

Regenerable Biosensor Platform: A Total Internal Reflection Fluorescence Cell with Electrochemical Control

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A new biosensor platform that provides simultaneous fluorescence detection and electrochemical control of biospecific binding has been developed and investigated using antibody–antigen and streptavidin–biotin interactions. Specifically, biotin was covalently bound to a transparent indium–tin oxide (ITO) working electrode, which also served as an integral part of a total internal reflection fluorescence (TIRF) flow cell. TIRF was used to monitor biospecific interactions, while electrochemical polarization was employed to control interactions between biotin and streptavidin or between biotin and anti-biotin antibodies. Both streptavidin and polyclonal anti-biotin antibodies bound kinetically irreversibly to the biotinylated surface. In the absence of electrochemical control, the assay exhibited an extremely slow release of the bound analytes, causing poor regeneration ability of the biosensor surface. However, electrochemical polarization was found to stimulate dissociation of kinetically irreversibly bound biotin–streptavidin and antibody–antigen complexes. A “square wave” polarization function stimulated dissociation more effectively than a “saw tooth” function over the same voltage range. Application of the square wave polarization resulted in regeneration of an active biotinylated surface. Electrochemical polarization also modified affinity and kinetics of protein adsorption, which could likely be used to promote biospecific interactions and/or to suppress nonspecific adsorption.

Interactions between biomolecules are of fundamental importance for living systems. An understanding of interaction mechanisms and the capability to control these interactions are crucial for determination of structure–function relationships and protein crystallography,¹ drug design and development of targeted drug delivery systems,² and biomolecular engineering and design of biosensors.³ Total internal reflection fluorescence (TIRF) has proven to be a well-suited technique for investigating interfacial biomolecular interactions. TIRF has been coupled with immu-

noassays to build various configurations of biosensors.^{3–6} Both planar and cylindrical TIRF waveguides have been used in conjunction with immunoassays. Recently, development of a planar TIRF immunosensor with a detection limit of 10^{-15} M has been reported.⁷ However, poor reversibility of this system hampers its application. Quantitative analytical measurements performed with immunosensors require either regeneration of the sensing surface or quantitation based on a series of measurements with disposable units.

To develop a sensitive biosensor, a high-affinity biospecific interaction is generally required. Association rate constants for most antibodies, k_a , have been shown to vary no more than 1 order of magnitude.⁶ However, the dissociation constants, k_d , vary 1000-fold. Therefore, the affinity constant, $K_f = k_a/k_d$, is determined primarily by the k_d value rather than by k_a . Additionally, surface immobilization of the immunoassay typically results in a decreased dissociation rate constant. Thus, a sensitive immunosensor with a large k_a is commonly not a true linear sensor but rather a simple binary detector, since it cannot respond rapidly to changes in analyte concentration.

Regeneration of the sensing surface is an important but difficult task. The use of extreme pH, temperature, and chaotropic agents to dissociate the antibody–antigen complexes is often followed by a significant loss of biospecific activity. On the other hand, a higher dissociation rate is associated with lower affinity and poor sensitivity. Consequently, the majority of sensitive biosensors are disposable devices, and quantitation is typically obtained using multiple single-use sensors.

The biotin–streptavidin bond, which is among the strongest noncovalent biospecific interactions known, gives an example of total kinetic irreversibility. The affinity constant of biotin–streptavidin in solution has been reported to be as high as $K_f \approx 10^{15} \text{ M}^{-1}$.⁸ To date, biotin–avidin and biotin–streptavidin

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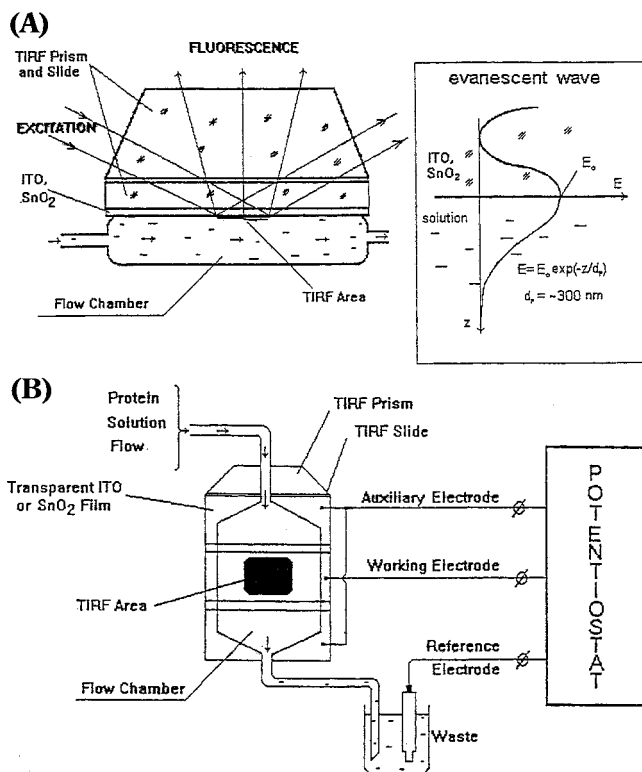


