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ARTICLE *in* ANALYTICAL CHEMISTRY · MARCH 2010

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Anal. Chem. **2010,** *82,* 1712–1719

Amperometric Immunosensor for Carcinoembryonic Antigen in Colon Cancer Samples Based on Monolayers of Dendritic Bipodal Scaffolds

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Detection of proteins that signal the presence or recurrence of cancer is a powerful therapeutic tool for effective early diagnosis and treatment. Carcinoembryonic antigen (CEA) has been extensively studied as a tumor marker in clinical diagnosis. We report on the development of an amperometric biosensor for the detection of CEA based on the immobilization of anti-CEA monoclonal antibody on a novel class of bipodal thiolated self-assembled monolayers containing reactive *N*-hydroxysuccinimide (NHS) ester end groups. The current variations showed a linear relationship with the concentration of CEA over the range of 0−200 ng/mL with a sensitivity of 3.8 nA·mL·ng^{-1} and a detection limit of 0.2 ng/mL, which is well below the commonly accepted concentration threshold (5 ng/mL) used in clinical diagnosis. Real time and accelerated stability studies of the reporter antibody under various storage conditions demonstrated that the enzymatic activity and antibody affinity of the conjugate is retained for long periods of time in commercial stabilizing buffers such as StabilGuard Biomolecule Stabilizer, and a prediction of the stability trends was carried out using the kinetic and thermodynamic parameters obtained from the Arrhenius equation. The developed immunosensor as well as a commercially available enzyme-linked immunosorbent assay (ELISA) kit were successfully applied to the detection of CEA in serum samples obtained from colon cancer patients, and an excellent correlation of the levels of CEA measured was obtained. Ongoing work is looking at the incorporation of the developed biosensor into a platform for multiplexed simultaneous detection of several breast cancer related biomarkers

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Cancer is one of the main causes of mortality worldwide whose cure has not yet been achieved. To date, detection at an early stage is the only tool to initiate appropriate treatment and potentially avoid a fatal outcome. Certain proteins can signal the presence or recurrence of cancer although only a few serum markers are truly specific for a disease in a defined organ. Carcinoembryonic antigen (CEA) is a glycoprotein of 200 kDa that has been extensively studied as a tumor marker for clinical diagnosis and is routinely used as part of an individual's annual medical checkup in many countries. It is present in about 95% of all colon tumors[1] and 50% of breast tumors[2] and is also associated with ovarian carcinoma,[3] lung cancer,[4] and other cancers. Therefore, it either can be part of a panel of cancer markers for different cancers or, more importantly, can be used as an independent prognostic factor.[5−7] The normal CEA levels in healthy adults lie in the range 3−5 ng/mL, although some benign diseases can increase these levels up to 10 ng/mL. When the CEA level is abnormally high before therapy, it is expected to fall to normal, following successful surgery or other treatment to remove the tumor. A rising CEA level indicates progression or recurrence of the cancer. In addition, levels of >20 ng/mL before therapy are commonly associated with cancer in metastatic state.[8,9]

Several strategies have been developed for the detection of this important marker in the past such as enzyme-linked immu-

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10.1021/ac902162e
Published on Web 01/29/2010

noassay[10] and fluoroimmunoassay.[11] These methods are time-consuming and require qualified personnel and sophisticated instrumentation, and hence, alternative methods at the point of care are desirable. Electrochemical biosensors offer the advantages of affordable instrumentation, ease of use, multiplexing ability, and rapid response and miniaturization, allow point-of-care measurements, and have, thus, been applied to the detection of cancer markers.[12] Recent examples of CEA detection using biosensors include the immobilization of anti-CEA on epoxysilane-modified core−shell magnetic Fe_3O_4/SiO_2 nanoparticles,[13] the covalent attachment of antibodies to a three-dimensional porous IrOx matrix using silane chemistry,[14] or the use of gold nanoparticle bioconjugates as labels for secondary antibodies.[15] Piezoelectric immunosensors[16] and flow injection with amperometric detection[17] have also been reported for the determination of CEA. These systems allow the detection of low concentrations of CEA, usually <1 ng/mL.

