way of reduction to the hydroquinone which does not participate in the D−A reaction.

We first investigated the kinetic behavior for the D−A reaction of cp with quinone attached to a monolayer (Figure 1). Cyclic voltammetry of a mixed SAM presenting hydroquinone (HQ) and hydroxyl groups ($\chi_{HQ} = 0.25$)[4] showed that the HQ undergoes oxidation at 220 mV to give the quinone (Q) and reduction at −150 mV.[5,6] Voltammograms over 50 consecutive cycles were indistinguishable and showed the oxidation was reversible. When cp was added to the electrolyte, consecutive voltammograms showed a decrease in the peak current for both reduction and oxidation (Figure 2). Several observations confirm that the loss in current was due to the D−A reaction of cp with Q.[7] The addition of other dienes (including cyclohexadiene and 1-cyclopentadienyl methyl acetate) to the electrolyte gave similar losses in current over consecutive cycles (although with different rates), but the structurally related cyclopentane and cyclopentene had no effect over the same number of scans. Immersion of a monolayer presenting HQ groups in electrolyte containing cp (15 mM) for 20 min had no effect on the voltammograms, demonstrating that only the Q underwent reaction with cp. Grazing angle FTIR spectroscopy further supported the reaction of Q with cp. A monolayer presenting Q groups showed a carbonyl stretching mode at 1663 cm^{-1}. After reaction with cp, this band was absent, and a band at 1653 cm^{-1} corresponding to the carbonyl stretching mode of the D−A adduct was observed.

Figure 3 shows a plot for the loss in peak current for the reduction of Q versus time for the data shown in Figure 2. Because the concentration of cp was much greater than that of immobilized Q, the data could be fit to an exponential decay to obtain a pseudo-first-order rate constant, k':

$$I_t = I_f + (I_o - I_f) \exp^{-k't}$$

where I_t is the peak current at time t, I_o is the initial peak current and I_f is the residual nonfaradaic current. The excellent fit of the experimental data to this equation indicates that the Q groups are sufficiently isolated on the monolayer that the reactivity is independent of the extent of reaction. We next repeated this experiment with concentrations of cp ranging from 0.76 to 58 mM and in all cases found that the loss in peak current was described well by an exponential decay and that the reaction always proceeded to completion. The pseudo-first-order rate constants increased linearly with the concentration of diene: the slope of the best-fit line (Figure 3, inset) provided a second-order rate constant of $k_{DA} = 0.26\ M^{-1}\ s^{-1}$, after adjustment for the fraction of time that Q was present. It is striking that this interfacial reaction is kinetically well-behaved.[8]

We used the association of streptavidin to immobilized biotin as a model system with which to demonstrate the D−A-mediated immobilization of protein.[9] To avoid problems with nonspecific protein adsorption, we instead used monolayers prepared from alkanethiolates terminated in tetra(ethylene glycol) groups (**1**), which resist entirely the adsorption of protein,[10] and an extended linker to the HQ (**2**).[11] Monolayers having $\chi_2 = 0.05$ were immersed in an aqueous solution of 1,4-benzoquinone for 10 min to oxidize the HQ groups to the Q.[12] These substrates were then treated with a conjugate of cp and biotin (**3**, 10 mM in 1:1 THF: H_2O) for 2 h to immobilize biotin. We used surface plasmon resonance (SPR) spectroscopy to characterize the binding of streptavidin to the immobilized biotin.[13] Figure 4 shows that streptavidin associated with a monolayer to which biotin was coupled and that this association was irreversible. When streptavidin was presaturated with biotin (0.8 mM), the binding of the

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(4) χ_{HQ} is the fraction of alkanethiolates in the monolayer that present HQ and is determined by integrating the waves in cyclic voltammograms of the monolayer.

(5) For previous reports of the electrochemistry of SAMs presenting HQ groups, see: Hickman, J. J.; Ofer, D.; Laibinis, P. E.; Whitesides, G. M.; Wrighton, M. S. *Science* **1991**, *252*, 688−691. Ye, S.; Yashiro, A.; Sato, Y.; Uosaki, K. *J. Chem. Soc., Faraday Trans.* **1996**, *92*, 3813−3821.

