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Reversible affinity interactions of antibody molecules at functionalized dendrimer monolayer: affinity-sensing surface with reusability

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Abstract

We described reversible affinity interactions of antibody molecules at a chemically functionalized electrode surface for a repeatedly renewable affinity–biosensing interface. Underlying biofunctionalizable monolayers were constructed with poly(amidoamine) dendrimers, whose surface chain-end groups were double-functionalized with biotinyl ligand and ferrocenyl groups for biospecific recognition and electron transfer reactions, respectively. Functionalized monolayers on gold electrodes provide platform surfaces for biospecific recognition reaction with monoclonal anti-biotin antibody molecules. Bound antibody molecules were dissociated from the surface via displacement reaction by the addition of free biotin in solution, enabling the affinity surface to be renewed and repeatedly utilized. Tracking of the association/dissociation reaction cycles were performed by registering the bioelectrocatalytic currents at the electrode using glucose oxidase (GO*x*) as a signal generator and ferrocenyl-tethered dendrimer (Fc-D) as an electron transferring mediator in electrolyte. Shielding of the affinity surface by biospecifically bound antibody molecules caused hindrance in electron transfer, resulting in reduced signal from cyclic voltammetry. By the displacement reaction using free biotin, bound antibody molecules were dissociated from the surface and the bioelectrocatalytic signal was restored. With the affinity surfaces constructed in this work, continuous association/dissociation reactions have been successfully accomplished, providing a possibility of reusable affinity biosensing interface. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: Affinity-sensing; Dendrimer; Electrochemical; Reversible

1. Introduction

Much effort has been devoted to the development of molecularly organized interfaces containing biomolecules for the applications as diagnostic tools (biosensors) [1,2], biomimetic membranes [3–5], and bioelectronic devices [6,7]. With the availability of highly controllable structuring and patterning techniques, especially with the self-assembled monolayers (SAMs) methodology [8–10], phase in the related research fields has greatly fastened. Pioneering works of Whitesides groups regarding mixed SAM [11,12] and microcontact printing [10,13] techniques have opened a new venue of application from electrical engineering to chemical and biological processes. Keeping in step with the development, there also

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have been numerous approaches for the fabrication of molecularly organized and active biocomposite assemblies on surfaces. The implementation of useful surface (bio-)functionalization methodologies as well as the introduction of novel materials having unique properties is an important subject of study in this viewpoint [14–20].

As one of the specially designed highly-branched polymers, dendrimers have been applied for the construction of interfacial layers ([21] and references therein). The unique characteristics of dendrimers such as structural homogeneity and integrity, internal void cavity, and dense functionalities at the molecule's surface have attracted great interests in areas including drug delivery, energy harvesting, ion sensing, catalysis, and information storage during the decade [22,23]. The utility of this molecule has been extended to the bio-related field: the G4 poly(amidoamine) dendrimer was adopted as the building block for the multilayered enzyme biocomposite films [21,24,25]. And we recently have devised affinity-sensing electrodes based on the functionalized dendrimer monolayers [26] and developed a surface regeneration methodology for avidin–biotin affinity interaction [27].

In the present paper, we describe a general approach regarding reversible affinity interactions of biomolecules at immunosensing surfaces, which is based on the biospecific association and displacement reaction between functionalized antigen ligands and antibody molecules. In an effort to demonstrate a wide applicability of the surface regeneration strategy, a biotin/anti-biotin antibody system was employed as a typical antigen/antibody couple. Underlying template layer for the affinity surfaces were constructed with G4 poly(amidoamine) dendrimer monolayers on SAM/Au electrode, whose dendritic surface groups have been double-functionalized with biotinyls and ferrocenyls for the biospecific recognition and electron transfer mediation reactions. The functionalized monolayers provide a platform for biospecific recognition with monoclonal anti-biotin immunoglobulin G (IgG), and the bound antibody molecules were dissociated by the addition of free biotin (displacement). The association of antibody molecules and renewal of the affinity surface were tracked with electrochemical method using glucose oxidase as a freely-diffusing signal generator and ferrocenyl-tethered dendrimers (Fc-D) as an electron transferring mediator in the reaction medium. The association/dissociation (OFF/ON) reaction cycles were observed based on the blockage of electrode surface with bound antibody molecules and subsequent hindrance in electron transfer by freely diffusing GO*x* and electron-transferring mediator and vice versa.