Figure 1. (A) Optical diagram of the TIRF flow cell with a transparent indium–tin oxide (ITO) or tin dioxide electrode deposited on the TIRF surface. Inset: Evanescent wave penetrating from ITO or SnO₂ into the aqueous solution. (B) Electrochemical scheme.

technology provides an advanced, versatile tool for designing numerous types of biosensors. However, systems based on biotin–avidin and biotin–streptavidin interactions are inherently single-use devices (with respect to the biotin–avidin and biotin–streptavidin bond), since biotin–avidin and biotin–streptavidin complexes are stable to extreme pH, temperature, and even chaotropic agents. However, a surprising example of lability of a biotin–streptavidin bond during transcription of biotinylated DNA bound to streptavidin beads has been recently reported.⁹

Recent investigations using a TIRF flow cell equipped with a transparent SnO₂ electrode have demonstrated the capability of electrochemical polarization (EP) to stimulate desorption of irreversibly adsorbed protein.¹⁰ EP imposed by steps was observed to be more efficient for surface regeneration in protein adsorption experiments than slow linear EP changes over the same voltage range. In this study, this approach has been adapted for regeneration of a TIRF immunosensor surface, as well as to stimulate dissociation of streptavidin bound to a biotinylated surface.

EXPERIMENTAL SECTION

Total Internal Reflection Fluorescence Coupled with Electrochemistry. Figure 1A depicts an optical diagram of the experiment. The principles of TIRF are well documented in the

literature.^{11–13} Penetration depth of the evanescent wave, d_p , in this work was approximately 300 nm. The TIRF cell was used in conjunction with an SLM-Aminco AB-2 fluorescence spectrophotometer. An excitation maximum wavelength of 493 nm with a 4-nm band-pass and a 522-nm emission maximum with a 8-nm band-pass (493/4/522/8) were used for FITC–IgG and BODIPY–IgG conjugates, and 520/4/550/8 was used for streptavidin–Oregon Green conjugates. Absolute values of IgG or streptavidin surface concentrations were estimated in this study using the TIRF signal calibration for bovine serum albumin (BSA) from previous work.¹⁵ Fluorescence intensities for adsorbed IgG and streptavidin are presented in Figures 4–6 in arbitrary units, where 1 unit roughly corresponds to a monolayer of IgG or streptavidin coverage, respectively.

For the TIRF electrochemistry (TIRF-EC) experiments, the TIRF flow cell was combined with a three-electrode system as described previously.¹⁰ A Bioanalytical Systems (BAS) cyclic voltammograph (model CV-27) controlled the electrochemical system as shown in Figure 1B. Electrode potentials were measured relative to a saturated Ag/AgCl reference electrode.

Transport of the analyte to and from the sensing surface is a critical aspect of this experimental design. Since the association process for biotin–streptavidin and antibody–antigen interactions was extremely rapid, reassociation of any unbound protein after disrupting these bonds was likely to occur if the diffusion and convection flow did not remove the analyte from the surface vicinity. Therefore, special attention was given to the characterization of transport conditions in the flow chamber. Gravity flows of protein solutions and pure buffer were directed into the TIRF cell by PTFE tubing and were switched by a PTFE three-way valve. The dead volume of the flow system was approximately 10 μ L, while the volume of the TIRF cell flow chamber (1 cm \times 2 cm \times 0.015 cm) was approximately 30 μ L. Due to the small thickness of the TIRF flow compartment ($b = 0.15$ mm), a relatively high wall shear rate, γ , was attained in the flow chamber with width $w = 1$ cm at a volumetric flow rate, V , of 10 mL/min:

$$\gamma = 6V/wb^2 = 4400 \text{ s}^{-1} \quad (1)$$

At this shear rate, convection and diffusion establish a steady-state concentration profile of protein at the TIRF surface within a short time, τ , given by eq 2:¹⁶

$$\tau = 3L^{2/3}\gamma^{-2/3}D^{-1/3} \cong 4 \text{ s} \quad (2)$$

where L is the distance from the flow chamber entrance and D is the translational diffusion coefficient of IgG ($D = 4 \times 10^{-7}$ cm² s⁻¹). When $L = 2$ cm, the steady-state profile of the IgG concentration develops during the transient time of approximately 4 s. Since the translational diffusion coefficient of streptavidin is

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greater than that of IgG, the transient time for this protein is less than 4 s. After the transient period, the concentration profile has a uniform cross section within the flow chamber, and diffusion–convection provides fast transport of the analyte to and from the TIRF surface. It was experimentally verified that the lag time of the TIRF flow system does not exceed 4 s at a solution flow rate greater than 10 mL/min. All kinetic data in the present work were obtained under conditions where increased shear rate did not result in an increase in the interfacial rate. This means that the rate of protein transport was always greater than the observed adsorption or desorption rates.