The modification of noble metals with thiols to form various types of self-assembled monolayers (SAMs) is one of the most exploited strategies for transducer activation in biosensors. SAMs are ordered molecular arrays formed by the spontaneous adsorption of an active molecule on a solid surface, often thiol molecules, which forms a covalent bond with gold by chemisorption through the sulfur group.[18] The thiol molecules can consist of a spacer chain usually terminated with a functional group that can be modified to obtain a reactive surface. In biosensors, this strategy is very often used to immobilize the biorecognition element (typically an enzyme, antibody, or nucleic acid sequence) on the transducer surface since it offers the possibility of controlling the orientation, distribution, and spacing of the sensing element.[19] For example, long-chain SAMs modified with polyethyleneglycol backbones have been used in optical biosensors with surface plasmon resonance detection due to their stability and effectiveness in reducing/eliminating nonspecific interactions.[20] Besides these interfacial properties, in electrochemical biosensors, it is also essential to have a surface with permeability to electron transfer in order to avoid a possible blocking of the electrochemical response.[21] This requirement can be fulfilled either using short-chain SAMs, which have the disadvantage of being relatively instable, or using alkanethiol mixtures with different chain lengths in which the relatively low biomolecule immobilization capacity of these surfaces negatively influences the performance of the sensor.[22]

The exploitation of thiol-based SAMs as electrochemical immunosensors has been widely reported.[21,23−27] Detection of CEA using SAMs has been achieved with a surface plasmon resonance biosensor modified with mixed monolayers of ethyleneglycol terminated alkanethiols in spiked serum samples with a detection limit of 25 ng/mL,[28] as well as mercaptoundecanoic acid/mercaptoundecanol[16] and phenyl boronic acid terminated alkanethiol monolayers.[29] In addition, anti-CEA antibodies have been attached to thiourea[30] and cysteamine monolayers[31] and have been used for the electrochemical detection of CEA in standard solutions with detection limits of 0.01 and 0.2 ng/mL, although no application to real samples of these systems was reported.

On the other hand, the integration of biosensing platforms with microfluidics remains challenging. This step is very important in the development of point of care analytical systems for early diagnostics, routine checks, and patient screening.[32] We have recently reported a simple and rapid approach for prototype microfluidics and sensor assembly to perform complex protein and genetic electrochemical assays with excellent reproducibility.[33] The microfluidic platform was realized by high precision milling of polycarbonate sheets, which offers flexibility and rapid turn over of the desired designs. Sixteen-electrode sensor arrays were fabricated using photolithographic deposition technologies in order to realize three-electrode cells comprising gold counter and working electrodes as well as a silver reference electrode. Fluidic chips and electrode arrays were assembled via a laser machined double-sided adhesive gasket, creating the microchannels necessary for sample and reagent delivery.

Additionally, we recently reported the application of dithiolated bipodal scaffold **1** (Chart 1) for the electrochemical detection of proteins such as the prostate specific antigen[21] and gliadin.[34] This dithiol features a central trisubstituted aromatic ring with two alkanethiol residues for gold linkage and a carboxylic acid terminated polyethyleneglycol chain.[35] This structure offers several design advantages such as increased stability due to the pair of attachment points, rapid chemisorption onto gold surfaces,

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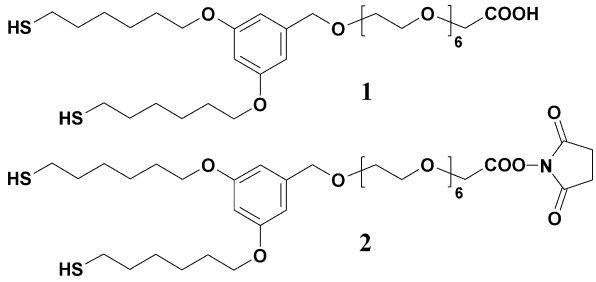
Chart 1. Structure of Dithiols Used in This Work



adequate antibody spacing avoiding the use of mixed monolayers, high resistance to nonspecific interactions, and facility to electron permeability, which allowed the use of both labeled (amperometric)[34] and unlabeled (impedimetric)[21] detection techniques.