2. Experimental

2.1. Chemicals and reagents

Amine-terminated fourth generation poly(amidoamine) (PAMAM) dendrimers are manufactured by Dendritech Inc. (Midland, MI) and were purchased from Aldrich. The syntheses of partial ferrocenyl-tethered dendrimers (Fc-D) have been described [28], and Fc(23%)-D was used in this study. 1-Ethyl-3-(3-dimethyl aminopropyl) carbodiimide hydrochloride (EDAC), D-(+)-glucose, and glucose oxidase type VII (EC 1.1.3.4, from *Aspergillus niger*) were purchased from Sigma. 11-Mercaptoundecanoic acid and pentafluorophenol were purchased from Aldrich. *N*-hydroxysulfosuccinimidyl ester-activated biotin (sulfo-NHS-biotin) was from Pierce. Anti-biotin MAb (mouse monoclonal 2F5, isotype $IgG_{1,\kappa}$, molecular probes) were used as received. All other materials used were of the highest quality available and purchased from regular sources. For the buffer solutions, doubly distilled and deionized water with the specific resistance over 18 MΩ cm was used throughout the work. A phosphate buffered saline solution (PBST, pH 7.4, 10 mM phosphate, 2.7 mM KCl, and 138 mM NaCl containing 0.05% (v/v) Tween 20) was used.

2.2. Construction of the dendrimer monolayers on thin-film gold surfaces

The process for the construction of a covalently modified dendrimer monolayer on SAM/Au electrode is essentially identical to our previous paper [27]. The affinity-sensing monolayer was made on evaporated Au/Ti/Si[1 0 0] surfaces. Freshly prepared gold surfaces were used for the affinity-sensing electrodes. Prior to the layer forming process, the surfaces were cleaned (5 min) by piranha solution (1:4 by volume of 30% H_2O_2 and concentrated H_2SO_4). CAUTION:

Piranha solution reacts violently with most organic materials and must be handled with extreme care. After the cleaning step, a SAM representing reactive carboxylate groups on gold surface was prepared from the chemisorption of 11-mercaptoundecanoic acid (MUA, 2 mM) in ethanol for 2 h on gold electrodes. After SAM formation and ethanol rinsing steps, the carboxylate groups were activated with pentafluorophenol to give reactive ester groups toward amine functionalities remaining at the chain-ends of Fc-D. The SAM modified electrodes were immersed in a DMF solution, containing EDAC (0.1 M) and pentafluorophenol (0.2 M). After the activation reaction for 20 min, the electrodes were rinsed with DMF, dried under an argon stream, and transferred to a Fc(23%)-D solution in methanol. The column purified Fc-D was reacted by dipping the electrode in 22 μM (based on the dendrimer concentration) of Fc-D solution. After the reaction for 30 min, the surfaces were rinsed with methanol, dried with a stream of argon, and immersed in a bicarbonate buffer (0.1 M, pH 9.5) for 20 min to hydrolyze and deactivate the remaining fluorophenyl esters.

2.3. Functionalization of dendrimer monolayers with biotin ligand groups

Prior to the biotinylation process, the electrodes were rinsed, air-dried, and clamped to Teflon electrode holders. The holders were designed to expose the defined electrode area of 0.148 cm^2 and the reaction well volume of 3 ml. Surface biotinylation reaction was performed with sulfo-NHS-biotin, an activated ester of biotin. An aqueous solution of sulfo-NHS biotin (2 mg/ml) was added to the electrode-immersed bicarbonate buffer solution (0.1 M, pH 9.5). The final concentration of biotin was adjusted to 2 mM. After 2 h reaction, the electrodes were rinsed with bicarbonate buffer and distilled water, and were stored in PBST.

2.4. Reversible affinity reaction with anti-biotin IgG antibody and surface regeneration via displacement with free biotin

Fig. 1 illustrates schematically the affinity binding and displacement reaction steps at the electrode