Reagents. Phosphate-buffered saline (PBS), pH 7.4, was purchased from Sigma Chemical Co. and used as a buffer in all experiments unless otherwise stated. Bovine serum albumin (BSA, product no. A-4378, crystallized, >97% purity), human γ -globulin (product no. G-4386, electrophoretic purity approximately 99%), and fluorescein isothiocyanate (FITC, isomer I, >90% purity) were also purchased from Sigma. The reagent for amination of the metal oxide surfaces, (3-((*N*-(2-aminoethyl)-amino)propyl)trimethoxysilane, was purchased from United Chemical Technologies, Inc. (Bristol, PA). The biotinylation agent, 6-((biotinoyl)amino)hexanoic acid, succinimidyl ester, sodium salt (sulfo-NHS-LC-Biotin), the streptavidin conjugate with fluorescent label (Oregon Green 500), and the pH-insensitive fluorescent label, BODIPY (*N*-(4,4-difluoro-5,7-diphenyl-4-bora-3a,4a-diaza-*s*-indace-3-propionyl)-*N*-(iodoacetyl)ethyleneamine) were purchased from Molecular Probes, Inc. (Eugene, OR). Polyclonal anti-biotin antibodies (product no. 31852) were purchased from Pierce (Rockford, IL).

Protein–Fluorophore Conjugation. FITC-labeled anti-biotin antibodies, IgG–FITC, and γ -globulin–FITC conjugates were prepared according to a modified procedure described earlier.¹ To prepare the antibody–BODIPY and γ -globulin–BODIPY conjugates, a 5-fold molar excess of BODIPY was dissolved in dimethyl sulfoxide, and 10 μ L of BODIPY solution was mixed with 1 mL of 1 mg/mL antibody solution in PBS. The labeling reactions were carried out for 30–50 min at room temperature. To remove unreacted FITC or BODIPY, the reaction mixtures were then dialyzed in excessive amounts of PBS using dialysis cassettes purchased from Pierce. The conjugate solutions were stored at 4 °C and used within 2 days of preparation. The molar ratios of FITC: γ -globulin, FITC:IgG, FITC:antibody, BODIPY: γ -globulins, BODIPY:IgG, and BODIPY:antibody were determined spectrophotometrically and ranged from 0.7 to 0.9. The streptavidin–Oregon Green conjugate was reconstituted in PBS from lyophilized powder as received.

Chemically Modified Electrodes. Indium–tin oxide (ITO) coatings of optical quality were produced by Optical Components, Inc. (Covina, CA). ITO films were deposited directly on TIRF quartz slides and were approximately 350 nm thick, with a resistivity of 12 Ω /cm², refractive index of 1.95–2.00, and a 9:1 In:Sn atomic composition, as specified by the manufacturer. The ITO film on the TIRF slides was divided by etching two separate sections to form areas of working and auxiliary electrodes, as shown in Figure 1B.

To prepare ITO surfaces for biotinylation, TIRF slides with ITO coatings were treated for 20 min in hot chromic acid (80 g/L K₂Cr₂O₇ in 96–98% sulfuric acid, 70 °C), rinsed in deionized water,

and soaked in 2 M NaOH for at least 24 h. After rinsing the surface with deionized water and air-drying, adsorbed water was removed by drying at 115 °C for 4 h. The silanization reaction was carried out according to the procedure described earlier.¹⁴ In brief, electrode surfaces were treated for several hours in a 10% solution of refluxing silane, under nitrogen. After amination, the electrodes were rinsed with water, followed by ethanol. Biotinylation of the electrodes was accomplished in 0.1 M sodium bicarbonate buffer, pH 8.0, at room temperature. The surface of each ITO electrode was exposed overnight to 1 mg/mL of the biotinylation agent, sulfo-NHS-LC-biotin. Cyclic voltammograms were obtained before and after amination and biotinylation reactions to verify surface modification. To prepare the alkyl-treated ITO surface which does not contain an amine group, ITO films were treated with chlorinated organopolysiloxane solution in heptane (SigmaCote, Sigma Chemical Co., product no. SL-2, lot no. 116H4369). SigmaCote-treated ITO surfaces were rinsed with chloroform and dried at 115 °C for 4 h.

The choice of biotin as a model antigen was determined by two factors. First, this relatively small antigen is well characterized, procedures for covalent attachment are well documented, and polyclonal anti-biotin antibodies are commercially available. Second, biotinylation of the ITO sensor surface incorporates the versatility of biotin–avidin and biotin–streptavidin technologies. A variety of biological molecules are available commercially in the form of biotin or avidin/streptavidin conjugates, along with biotin and avidin/streptavidin derivatives containing different reactive groups. However, poor reversibility of the biotin–avidin and biotin–streptavidin bonds limits application of this technology to single-use devices. The coupling of electrochemistry with TIRF offers the opportunity to better study streptavidin–biotin interactions and allows electrochemical perturbation, which promotes disruption of these complexes without deterioration of at least one of its components. Furthermore, electrochemistry provides additional analytical and preparative capacity to detect and to control interfacial biospecific and nonspecific interactions, as well as to control different stages of the surface design assembly. In this study, the preparative capabilities of electrochemistry were primarily used to change physical and chemical properties of the ITO surface. In future studies, analytical electrochemical techniques will be used to better characterize the sensor surface and to control the process of surface modification and suppression of nonspecific interactions.