In the present paper, we report on the development of a packaged amperometric immunosensor for the detection of CEA in real samples in which surface chemistry and analytical parameters are evaluated in conjunction with other operational and manufacturing biosensor parameters commonly neglected in previous reports such as biomolecule stability. The immunosensor is based on SAMs of dithiol **2**, which is the *N*-hydroxysuccinimide (NHS) ester of **1**. This amine-reactive NHS ester was found to be particularly suitable for work in electrode arrays, which avoid carboxylate activation steps. Highly reproducible levels of immobilized capture antibody were attained using an "in-house" constructed spotting device that required very low volumes of solution. Since for true application of the developed immunosensor it is necessary that the reagents used and coating antibodies are highly stable, an extensive investigation of the stability of the reporter antibody in solution and of the affinity of coated antibodies in the electrode arrays under various storage conditions was also carried out. The developed immunosensor has been successfully applied to the detection of CEA in serum samples obtained from colon cancer patients, and the results were compared with those obtained using a commercial enzyme-linked immunosorbent assay (ELISA) kit, with an excellent correlation obtained.

EXPERIMENTAL SECTION

Reagents and Materials. Dithiols (Chart 1) 22-(3,5-bis((6-mercaptohexyl)oxy)phenyl)-3,6,9,12,15,18,21-heptaoxadocosanoic acid (**1**) and 22-(3,5-bis((6-mercaptohexyl)oxy)phenyl)-3,6,9,12,15,18,21-heptaoxadocosanoic acid *N*-hydroxysuccinimide ester (**2**) were purchased from SensoPath Technologies (Bozeman, MT). Stock solutions (1 mM) were prepared in absolute ethanol (for **1**) and CaH_2-dried acetonitrile (for **2**), purged with argon, and kept at −20 °C when not in use. Carcinoembryonic antigen (CEA) and prostate specific antigen (PSA) were purchased from Scipac Ltd. (Kent, UK). Monoclonal anti-CEA antibodies (12-140-10 and 12-140-1) and anti-PSA (PSA66) were kindly provided by Fujirebio Diagnostics AB (Göteborg, Sweden). The affinity column Freezyme Conjugation purification kit, and *N*-succinimidyl-*S*-acetylthioacetate (SATA) were purchased from Pierce. StabilGuard Biomolecule Stabilizer, StabilZyme Select Stabilizer, and StabilCoat Immunoassay Stabilizer were obtained from Surmodics Inc. (Eden Prairie, MN).

Instrumentation. Electrochemical measurements were performed on a PC controlled PGSTAT12 Autolab potentiostat (EcoChemie, The Netherlands) with an in-built frequency response analyzer FRA2 module. Electrochemical impedance measurements were performed using a standard three-electrode configuration (reference electrode, Ag/AgCl (sat); counter electrode, Pt wire) in 1 mM $Fe(CN)_6^{3-/4-}$ in 0.1 M KCl. The impedance spectra were recorded over the frequency range of 10 kHz to 0.1 Hz at a bias potential of +0.22 V and AC amplitude of 5 mV. The impedance data was represented in the complex impedance plot (Nyquist plot), and the electrochemical parameters were obtained from simulation using the Autolab FRA software.

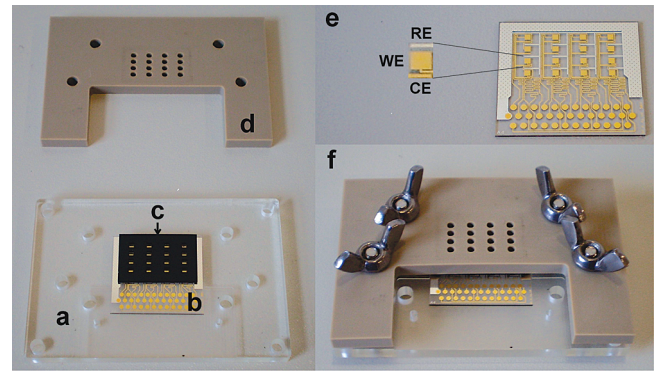


Figure 1. Photographs of the spotting device used in this work: (a) support, (b) electrode array, (c) structured gasket, (d) spotting mask, (e) detail of the electrode array (WE: gold working electrode; CE: gold counter electrode; RE: solver reference electrode), (f) view of the assembled device.

Spectrophotometric analyses were carried out with a Cary 100 Bio spectrophotometer from Varian, and ELISA plates were read in a multiplate reader Wallac Victor2 1420 Multilabel counter from Perkin-Elmer.

Spotting Device Fabrication. A spotting device was fabricated on highly resistant polymeric materials to allow easy deposition of solutions onto the chip during electrode coating (Figure 1). The device is composed of a spotting area, with 16 holes of 1×1 mm^2 on top of a structured rubber gasket as a sealing element (also displaying 1×1 mm^2 square openings), and a support with a dedicated area for the electrode array. After appropriate alignment of the parts, provided by guides on the support and spotting mask, the components are fastened together with four screws and nuts. The device requires only 5 μL of sample per electrode spot and is sealed with Parafilm to avoid evaporation.