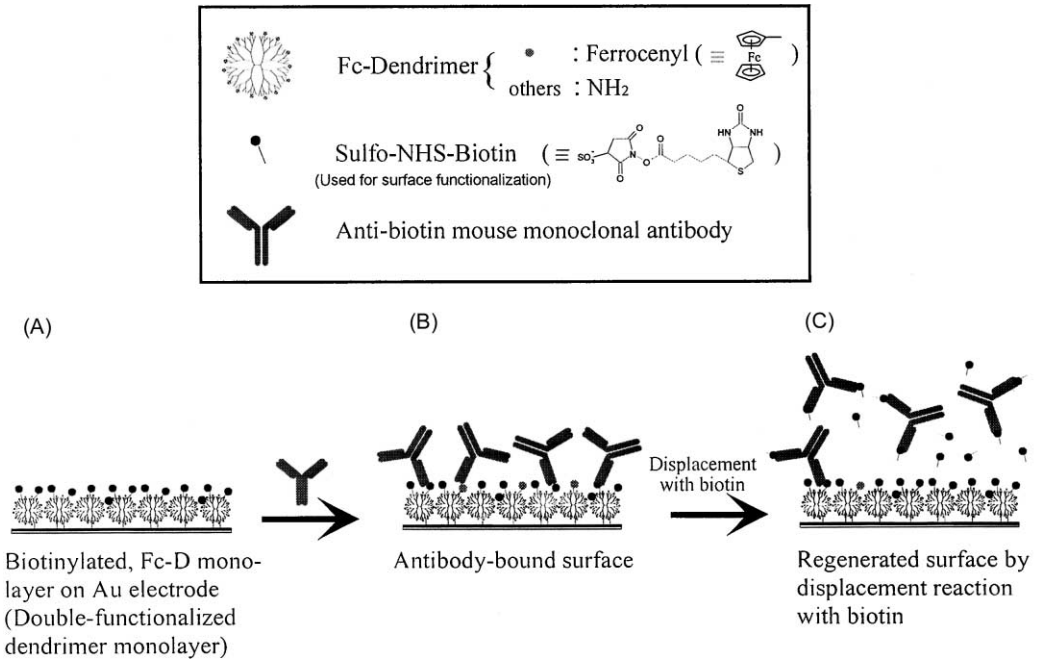

Fig. 1. Schematic representation of the compounds used (in box) and the procedure for the biospecific association/dissociation of antibody molecules at the affinity-sensing electrode surface (A–C). The dimensions of the components are not drawn to scale for simplicity.

surface (vide infra). First, the biospecific association reaction at the surface was performed with monoclonal anti-biotin IgG antibody. Aliquots of antibody samples (100 μl, 20 μg/ml) were prepared in PBST and incubated at the electrode for 30 min at room temperature. After rinsing the surfaces thoroughly with PBST, the antibody-associated surfaces were subjected to the signal measurement step. The surface regeneration process via dissociation reaction proceeded after signal registration and washing of the electrodes. The displacement reaction was performed by exchanging the rinsing buffer with the solution of biotin (2 mM, in PBST) and incubating for 1 h.

2.5. Electrochemical tracing of the biospecific association/displacement reaction cycles

Cyclic voltammetry was employed to measure the biospecific reactions at the Fc-D associated and biotin functionalized SAM/Au electrodes. The principle of the tracking of association/displacement cycle relies on the shielding of the electrode surface with biospecifically bound antibody molecules, by which the accesses of electroactive molecules to the electrode surface and charge transfer are hindered. By using GO*x* as a free-diffusing signal generator and Fc-D as an electron-transferring mediator in electrolyte, the registration of bioelectrocatalytic currents correlating to the level of surface coverage was possible. A phosphate buffer (0.1 M, pH 7.2) containing 0.51 μM GO*x*, 50 mM glucose, and 7.7 μM Fc(23%)-D (dendrimer concentration) was used as an electrolyte. The glucose solutions were allowed to mutarotate overnight before use. The electrolyte solutions used were deoxygenated with argon bubbling (20 min) before each voltammetric run.

2.6. Instrumentation

Cyclic voltammetric measurements were carried out with a BAS CV-50 W potentiostat. A standard three-electrode configuration with a gold working electrode, a platinum wire counter electrode and an Ag/AgCl (BAS) reference electrode was used. All measurements were performed at room temperature ($25 \pm 2\,^{\circ}C$) under argon atmosphere unless otherwise specified.

3. Results and discussion

3.1. Construction of an affinity interface using functionalized dendrimers on SAM/Au electrode

The construction process of affinity interaction surfaces on gold electrodes is essentially similar to our previous investigation [27] as described in the Section 2. Amine-terminated poly(amidoamine) dendrimers (generation 4, 64 surface NH_2), whose chain-ends groups had been partially functionalized with ferrocenyls (23% modification, ca. 15 ferrocenyls per dendrimer molecule, denoted as Fc-D), were used for the construction of template for the ligand modified interface. Procedure for the construction of affinity surfaces and the association/dissociation reactions at the functionalized surfaces are illustrated in Fig. 1.