Format of TIRF-EC Biosensor Platform. Figure 2 illustrates a fluoroimmunosensor with biotin as model antigen based on TIRF detection and electrochemical control. An indirect immunoassay format is shown in which the analyte antibodies replace previously bound fluorescent-labeled antibodies. The sensor responds to the presence of antibodies by a decrease of fluorescence signal. The goal of this line of research is to develop a platform for simultaneous sensing and control of interfacial biospecific interactions. The fact that TIRF is a sensitive, surface-selective, low-volume, in situ, real-time, and nondestructive technique suitable for investigation of interfacial kinetics determined the choice of TIRF as a technique for detecting interfacial binding. The main disadvantage of TIRF is that this technique requires that the analyte possess either intrinsic or extrinsic fluorescence. However, most proteins contain fluorescent amino acids (i.e., tryptophan and tyrosine).

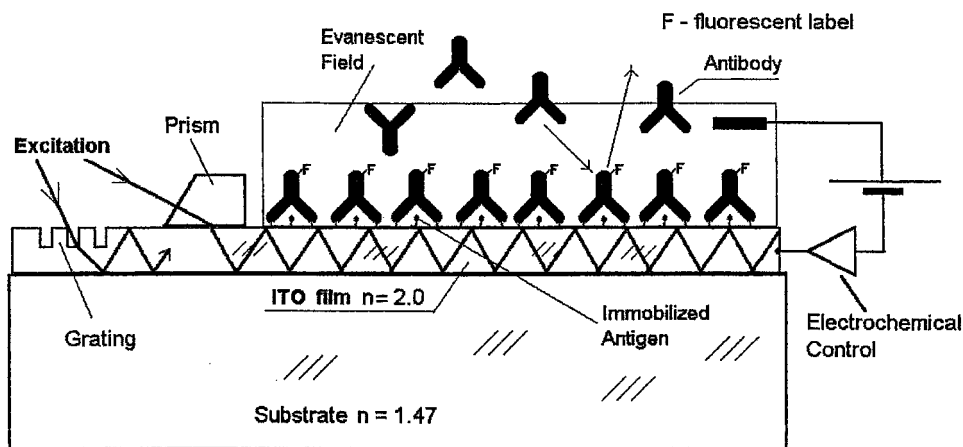


Figure 2. Regenerable fluoroimmunosensor platform using TIRF with electrochemical control.

tophan, tyrosine, and phenylalanine). In the case of a nonfluorescent analyte, there are a variety of extrinsic labels and probes and well-developed techniques of fluorescent conjugate preparation.

Other commonly used surface-selective techniques, such as ellipsometry and surface plasmon resonance (SPR), do not require extrinsic labeling of analyte. However, lower sensitivity and poor selectivity limit practical application of these techniques, especially for multicomponent biological systems. Although a detection limit of 10^{-11} M has been reported for a single-component system,¹⁷ fluctuations of the sample's refractive index in multicomponent biological samples causes SPR and ellipsometry severe background problems. Thus, from the standpoint of analytical capabilities and prospective applications, TIRF seems more acceptable for building the biosensor platform than these other techniques.

The choice of ITO transparent electrodes was dictated by several criteria. First, due to a high refractive index (~ 2.0) and mechanical and chemical stability, ITO is a perfect material for manufacturing a planar optical waveguide with multiple reflections of the excitation beam. Coupling of the excitation light into the waveguide can be done through either a prism or grating, as shown in Figure 2. Integrated optical waveguides with planar geometry are currently under investigation by several research groups, particularly utilizing TIRF coupled with immunoassays.^{4,6,7,13} The differences between ITO/quartz and ITO/water refractive indexes (2.0/1.47 and 2.0/1.33, respectively) yield such small critical angles for total internal reflection as 47.3° and 41.7° , respectively. The incident light can undergo multiple reflections in the ITO film between the quartz substrate and the aqueous solution for a wide range of incident angles ($47.3\text{--}90^\circ$). In this study, it was observed that thick ITO films ($>1\ \mu\text{m}$) effectively captured the excitation beam coupled through grating or prism as shown in Figure 2. However, to minimize the number of parameters which affect TIRF sensitivity, thin ITO films providing a single reflection were used for all kinetic measurements reported here. Second, numerous methods for chemical modification of metal oxide surfaces such as ITO have been documented. The choice of the primary amine reactive group covalently attached

to the surface through a spacer was dictated by the need for further surface derivatization to create a platform for numerous types of biosensors.

RESULTS AND DISCUSSION

Electrochemistry of ITO Electrodes. Figure 3 depicts cyclic voltammograms of bare and chemically modified ITO electrodes. Currents observed at $+1.4$ and -0.9 V correspond to water electrolysis with oxygen and hydrogen evolution, respectively. For an untreated electrode (curve 1), the cathodic wave between -0.5 and -0.8 V was diminished but not eliminated following oxygen removal from the solution. Although no deterioration of optical properties of the ITO electrodes was observed with short-term cathodic polarization, prolonged polarization at <-0.8 V resulted in reduction of the ITO film. The metallic film exhibited a mirrorlike reflection unsuitable for TIRF experiments. However, alternating cathodic-anodic or prolonged anodic polarization did not degrade the optical properties of the ITO electrodes. Electrodes maintained original optical quality for more than 100 h of experimentation.