(6) Cyclic voltammetry was performed with a Bioanalytical Systems CV-50W potentiostat using a cell with the gold/SAM as the working electrode, platinum wire as the counter electrode, and Ag/AgCl as the reference electrode.

(7) The tautomerization of the D−A adduct, which would yield a redox-active quinone, requires strongly acidic or basic conditions and does not proceed under the conditions employed here. See: Meinwald, J.; Wiley: G. A. *J. Am. Chem. Soc.* **1958**, *80*, 3667−3670.

(8) The solution reaction of 1-methyl-benzoquinone with cp in 1:1 THF: H_2O proceeded at least 300 times faster than did the interfacial reaction. We are exploring the basis for the difference in rates.

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(10) Mrksich, M.; Whitesides, G. M. In *Chemistry and Biological Applications of Polyethylene Glycol*; ACS Symposium Series 680, American Chemical Society: Washington, DC, 1997; p 361 and references therein.

(11) Alkanethiols **1** and **2** were synthesized in three and six steps, respectively. Conjugate **3** was synthesized in seven steps. All intermediates gave satisfactory ^1H NMR spectra. Details will be described in a subsequent full report.

(12) We used a chemical oxidant because the instrument for SPR is not compatible with electrochemistry. The ability to use chemical oxidants and reductants to interconvert the HQ and Q also expands the utility of this method to nonconducting substrates.

(13) For examples of the use of SPR to measure the association of proteins with monolayers, see: Mrksich, M.; Grunwell, J. R.; Whitesides, G. M. *J. Am. Chem. Soc*. **1995**, *117*, 12009−12010. Houseman, B. T.; Mrksich, M. *Angew. Chem., Int. Ed.* **1999**, *38*, 782−785.

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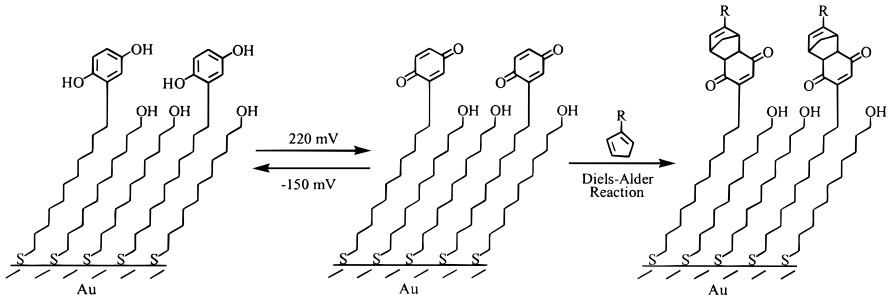


Figure 1. Reversible electrochemical oxidation of a monolayer that presents the HQ group affords a monolayer that presents the Q group, which can in turn react with cp to give the D−A adduct. The figure shows only one possible product. Other products can arise through sigmatropic isomerization of the cp prior to D−A reaction.

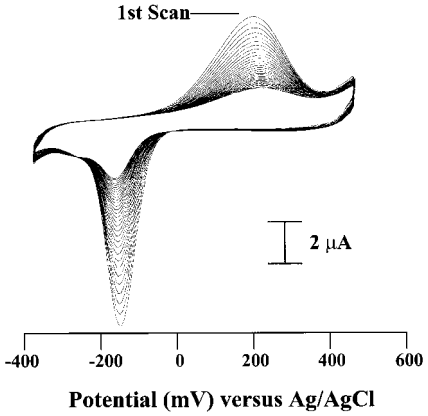
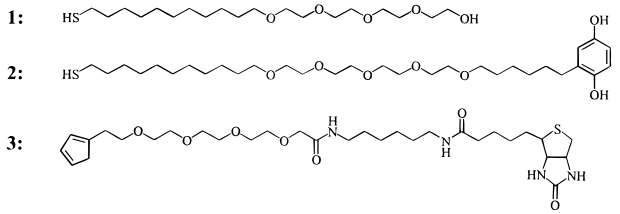