The covalent immobilization and the formation of Fc-D monolayer were verified from the electrochemical measurement of the Fc-D/SAM/Au electrodes (Fig. 2). From the cyclic voltammetric measurements, we found a ferrocene/ferricinium redox wave with a peak potential of +380 mV versus Ag/AgCl and a ΔE_p of about 25 mV at the sweep rate of 0.05 V/s, which is typical for the surface immobilized electro-active groups. The electrodes exhibited direct proportionality of peak currents to potential sweep rates, also proving the surface immobilization of Fc-D on electrode (data not shown). From the coulometric data analysis, the amount of immobilized ferrocenyl functionalities (surface density of immobilized ferrocenyl groups) was estimated to be $\Gamma_{Fc} = (2.3 \pm 0.2) \times 10^{-10}$ mol/cm^2, given that the diameter of G4 dendrimer is about 45 Å [29]. From this result, it is likely that dendrimers are immobilized covalently onto the preactivated mercaptoundecanoic acid SAM, forming a compact monolayer [28].

After the formation of dendrimer monolayer, the remaining surface amine groups from dendrimers were further functionalized with sulfo-NHS-biotin, a water-soluble succinimidyl ester of biotin, which led to the construction of an affinity surface for the biospecific recognition.

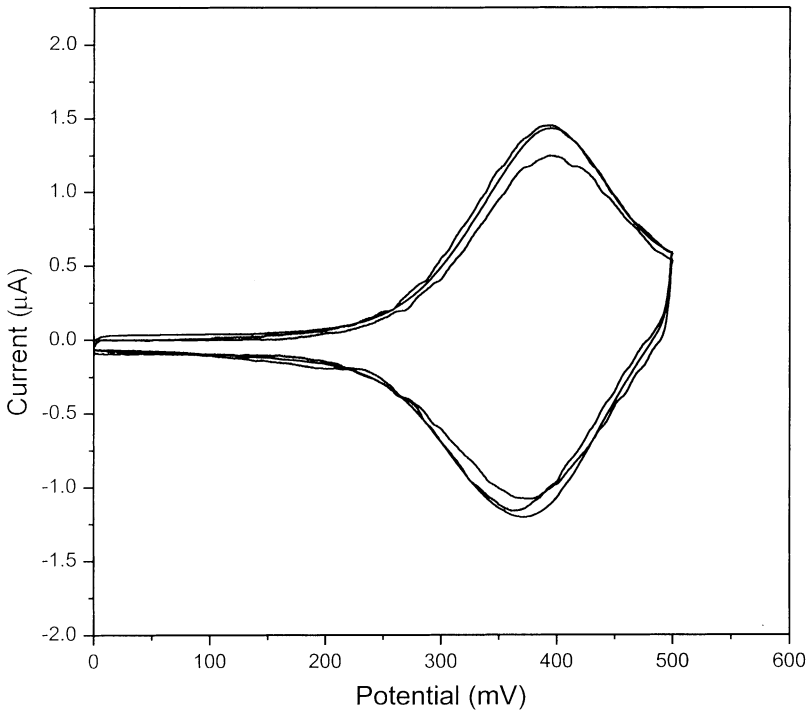

Fig. 2. Immobilization of electrochemically active ferrocene-tethered dendrimer monolayer on gold electrode surfaces (Fc[23%]-D/MUA-SAM/Au), which was verified with cyclic voltammetry. Curves were registered in a dedioxygenated 0.1 M phosphate buffered (pH 7.2) electrolyte, and potential scan rate was 50 mV/s.

3.2. Electrochemical signal generation at the functionalized electrode

Biospecific interactions at the affinity surface were monitored by tracing the signal generated from the bioelectrocatalytic reaction of glucose oxidase (GO*x*). The working principle of the tracking of reaction cycles (i.e. association of antibodies and dissociation with free biotin) relies on the shielding of the electrode surface with biospecifically bound antibody molecules, by which the accesses of electroactive molecules to the electrode surface are hindered. On the other hand, by the addition of biotin to the reaction cell, bound antibody molecules are dissociated from the functionalized surface and the bioelectrocatalyic signal is restored. The idealized schematic representation of the procedure employed for the biospecific association/displacement of antibodies at the electrode surfaces is shown in Fig. 1A–C.