Electrochemical polarization affects a number of physical and chemical properties of the electrode surface. Electrostatic properties are among the most obvious and are frequently considered as the most important for interfacial behavior of adsorbates, since there is strong correlation between surface charge and adsorption affinity to small ions, ionized polymers, and proteins with molecular mass up to 20–25 kDa. However, for larger protein molecules, other surface properties can be more important than net charges of macromolecules and surfaces. For example, insensitivity to surface and protein charges was found for BSA adsorption on SnO_2 electrodes.¹ Since hydrophobic interactions are a primary driving force for BSA adsorption, changes of surface hydrophobicity upon electrochemical polarization affected adsorption more significantly than variations in electrostatic conditions. The zero charge point for SnO_2 electrodes is approximately $+0.3$ V at neutral pH.¹⁸ Since the electrochemical properties of ITO electrodes are close to those of SnO_2 , the ITO surface is likely positively charged at >0.3 V and negatively charged at <0.3 V. It

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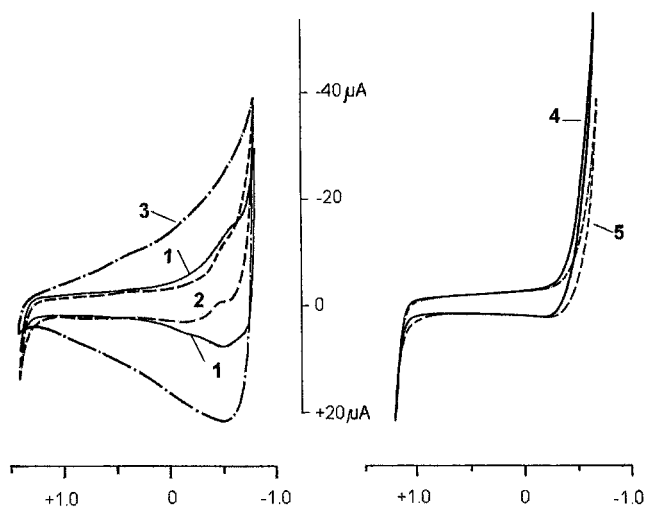


Figure 3. Cyclic voltammograms of chemically modified ITO electrodes. Curve 1, bare ITO, PBS, pH 7.4; curve 2, aminated ITO, PBS, pH 7.4; curve 3, alkyl-treated ITO, PBS, pH 7.4; curve 4, aminated ITO, 0.1 M NaHCO₃, pH 8.6; curve 5, biotinylated ITO, 0.1 M NaHCO₃, pH 8.6. Scan rate, 0.1 V/s. The electrode surface area was 1.2 cm².

has been shown for SnO₂ electrodes that cathodic polarization increases hydrophobicity of the electrode surface.¹ This is probably due to the reduction of the upper layer of the metal oxide to form either the zero-valence metal or nonstoichiometric oxide, which are both hydrophobic. Electrochemical polarization changes the chemical composition of the electrode surface and affects the surface charge and the potential drop across the double electric layer (DEL). At physiological ionic strength, the thickness of the DEL (Debye screening length = ~1.3 nm) is significantly less than the size of a globular protein like IgG (~10 nm). As a result, adsorption behavior of large protein molecules that contain on their surfaces hydrophilic, hydrophobic, positively and negatively charged patches can be relatively insensitive to net charges of both the protein and the surface but rather may be affected by changes of other physical and chemical properties of the electrode surface. Thus, for protein adsorption, behavior changes of other than electrostatic properties of the electrode surface should be taken into account.

Amination of ITO electrodes resulted in reduction of cathodic waves in the cyclic voltammograms with respect to untreated surfaces. Aminated electrodes revealed an additional reversible current wave at approximately -0.5 V (Figure 3, curve 2). This wave can be attributed to the protonation of an amine surface moiety. Interestingly, under cathodic polarization, aminated ITO electrodes were more stable than untreated ITO electrodes with respect to the oxide to metal film reduction. At more basic pH (>8.6), cyclic voltammograms of aminated ITO electrodes did not exhibit this wave (curve 4). Biotinylated electrodes did not reveal this wave at either basic or neutral pH (curve 5). The wave at -0.5 V was also not observed for alkyl-treated ITO surfaces which did not contain the amine moiety (Curve 3). Thus, the presence of a wave at approximately -0.5 V was used to verify the presence of a primary amine at the surface, while the absence of the wave after incubation with a biotinylation reagent was used as indirect evidence for surface biotinylation.