Surface Plasmon Resonance Studies. Surface plasmon resonance (SPR) experiments were performed in a Biacore 3000 instrument. A Biacore SIA gold chip was first treated by three cycles of 10 min exposure to an ozone atmosphere using a PSD-UVT cleaning instrument (Novascan Technologies, Ames, IA), followed by rinsing with ethanol and drying in an argon stream. The running buffer was 10 mM HEPES buffered saline (HBS), 150 mM NaCl, 3.4 mM EDTA (pH 7.4). Covalent immobilization of CEA monoclonal antibody was achieved on dithiol-modified chips at a flow rate of 5 μL/min using a 0.5 mg/mL solution of antibody in 10 mM acetate buffer (pH 5.0), followed by injection of 50 μL of 1.0 M ethanolamine (pH 8.5) in order to block remaining NHS ester active groups. Physically adsorbed molecules were removed by two 10 μL pulses of 10 mM glycine (pH 2.2). Antigens and proteins were diluted in PBS buffer (pH 7.4) to the concentrations applied and were then passed over the

antibody-immobilized surface at a flow rate of 5 μL/min for 5 min. Regeneration of the surfaces was performed with 2×10 μL pulses of 10 mM glycine. The equivalence 1000 RU = 1 ng/mm^2 was used to evaluate the amount of immobilized biomolecules on the surface.

Preparation of Anti-CEA HRP Conjugate. Anti-CEA monoclonal antibody 12-140-1 (1 mg) was dissolved in 0.1 M MES and 0.15 mM NaCl (pH 7.2) to obtain a final volume of 1500 μL. A 10 μL aliquot of a solution containing 1 mg of SATA was then dissolved in 100 μL of dried DMSO and added to the antibody solution, and the mixture was gently stirred for 30 min at room temperature, protected from light. A deacetylation solution (100 μL) consisting of 0.5 M hydroxylamine hydrochloride in 0.15 mM NaCl (pH 7.2) was added to the SATA−antibody mixture and allowed to react for 2 h at room temperature, again protected from light. Subsequently, 1 mg of maleimide activated HRP was added, and the solution was incubated for 90 min at 37 °C. To deactivate the maleimide groups and avoid further nondesired reactions, an aliquot of 2-mercaptoethanol was added to the solution to a final concentration of 0.15 mM and stirring continued for 15 min.

Purification of the resulting conjugate was carried out with the metal chelate affinity chromatography Freezyme Conjugation purification kit. Finally, the pure conjugate was concentrated by passing through a molecular weight cutoff filter of 50 kDa at 7500 rpm for 5−10 min, and the conjugates were washed with Milli-Q water. Protein concentration was calculated using the relationship [protein] (g/L) = $A_{280} \times 1.38$.

Electrochemical Measurements. Electrode arrays were fabricated as previously described[28] consisting in 16 gold working electrodes (dimensions: 1×1 mm^2) in a 4×4 arrangement. Each working electrode is placed between a silver pseudo reference 0.2×1 mm^2 and a gold counter electrode of the same size. The working electrodes were spotted with 5 μL of a stock solution of **2** for 3 h, using the spotting device depicted in Figure 1. After rinsing with acetonitrile, capture anti-CEA antibody was covalently immobilized on the NHS-activated SAM by spotting with 5 μL of a 0.5 mg/mL solution of anti-CEA in 10 mM acetate buffer (pH 5.0) for 1 h at 37 °C. The remaining NHS active ester sites were blocked with 1.0 M ethanolamine (pH 8.5) for 30 min at 37 °C. The modified arrays were then assembled in the microfluidic cell as previously described. Reagents and washing buffers were manually injected through a lateral inlet.

CEA antigen was diluted in phosphate buffer (pH 7.2) to the desired concentration, while real serum samples were applied without any dilution for 2 min at room temperature. After rinsing with PBS, a 1 μg/mL aliquot of the prepared HRP-labeled antibody was added and left to incubate to form a sandwich immunocomplex for 2 min. The amperometric measurements were carried out by first recording the background response at −0.2 V in PBS followed by injection of a mixture of 1 mM hydroquinone/1 mM H_2O_2 in PBS (pH 6).