At the initial phase of the study, GO*x* was used as a free-diffusing signal generator in the electrolyte for the tracking of reaction cycles. An electrolyte containing GO*x* and glucose (as a substrate for enzyme reaction) was prepared and subjected to analysis. In this case, the electrodes exhibited typical voltammetric traces for the ferrocenyl-mediated and enzyme-catalyzed reaction, as shown in Fig. 3A, but the anodic peak signal was relatively small and gradually decreased as the potential cycling was iterated. Furthermore, when the same electrode was subjected to the association reaction of antibiotin IgG antibody and displacement reaction with biotin, the electrodes did not respond adequately. This was not due to the consumption of glucose substrate or inactivation of GO*x* in the electrolyte, because the same trend was observed for the freshly prepared electrolyte. We reasoned that the above result might be due to the nonspecific and irreversible adsorption of GO*x* to the surface and subsequent deterioration of affinity

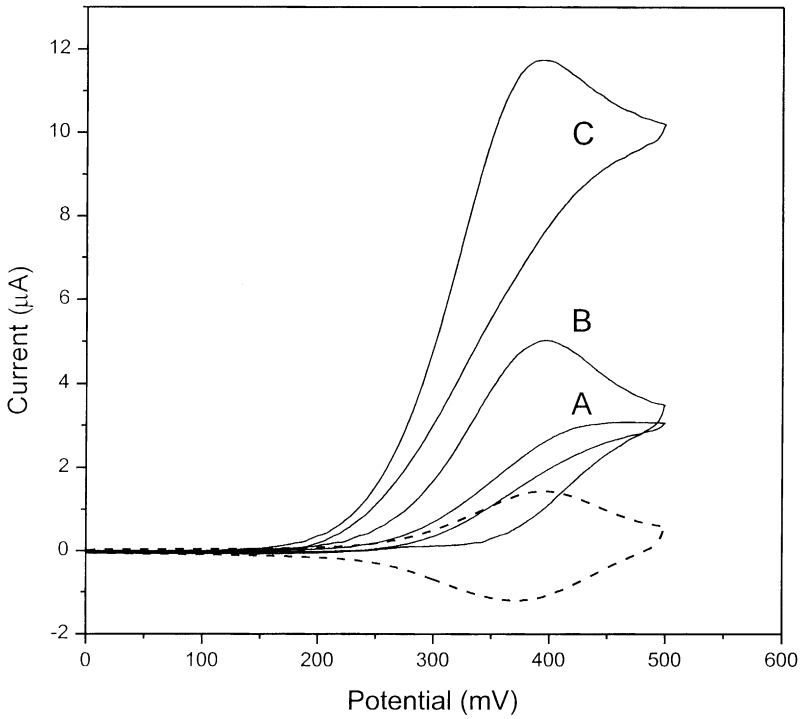

Fig. 3. Bioelectrocatalyzed voltammograms of the Fc-D/SAM/Au electrodes at different signaling conditions: (A) with only GO*x* (0.51 μM) as the signal generator, (B) with Fc(23%)-D (4.0 μM, dendrimer concentration) and GO*x*, (C) with Fc(23%)-D (7.7 μM) and GO*x*. Background trace was shown as dashed line. Glucose concentration was 50 mM, and potential scan rate was 50 mV/s.

monolayer. Amine-terminated dendrimer has a high pK_a (p$K_a \sim 9.5$) [29], and the dendrimer monolayer should exhibit positive surface charges under the reaction condition of pH 7.2. Meanwhile, GO*x* possesses relatively low isoelectric point (pI = 4.05) that renders it negatively charged, which causes nonspecific adsorption of enzyme molecules to the dendrimer monolayer by charge attraction. For the generation of bioelectrocatalytic current at the electrode under the condition of Fig. 3A, GO*x* molecules in electrolyte should access to the functionalized monolayer surface, transferring electrons to the immobilized ferrocenyl groups of Fc-D that are electron acceptors and only present at the electrode surface. Subsequently, enzyme molecules would adsorb irreversibly to the surface by charge attraction and gradually deactivate, which should result in the reduction of bioelectrocatalytic current. This reasoning is supported by the previous work of Seigel et al describing the nonspecific protein adsorption at SAM surface and subsequent deactivation by protein unfolding [30].