Discrimination of Biospecific Interaction from Nonspecific Adsorption. To distinguish the specific binding of an anti-

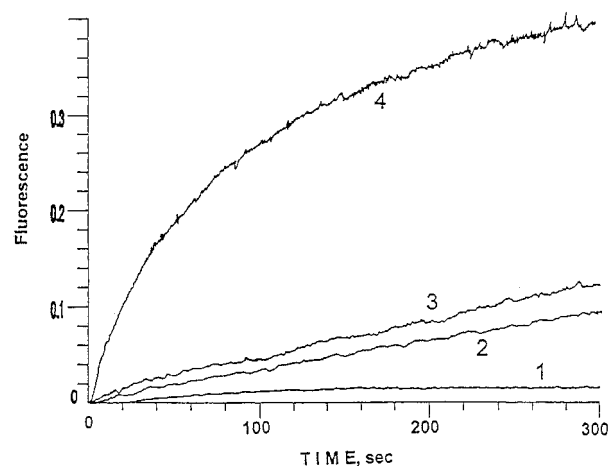


Figure 4. Initial nonspecific and biospecific adsorption on biotinylated ITO surface. Curve 1, 0.03 mg/mL IgG-FITC adsorption in the presence of 0.01 mg/mL BSA; curves 2 and 3, 0.03 mg/mL IgG-FITC and 0.03 mg/mL γ -globulin-FITC adsorption, respectively, in the absence of BSA; curve 4, association of 0.03 mg/mL anti-biotin antibodies-FITC to the biotinylated ITO surface in the presence of 0.01 mg/mL BSA. Conditions: PBS, pH 7.4; room temperature; wall shear rate, 4400 s⁻¹.

biotin antibody to the surface-immobilized biotin from the nonspecific adsorption of antibodies, the antibody interactions were compared with nonspecific adsorption of IgG and γ -globulin. These experiments were accomplished without electrochemical polarization under open-circuit conditions. Figure 4 depicts kinetics of nonspecific and specific interfacial binding. Over the same time interval, the nonspecific adsorption (curves 1–3) demonstrates lower affinity and slower kinetics than the attachment of anti-biotin antibodies to the biotinylated ITO surface (curve 4). Untreated ITO surfaces (kinetics not shown) demonstrate higher adsorption affinity and faster kinetics for IgG and γ -globulin adsorption compared to either biotinylated or aminated surfaces.

The common method to minimize nonspecific adsorption is to treat the sensing surface with an agent that blocks the sites of nonspecific adsorption and does not interfere with antibody-antigen interactions. BSA has been found to suppress nonspecific adsorption in most immunoassay systems utilizing a polystyrene substrate.¹⁹ In this work, it was found that, for untreated ITO electrodes, preadsorption or addition of 0.01 mg/mL BSA drastically reduced IgG and γ -globulin adsorption. In the case of aminated ITO electrodes, preadsorption of BSA or addition of 0.01 mg/mL BSA to the IgG and γ -globulin solutions resulted in suppression of nonspecific adsorption to approximately 3% of the specific binding. Since BSA effectively blocked sites of nonspecific interactions, all our experiments on biospecific binding were conducted in the presence of 0.01 mg/mL BSA, unless otherwise noted.

Additionally, a kinetic criterion can be used for discrimination between specific and nonspecific interactions. Antibody-antigen and biotin-streptavidin interactions in this work exhibited faster binding kinetics than nonspecific adsorption. The difference in kinetics was used as a supplementary experimental factor to discriminate nonspecific and specific interactions.

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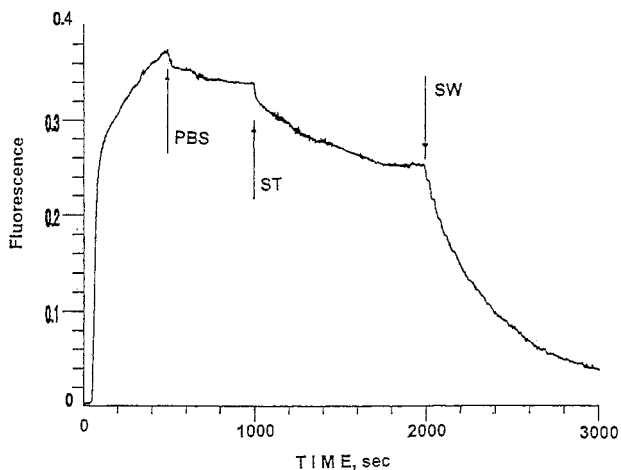


Figure 5. Kinetics of anti-biotin binding to the biotinylated ITO electrode and the effect of electrochemical polarization on dissociation of antibody–antigen complex. Time interval, 0–1000 s, open circuit condition; 0–60 s, background; 60–500 s, 0.02 mg/mL antibody-FITC flow; 500–1000 s, PBS flow; 1000–2000 s, application of “saw tooth” polarization (cyclic scan -0.7 to $+1.1$ V, 0.1 V/s); 2000–3000 s, “square wave” polarization, -0.7 to $+1.1$ V; time period 5 s. Conditions: PBS, pH 7.4; room temperature; wall shear rate, 4400 s^{-1} .

Electrochemical Stimulation of Antibody–Antigen Dissociation. Association rates of antibody–antigen interactions have been shown to fall within a relatively narrow slot between 10^6 and 10^7 $M^{-1} s^{-1}$, while the dissociation rate constants for different antibody–antigen pairs differ by a factor of $\sim 10^3$.⁶ Therefore, for the development of a reversible immunosensor, the dissociation lifetime, $t_d = 1/k_d$, is a parameter of paramount importance. A true immunosensor requires that the system respond to any increase in analyte concentration, as well as to a decrease. As previously noted, most of the immunosensors developed to date are actually detectors with “yes” or “no” response rather than true sensors, since an antibody–antigen bond is typically too strong to allow rapid reversibility. This study pursues the development of immunosensors which are inherently reversible or which can be quickly regenerated in situ to detect a continuous stream of analyte.