Figure 2. Consecutive cyclic voltammograms of a monolayer presenting HQ groups ($\chi_{HQ} = 0.25$) and hydroxyl groups in 1:1 THF:H_2O containing Na_2PO_4 (2mM), NaCl (75 mM), and cp (7.6 mM) at a scan rate of 25 mV/s.

protein to the surface was prevented and demonstrated that the association was biospecific. Furthermore, streptavidin did not immobilize to monolayers that presented either Q or HQ groups mixed with glycol groups.[14]



The D−A reaction described here provides an attractive and flexible method for bio-immobilization and is especially well-suited for tailoring monolayers with peptides, carbohydrates, and other low-molecular weight ligands.[15] Because the reaction is kinetically well-behaved, this method can be used to quantitatively attach groups in low densities (<1%) where the direct determination of density is not straightforward. This method also allows for the sequential immobilization of several ligands to a common substrate, with independent control over the density of each ligand. This chemistry further makes possible a class of dynamic substrates for attached cell culture, wherein the immobilization of biologically active ligands can be turned on at discrete times. This and related electroactive substrates[16] will provide an exciting range of tailored substrates for studies in cell biology and applications in biotechnology.

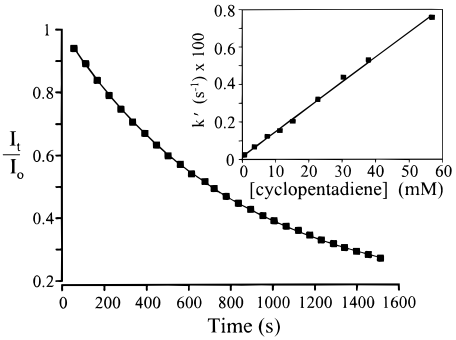


Figure 3. A plot of the peak current for reduction of immobilized Q versus time (I_t is the peak current at time t and I_o is the peak current for the first scan). The loss of current is due to conversion of the Q to the D−A adduct, which is not redox active. The data were fit to an exponential decay (see text) to provide a pseudo-first-order rate constant (k'). The inset shows the relationship of k' and concentration of cp. The slope of the best-fit line gives the second-order rate constant (k_{DA}).

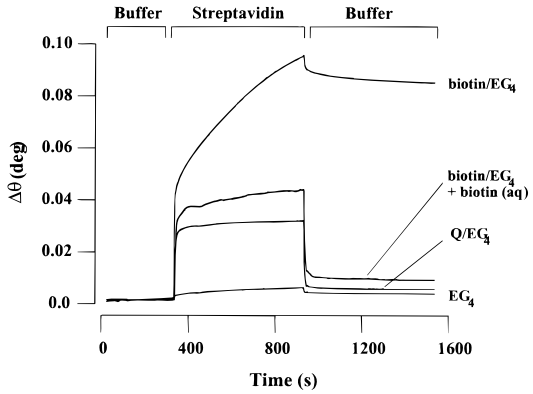


Figure 4. Data from SPR for the immobilization of streptavidin to a monolayer presenting tetra(ethylene glycol) groups and biotin (see text for details). A solution of PBS was flowed over the monolayer for 5 min and replaced with a solution of streptavidin (5 μg/mL) for 10 min and then with PBS for 10 min. The addition of biotin to the buffer containing streptavidin prevented immobilization (biotin/EG_4 + biotin). Streptavidin did not immobilize to monolayers presenting quinone and glycol groups (Q/EG_4) or glycol groups alone (EG_4). The data are reported as the shift in resonance angle (θ). An increase in θ of 0.10° corresponds to a density of adsorbed protein of 1 ng/mm^2.

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(14) The magnitude of change in θ when streptavidin is flowed over the monolayer presenting quinone and glycol groups is greater than that observed with monolayers presenting only glycol groups and shows that the protein adsorbs to the mixed monolayer. This adsorption does not result in immobilization of streptavidin, since it is weak and rapidly reversible when the protein-containing solution is replaced with buffer.

(15) SAMs are currently the best available class of model substrates for studies in biointerfacial science. For reviews, see: Mrksich, M. *Cell. Mol. Life Sci.* **1998**, *54*, 653−58. Mrksich, M.; Whitesides, G. M. *Annu. Rev. Biophys. Biomol. Struct.* **1996**, *25*, 55−78.

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