As an approach to reduce the adsorption of GO*x* to the affinity surfaces, we attempted to make the electron transferring (mediation) reaction to take place in electrolyte. For this, we added electrochemically active ferrocenyl-tethered dendrimers (Fc-D) directly to the electrolyte (Fig. 3B and C). The rather bulky ferrocenyl-tethered dendrimer was used instead of small ferrocenyl monomers. In this case, adsorption of enzyme molecules and surface fouling could be reduced by the presence of Fc-D in electrolyte, mediating electrons between GO*x* and ferrocenyl groups at the electrode surface and reducing the direct contact of enzyme molecules to the electrode surface. In addition, the shielding effect of the surface with biospecifically bound antibody molecules can be maximized by the use of Fc-D, which appears to be a critical factor for development of affinity-sensing electrodes. When ferrocenyl monomer (ferrocenemethanol) was

used, for example, the signal difference registered for each association/dissociation cycle was very small, making the discrimination of interaction difficult. It is assumed that small ferrocenyl monomers, comparing to Fc-D having large dimension, readily penetrate even the antibody-associated surface.

On the other hand, when Fc-D was added to electrolyte as an electron-transferring mediator, cyclic voltammograms from the electrodes were maintained stable during and after repetitive sweep cycles; 5 cycles with another cycle after 5 min storage in buffer solution have been registered (Fig. 3B and C), suggesting that nonspecific binding of GO*x* has been reduced. Also, by increasing the Fc-D concentration in electrolyte, amplification in the catalytic signal was observed (Fig. 3B and C). However, the tests for higher concentration of Fc-D above 7.7 μM (based on the dendrimer concentration, which was used in Fig. 3C) were not possible because of the limited solubility of Fc-D in electrolyte. Therefore, the following experiments were conducted under the condition of Fig. 3C. The idealized schematic illustrations of the enzyme reaction, electron mediation with Fc-D in electrolyte, and the charge-transfer to the electrode surfaces for antibody association and displacement reaction steps are shown in Fig. 4.

3.3. Characterization of the biospecific interactions: affinity binding of antibodies and surface regeneration via the displacement reaction with biotin

On the basis of the above observations, a set of affinity electrodes were prepared and subjected to the tracking tests of association/displacement reaction cycles. Cyclic voltammetry was conducted after each reaction step, and the voltammograms are shown in Fig. 5. Fig. 5A represents cyclic voltammograms for the newly prepared electrode as in Fig. 1. An amplified cyclic voltammogram for the bioelectrocatalysis (solid line) and a background one (dashed line) are shown. The voltammetric traces from the electrodes exhibited a typical pattern for the bioelectrocatalysis, an anodi-