Figure 5 illustrates the typical kinetics of specific antibody binding to a biotinylated ITO surface. The observed kinetics of interfacial binding was non-Langmuir, comparable to logarithmic kinetics ($\Gamma = k \ln t$) typical for heteroenergetic interactions described earlier for the case of protein adsorption.¹ Dissociation kinetics also demonstrated heteroenergetics. By definition, the dissociation half-life is the period during which the signal decays by 50%. In the case of monoexponential kinetics, the half-life characterizes the entire decay process. However, in the case of “tailed” kinetics, which is typical for heteroenergetic interactions, the time necessary for a complete regeneration should be characterized by the half-life time and an additional parameter or parameters which reflect heteroenergetics. In the case of heteroenergetic kinetics, the time necessary for a complete regeneration can be unproportionally longer than that in the case of monoexponential decay. In this study, antibody–antigen and streptavidin–biotin interactions demonstrated heteroenergetic kinetics. Energetic heterogeneity for these interactions can be caused by inherited inhomogeneities of chemically modified electrodes, which have been previously reported.¹⁴ Detailed

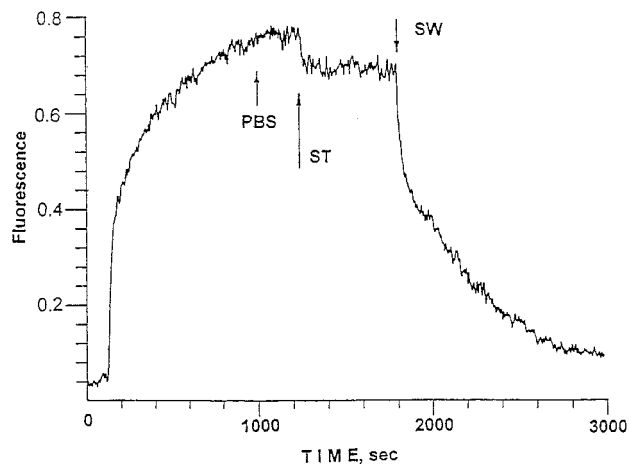


Figure 6. Kinetics of streptavidin binding to the biotinylated ITO electrode and the effect of electrochemical polarization on streptavidin–biotin dissociation. Time interval, 0–1250 s, open circuit condition; 0–110 s, background; 110–1000 s, 0.01 mg/mL streptavidin–Oregon Green conjugate flow; 1000–1250 s, PBS flow; 1250–1800 s, application of “saw tooth” polarization (cyclic scan -0.9 to $+1.3$ V, 0.1 V/s); 2000–3000 s, “square wave” polarization, -0.9 to $+1.3$ V; time period 5 s. Conditions: PBS, pH 7.4; room temperature; wall shear rate, 4400 s^{-1} .

investigation of heteroenergetic kinetics for antibody–antigen and biotin–streptavidin interactions is currently in progress. This study was limited to the dissociation process.

In the absence of electrochemical polarization, the decrease of antibody surface concentration upon rinsing with a pure buffer was extremely slow (Figure 5, time interval 500–1000 s). Under these conditions, the dissociation half-life was greater than $\sim 10^5$ s, which is typical for high-affinity antibodies. Application of “saw tooth” polarization changes from -0.7 to $+1.1$ V (time interval 1000–2000 s) resulted in a notable increase in the rate of dissociation. An estimated half-life for this dissociation kinetics was less than 10^4 s. Furthermore, application of a square wave polarization function over the same voltage range (time interval 2000–3000 s) promoted dissociation more effectively and reduced the dissociation half-life to a practically reasonable number of ~ 300 s.

The first cycle of electrochemical regeneration of a freshly prepared biotinylated ITO surface reduced biospecific activity of the sensor surface to 20–25% of its original affinity. However, during the next 10 regeneration cycles, the affinity decreased less than 10%. In fact, utilization of a square wave polarization treatment provided reproducible conditions at the biotinylated sensor surface, suitable for construction of a reusable immunosensor.

Electrochemical Stimulation of Biotin–Streptavidin Dissociation. Figure 6 shows kinetics of biospecific interaction in the case of streptavidin binding with a biotinylated surface and electrochemically stimulated dissociation of the surface complex. As in the case of antibody–antigen interactions, streptavidin binding to a biotinylated surface is controlled by the interfacial kinetics. Untreated ITO surfaces exhibited relatively high affinities to streptavidin. However, amination of the ITO surface resulted in suppression of nonspecific adsorption to approximately 2% of the untreated surface affinity. Subsequent biotinylation of the aminated ITO surface could possibly lead to an increase of

nonspecific adsorption. With this potential in mind, a test experiment was conducted to verify interference of nonspecific adsorption of streptavidin to a biotinylated surface. In this experiment, biospecific activity of streptavidin was blocked prior to adsorption by addition of an excess of biotin to the streptavidin solution. Adsorption of deactivated streptavidin was less than 3% of the TIRF signal, which can be attributed to biospecific binding.