Stability Studies of HRP-Conjugate and Coated Electrodes. Pure conjugate aliquots of 50 μg/L were prepared in the following media: PBS (0.1 M sodium phosphate + 0.15 M NaCl, pH 7.2), 2% w/v bovine serum albumin, 1% w/v trehalose, StabilGuard Biomolecule Stabilizer, StabilZyme Select Stabilizer, and 50% v/v glycerol in PBS. From each sample solution, one aliquot was kept refrigerated at 4 °C (glycerol samples were kept at −20 °C) and another aliquot was kept separately in an incubator at 37 °C for the accelerated aging studies.

To determine the stability of the conjugates over time, triplicate kinetic enzyme activity assays were carried out colorimetrically by measuring the absorbance 2 min after the addition of a 0.5 μL aliquot of the conjugate to 49.5 μL of 3,3′,5,5′-tetramethylbenzidine (TMB) liquid substrate system (Aldrich). Accelerated aging measurements were followed for >160 days on a weekly basis. Real time measurements were monitored for >210 days every 10−20 days.

The absorbance values were then normalized to relative activity (%) and adjusted to a first-order reaction equation (eq 1) in order to evaluate the kinetic constant of the process:

$$\ln\left(\frac{\alpha_t}{\alpha_0}\right) = -k_T t \tag{1}$$

where α_t is activity at time t, α_0 is the initial value to be examined, and k_T is the kinetic constant at a certain temperature. Since data was collected at two temperatures, the activation energy E_a could be calculated by combining the Arrhenius equation ($k_T = Ae^{-E_a/RT}$) with eq 1.

$$\ln\left(\frac{k_{T_1}}{k_{T_2}}\right) = \frac{E_a}{R}\left(\frac{1}{T_2} - \frac{1}{T_1}\right) \tag{2}$$

where R is the gas constant, T_1 and T_2 are the absolute temperatures of refrigerated (277.15 K) and accelerated study samples (310.15 K).

The half-life time of the samples takes into account the time in which the activity decayed to half of its initial value and was calculated according to eq 3:

$$t_{1/2} = \frac{\ln 2}{k_T} \tag{3}$$

The correlation between the accelerated aging and the real time degradation was calculated using eq 1. When the activities at both temperatures were equalized, the relationship between times was obtained and related to the quotient between k_T at the studied temperatures:

$$t_4 = \frac{k_{T_2}}{k_{T_1}} t_{37} \tag{4}$$

where t_4 and t_{37} are the times at which the activities at 4 and 37 °C are equalized. This relationship was then used to predict the trend of the refrigerated samples for long-term storage under refrigerated conditions.

For the stability study of coated electrodes, array chips were modified with anti-CEA as described in the previous section. The stabilizing agents tested were PBS (0.1 M sodium phosphate + 0.15 M NaCl, pH 7.2), 1% w/v trehalose, StabilGuard Biomolecule Stabilizer, and StabilCoat Immunoassay Stabilizer. After the ethanolamine blocking step, the chips were rinsed with water and a drop of the stabilizing agent was quickly spotted over the wet working electrode, avoiding drying of the coated components. The arrays were incubated at room temperature for 1 h, dried in

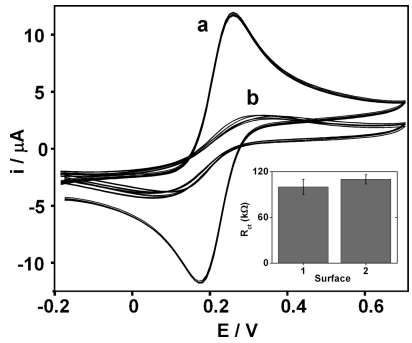


Figure 2. Typical overlay of cyclic voltammograms (in 1 mM $K_3Fe(CN)_6$ in 0.1 M KCl) obtained with the electrode array at bare gold (a) and SAM of **2** (b). Inset: Impedance responses obtained after derivatization of the surface with anti-CEA antibody at SAMs of **1** and **2**.

vacuum at room temperature overnight, and stored at 25 or 37 °C in a sealed container. The stability study was carried out weekly in rows of four working electrodes. The chips were reconstituted with PBS, and the affinity was evaluated by amperometrically detecting 100 ng/mL CEA as described in the previous section.