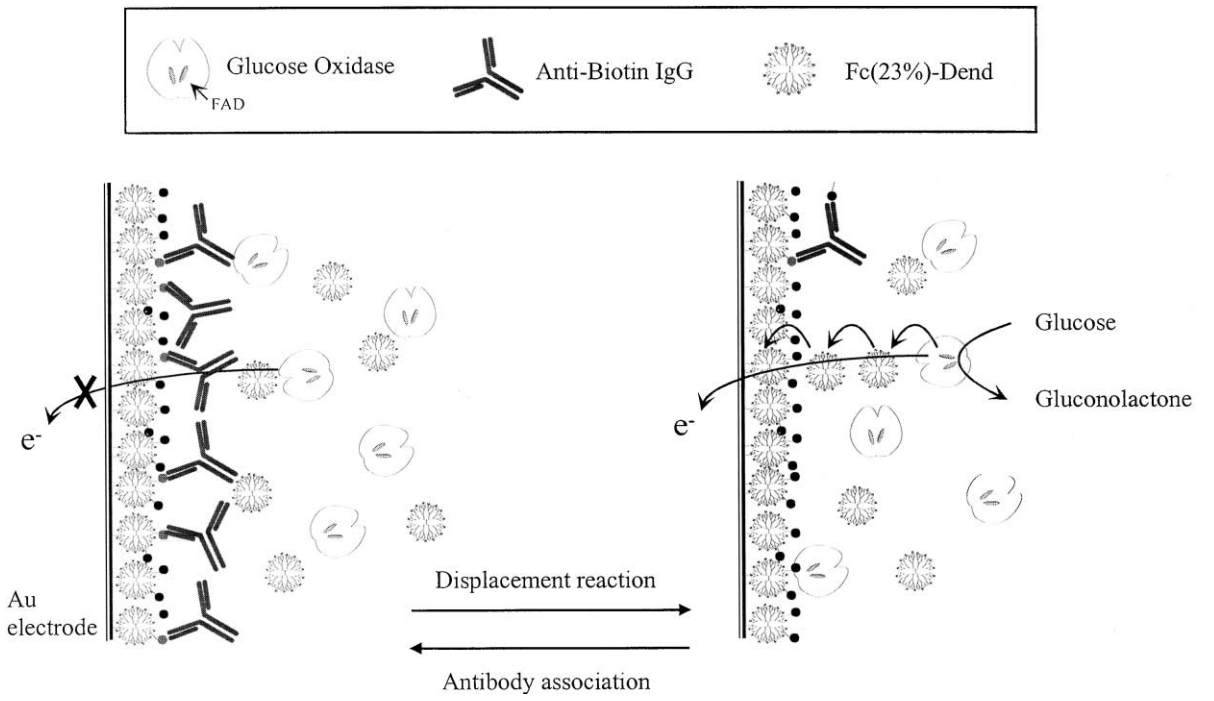

Fig. 4. Schematic representation of the idealized reactions in electrolyte and at the affinity-sensing electrodes for antibody-associated and regenerated surfaces: enzymatic catalysis, electron mediation with Fc-D in electrolyte, and charge transfer to Au electrodes. The dimensions of the components are not drawn to scale for simplicity.

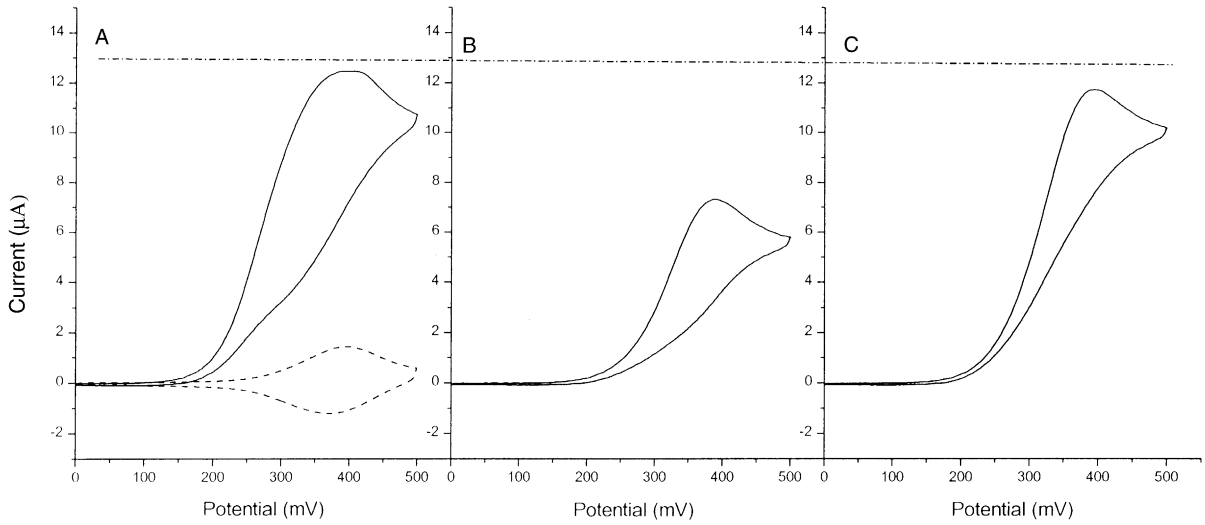

Fig. 5. Cyclic voltammetric traces for the association/dissociation process at affinity surfaces for: (A) newly prepared and biotin functionalized surfaces which was subjected to first bioelectrocatalytic reaction "ON 1" with a background one (dashed line); (B) electrodes which were treated with antibody "OFF *n*" and (C) electrode surfaces after displacement reaction with biotin "ON *n*".

cally amplified sigmoidal curve, and the peak current reached a plateau at ca. +385 mV versus Ag/AgCl.

After the anti-biotin IgG binding step, the peak current in voltammograms significantly diminished, which reflects the surface shielding by associated antibody molecules (Fig. 5B). The shape of voltammograms and peak potential were conserved. Through the antibody association step, signal retardation of about 55% of the initial level was registered. This was a typical value obtained under the reaction condition used in this work, indicating the limited yield of antibody association to the surface and consequent surface shielding level. Fig. 5C represents cyclic voltammogram for the electrodes after the displacement reaction with free biotin. The voltammogram has almost restored to the original one as in Fig. 5A, which shows the biospecific dissociation of antibodies from the affinity surface by the addition of biotin.