The interfacial biotin–streptavidin complex is extremely stable. No detectable desorption into circulating PBS was observed after streptavidin was bound to the biotinylated surface (Figure 6, time interval 1000–1250 s). Application of a saw tooth electrochemical polarization function from -0.9 to $+1.3$ V resulted in a drop to approximately 90% of the original signal. This decrease was observed immediately following the initiation of polarization (time interval 1250–1800 s). Prolonged electrochemical perturbation with the saw tooth polarization function had little, if any, effect on the streptavidin–biotin dissociation. Interestingly, square wave polarization (time interval 1800–3000 s) over the same voltage range resulted in a relatively fast dissociation, with a half-life of approximately 200 s. This electrochemical treatment ultimately led to a complete regeneration of the biotinylated surface. Binding–release cycles were repeated more than 30 times without significant loss of biospecific activity at the surface. Thus, electrochemically stimulated dissociation of streptavidin–biotin complexes demonstrate one of few examples of lability of the biotin–streptavidin bond.

One of the possible explanations of the electrochemical stimulation of dissociation of biospecific complexes can be based on the earlier proposed model¹⁰ which assumes the absence of redox reactions involving the protein. It also assumes that a protein molecule at a surface with a variable DEL has insufficient time to adjust its orientation with respect to the surface and its conformation to the new parameters of its surroundings and, thus, rapidly desorbs from the surface. However, during slow changes of the polarization, a flexible protein molecule has enough time to modify its structure and orientation to accommodate the new surface conditions and the changed conditions in the DEL. Due to the adjustment, it remains in the surface-bound state. It has been previously shown that the rotational correlation time of adsorbed protein depends on the electrode polarization,¹⁰ which provides evidence in favor of the proposed model. It seems reasonable that similar mechanisms can also work in the case of disruption of biospecific interactions.

Varying electrode polarization results in changes of a number of surface properties and parameters in the DEL. These surface properties and parameters include sign and surface density of charges, variation of surface chemistry, intensity of the electric field in the dense and diffuse parts of the DEL, nonspecific and specific adsorption of small ions at the surface, changes of the dielectric constant, and changes of ion concentrations in the DEL.^{20,21}

Besides their effect on protein adsorption behavior, local changes of pH in the DEL might also affect FITC fluorescence, as FITC is known to be a pH-sensitive fluorescent label. To detect this possible influence, the effect of electrochemical polarization

on FITC conjugates was compared with that for BODIPY conjugates, as BODIPY is known to be a *pH-insensitive* label. In the case of FITC conjugates, small oscillations of the TIRF signal (within 5%) were observed under alternating electrochemical polarization. TIRF signal monotonically decreased during the anodic phase of polarization and did not decrease or slightly increased back for less than 5% during the cathodic phase of polarization. These small oscillations can be attributed to pH effects, which might affect the fluorescence of FITC. Changes of other parameters in the DEL can also affect FITC fluorescence. Detailed investigation of the effect of parameters in DEL on FITC fluorescence will be undertaken in future studies. Experiments conducted with a pH-insensitive fluorescent label, BODIPY, indicated the same integral kinetics of dissociation. Oscillation of the TIRF signal was not observed with this label. These differences in results for FITC and BODIPY conjugates can be attributed to the small effect of pH changes in the DEL on FITC conjugates and no pH effect on BODIPY conjugates' fluorescence.

CONCLUSION

The goal of this research is to develop a platform for simultaneous sensing and control of interfacial biospecific interaction. The application of electrochemistry along with TIRF, combined with a suitable immunoassay, provides the analytical and preparative power of electrochemistry to detect and control interfacial biospecific interactions. In this study, preparative capabilities of electrochemistry were primarily used to control the physical–chemical properties of the ITO electrode surface. In future studies, analytical electrochemical techniques will be used to better characterize the sensor surface and to control the process of surface modification and suppression of nonspecific interactions.

This study has successfully demonstrated the capability of electrochemical polarization to regulate the antibody–antigen and streptavidin–biotin binding. Electrochemistry alone or in combination with selective surface chemistry can likely solve the irreversibility problem, which hampers the practical use of immunoassays for biosensing. A TIRF-EC platform can potentially be used for development of a wide range of fast, sensitive, and *reusable* fluoroimmunosensors. The investigation of the electrochemical effect can also provide new insight into fundamental mechanisms of protein–protein, protein–membrane, protein–ligand, and protein–DNA interactions. Potentially, the TIRF-EC technique can be used in numerous applications based on avidin–biotin and immunoassay technologies to build reusable biosensors.

A logical extension of this work is to incorporate solid-phase peptide synthesis, which provides a versatile tool for sensor surface design with biologically significant compounds. For example, short or long synthetic peptides which mimic native antigen epitopes and bind to antibodies with an affinity comparable to that of the native antigen³ can be synthesized directly on the sensor surface and used for numerous clinical applications. An advantage is that the aminated ITO TIRF-EC platform is not limited to naturally occurring molecules. Synthesis of various amino acid sequences directly on a sensor surface has already been described and used for metal–ion coordination.²² Solid-

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phase peptide synthesis also facilitates combinatorial chemistry to construct a specifically desired surface receptor molecule.

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