Serum Samples Collection. Serum samples from colon cancer patients who underwent surgery at Robert Roessle Hospital with complete tumor removal (R0) were evaluated. All patients were free of distant metastasis (M0) at surgery. One-third of the cases were diagnosed as colon adenocarcinoma, as sigma adenocarcinoma, or as mucinous colon carcinomas. A trained nurse carried out collection of serum samples on the surgical ward or the acute day ward. Serum was collected in specific cuvettes (about 5 mL), stored at 4 °C when not processed, and centrifuged at 3000 rpm for 10 min. Thereafter, the supernatant was collected and stored at −80 °C. Patient specimens were collected after informed consent and only used after the Ethics Committee approval of Charité, Berlin.

The CEA levels of these samples were determined using a commercial ELISA kit (CanAg CEA EIA) from Fujirebio Diagnostics AB (Göteborg, Sweden) and using the developed immunosensor.

RESULTS AND DISCUSSION

Surface Chemistry for CEA Detection. NHS-preactivated SAMs were prepared by exposing the working electrodes of the array with a solution of **2** in acetonitrile for 3 h. The use of this aprotic solvent instead of most commonly used alcohols avoids decomposition of the NHS ester group. The cyclic voltammograms of the $Fe(CN)_6^{3-/4-}$ redox couple at bare and SAM-modified electrodes were recorded, revealing the characteristic blocking effect on the cyclic voltammetry (CV) response due to the formation of packed SAMs of **2** on the gold surfaces (Figure 2). The peak-to-peak separation was 90 ± 1 mV for the bare electrode and 265 ± 12 mV ($n = 16$) for the SAM of **2**, indicating a highly reproducible deposition on the array of working electrodes. The estimated surface coverage of **2** was $\Gamma_2 = 2.4 \times 10^{-10}$ mol/cm^2 as determined by reductive desorption experiments in 0.5 M KOH, which compares well with the value obtained for a SAM of **1** ($\Gamma_1 = 2.8 \times 10^{-10}$ mol/cm^2).

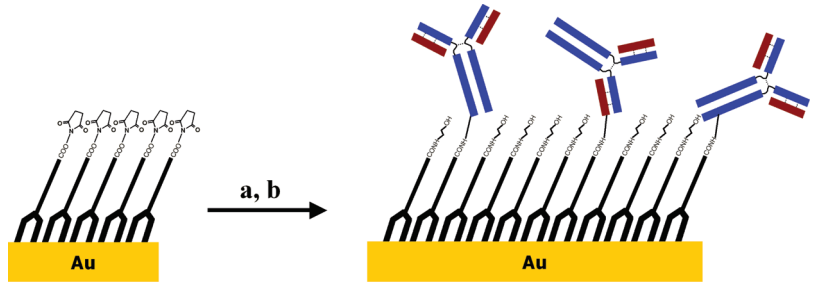
The array was subsequently spotted with a solution of anti-CEA antibody for 1 h, followed by backfilling with ethanolamine (Scheme 1). The immobilization of anti-CEA on each working electrode was confirmed by observing an increase (typically 50 kΩ) in the faradaic impedance response of the electrode following modification. Figure 2 (inset) shows a comparison between the average impedance responses obtained after anti-CEA immobilization at SAMs of **1** (activated by treating the surface with an aqueous solution of EDC/NHS) and **2** in the 16-electrode array. The response obtained using **2** is 9% higher and more reproducible than in the case of **1**. If we consider the observed impedance values as an indirect measure of antibody immobilization degree, the SAM of **2** shows a higher capture efficiency, highlighting the advantages of the use of the preactivated SAM in which the coverage of amino-reactive NHS groups should be higher than when the activation is carried out in solution. In addition, the number of steps and the time required for electrode preparation is reduced in ∼60 min, since no carboxylic group activation is required.

Surface plasmon resonance (SPR) was used to evaluate the optimum surface chemistry for CEA detection. Anti-CEA monoclonal antibody was covalently linked to the bipodal thiolated chemisorbed linker using standard EDC/NHS chemistry followed by ethanolamine backfilling to prevent nonspecific binding. The antibody immobilization level was 430 ng/cm^2, which represents an interfacial concentration of 2.1 pmol/cm^2 assuming a molecular weight of 200 kDa for the anti-CEA antibody. This value is in the same order of magnitude as reported previously for human anti-IgG on mixed SAMs but considerably lower than the theoretical surface density of a packed monolayer of a whole antibody (∼30 pmol/cm^2).[22] This value is particularly important, since a dense monolayer would hinder antigen recognition and electron transfer, which is the basis of the amperometric detection method used here. Table 1 summarizes the SPR responses obtained for CEA alone and when nonspecific antigen was added to test the cross reactivity of CEA antibody using prostate specific antigen (PSA) and bovine serum albumin (BSA). As can be seen, low RU values were obtained upon addition of the nonspecific analytes, as well as for the detection of CEA at an anti-PSA modified surface, highlighting the usefulness of the dithiol-modified surface to prevent nonspecific binding.