For biospecific recognition reactions that occur at surfaces, especially, the steric limitation between binding couples would be a critical factor. In this study, the surface was functionalized with sulfo-NSH-biotin, rendering four-carbon distal chain at the dendrimer monolayer surface, and the resulting electrode showed successful biospecific reaction cycles. On the contrary, with the system using (strept-)avidin as the recognition couple, only biotin analogs containing extended distal chain (amidocaproate form) exhibited biospecific interactions [27]. This phenomenon can be attributed to the rather shallow recognizing epitope at the F_{ab} part of antibody, in comparison to the deep binding pocket from avidin molecule [31,32]. This reasoning is also supported by the fact that the binding constant of biotin/anti-biotin IgG is usually several orders of magnitude lower than the biotin/avidin binding constant ($K_a = 1 \times 10^{15}\,M^{-1}$) [33]. We, therefore, believe that the steric problem is not so significant for the system employed in this study, while the steric limitation is usually considered critical for the biospecific interactions adopting biotin/(strept-)avidin couples.

3.4. Repetitive association/displacement reaction cycles at the affinity-sensing surface

A set of affinity surfaces were prepared and tested for the repeated association/displacement processes. For the ease of analysis, signals were registered after normalization. The anodic plateau signal from the first voltammogram for the newly prepared electrode "ON 1" was denoted as I_{peak}. The peak current from the background voltammogram was measured as I_{back}. And the anodic peak currents from the cyclic voltam-

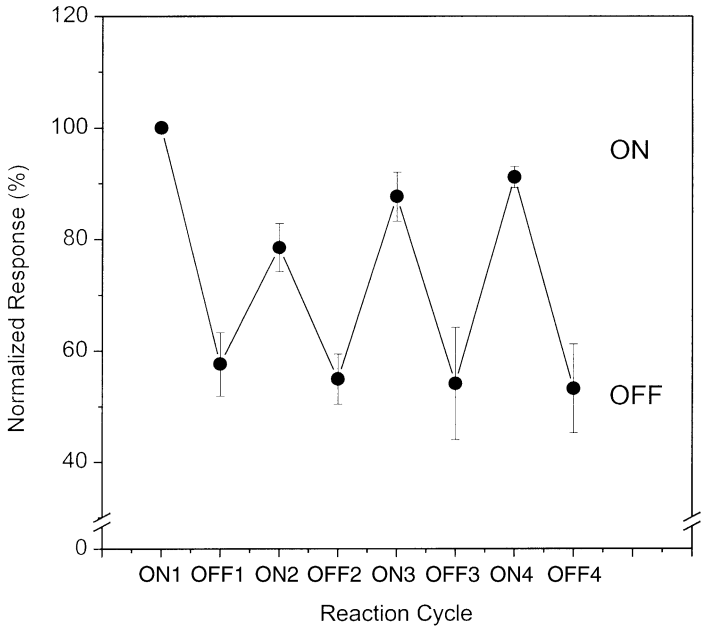

Fig. 6. "ON" and "OFF" signal traces from the affinity-sensing surfaces via biospecific association/displacement reaction cycles. Normalized signal levels were registered as $[(I_{sig} - I_{back})/(I_{peak} - I_{back})] \times 100\%$ (see text).

mograms at each measurement step were registered as I_{sig}. The normalized responses from the electrode were calculated using the following equation:

$$\text{normalised response (\%)} = \frac{I_{sig} - I_{back}}{I_{peak} - I_{back}} \times 100$$

Signal traces for the affinity electrodes through iterative biospecific association/dissociation cycles were plotted as shown in Fig. 6. The electrodes exhibited well-developed responses from the blocking of the monolayer surface by antibody association "OFF" and regeneration of the surface by displacement reaction with biotin "ON", confirming the occurrences of efficient biospecific interactions. The normalized responses oscillated between ca. 45 and 90%, for shielded and regenerated surfaces, respectively, and the signal fluctuations were found within 15% levels. For the first cycle of association/dissociation, signal retardation of about 55% of its initial level was found, which might be due to the limited reaction at surfaces. And for the dissociation step "ON", some degree of signal drift was registered. However, during the following successive reaction cycles, the trend of signal switching was evident and the level of signal oscillation was stabilized, suggesting that the active portion of affinity surface responded appropriately throughout the test. In addition, a set of electrodes were subjected to the test after having been used and stored in buffer for 24 h under room temperature, and as a result, similar responses were obtained for subsequent association and displacement steps.

We, therefore, concluded that the affinity interface developed in this work satisfies the objective of repeatedly renewable affinity interface through biospecific interactions: association of antibody molecules to the surface, which was functionalized with antigen (ligand), and regeneration of the surface by displacement reaction with free antigen (ligand). The present method, we believe, could be applied to the reversible affinity-sensors for other antigen-antibody or ligand-receptor couples, and would find applications to the biomolecular switches.

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