The SAM modified with anti-CEA capture antibody was incubated with 100 ng/mL CEA antigen alone and in the presence of fetal calf serum (10 mg/mL). As can be seen in Figure 3, the system is able to selectively detect the target antigen in this complex matrix. This result is especially relevant since real samples often contain very high concentrations of other globulins, enzymes, hormones, and nutrients, which are potential sources of nonspecificity and false positives.

Amperometric Detection of CEA. Figure 4 represents the amperometric calibration plot obtained for CEA detection using the developed immunosensor. The current variations showed a linear relationship with the concentration of CEA over the range of 0−200 ng/mL with a sensitivity of 3.8 nA·mL·ng^{-1} and a detection limit of 0.2 ng/mL, which is below the commonly accepted concentration threshold (5 ng/mL) used in clinical diagnosis. It should be noted that this low detection limit has been achieved on a surface modified with randomly oriented

Scheme 1. Strategy for Electrode Modification and Antibody Immobilization[a]



[a] See Experimental Section for details. (a) Anti-CEA immobilization; (b) ethanolamine backfilling.

Table 1. SPR Responses (RU) Obtained for the Detection of CEA and Nonspecific Targets

coating antibody	analyte	RU	NSB (%)[a]
anti-CEA	CEA (5 μg/mL)	274	
anti-CEA	PSA (10 μg/mL)	27	9.8
anti-CEA	BSA (2 mg/mL)	10	3.7
anti-PSA	CEA (5 μg/mL)	40	14.8

[a] NSB = $100(\Delta RU_{nonanalyte}/\Delta RU_{analyte})$, where NSB is the degree of nonspecific binding and ΔRU represents the change in SPR response for both nonspecific and specific analyte.

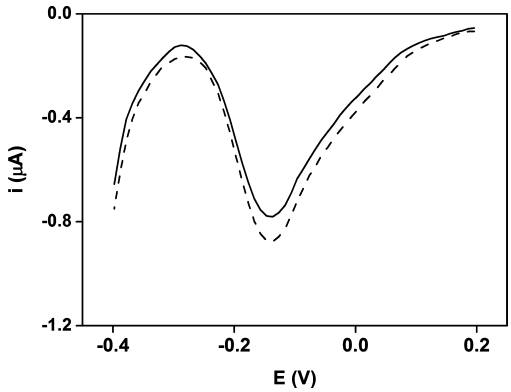


Figure 3. DPV responses for the detection of 100 ng/mL CEA in the absence (—) and in the presence (---) of 10 mg/mL fetal calf serum.

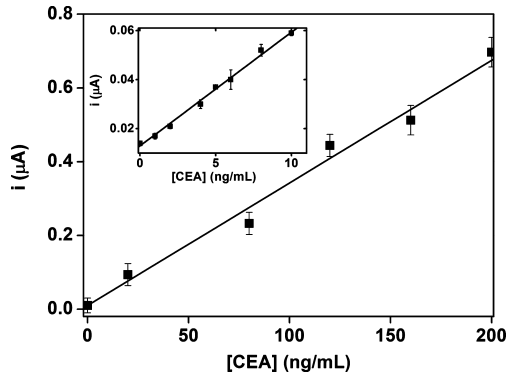


Figure 4. Calibration plot for the amperometric detection of CEA.

antibodies (see Scheme 1) and it could be expected that even lower values could be attained if methods for oriented immobilization are used (i.e., use of antibody fragments).[26]

To study the reproducibility of the immunosensor, a 16-electrode array was identically modified with **2**/anti-CEA/ethanolamine and applied in the detection of 10 ng/mL CEA. The relative standard deviation of the measurement was 7%, indicating an excellent reproducibility of the immunosensor.

Arrhenius Accelerated Stability Studies of Anti-CEA HRP Conjugate in Different Stabilizing Agents. The electrochemical assay under study is, as previously described, based on the formation of a sandwich immunocomplex between a capture antibody, the sample, and an HRP-labeled reporter antibody. Long-term stabilities of labeled reporter antibody and coating capture antibody are essential for the clinical application of biosensors since they can determine the accuracy of the assay, and thus, we carried out a stability study of anti-CEA coated electrode arrays and in-house prepared anti-CEA HRP conjugate under different conditions of storage buffer and temperature.

Protein inactivation is a combination of several complex processes such as unfolding leading to a disruption of the quaternary structure, autoaggregation or aggregation to other surfaces, loss of prosthetic group, or chemical modifications.[36] In the biodiagnostics industry, it is common to use accelerated aging studies as an empirical indication of real time stability. This method for estimating the shelf life of a product is based on the Arrhenius equation, termed as an "Arrhenius accelerated stability study".[37] Figure 5 shows the plots of the residual activity of the conjugate in the different conditions after several months of storage. The best stabilizing agent was found to be StabilGuard Biomolecule Stabilizer, in which the HRP conjugate retained more than 85% of its enzymatic activity after 222 days at 4 °C and ~60% after 165 days at 37 °C.

Table 2 shows the kinetic parameters obtained from the data depicted in Figure 5 using eqs 1−3. As expected, the inactivation kinetic constants obtained in the presence of the stabilizing buffers are lower than that obtained in PBS. In StabilGuard buffer, the inactivation rate was 10 times lower at 37 °C, with a half-life of 2.1 years at 4 °C, indicating an excellent resistance to denaturation of the conjugate in this buffer. The activation energy (E_a), which is also related to the resistance to denaturation, increased by 20−22 kJ/mol in the commercial stabilizing buffers. These values are in the range of activation energy (20−40 kJ/mol) required to preserve the native conformation of proteins,[38] further demonstrating the stabilizing effect conferred by these buffers. However, the absolute values of activation energy obtained (48−50 kJ/mol) are ~2 fold lower than that reported for the unfolding of native peroxidase at 37 °C and pH 7.8, which is 112 kJ/mol.[38] The possibility of dissociation of the antibody and enzyme components in the conjugate was tested

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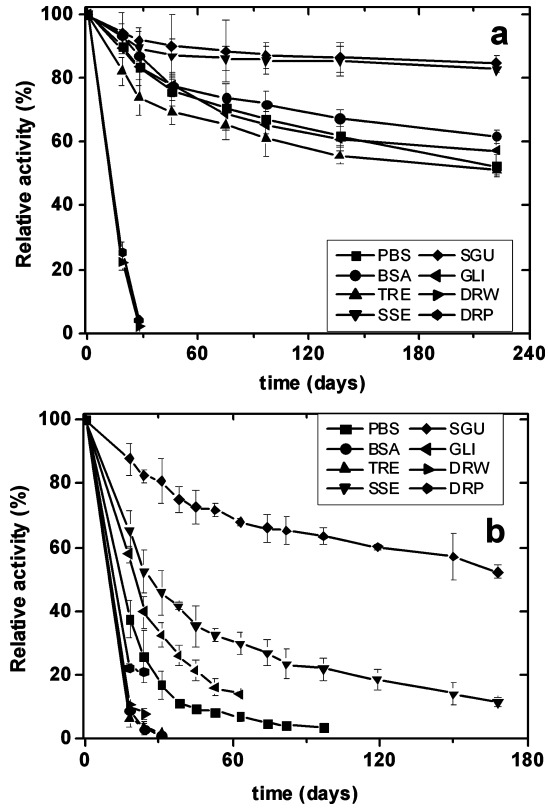

Figure 5. Residual enzymatic activity of anti-CEA-HRP conjugate stored in different preservative solutions at 4 °C (a) and 37 °C (b). Legend: PBS, 0.1 M sodium phosphate + 0.15 M NaCl, pH 7.2; BSA, 2% bovine serum albumin; TRE, 1% trehalose; SGU, StabilGuard Biomolecule Stabilizer; SSE, StabilZyme SELECT Stabilizer, GLI, 50% glycerol in PBS; DRW, dried from aqueous solution; DRP, dried from PBS.

Table 2. Kinetic Parameters for Real Time and Arrhenius Accelerated Stability Studies of Anti-CEA HRP Conjugate in Different Stabilizing Agents

storage conditions	$k_T \times 10^7$ (s^{-1})		$t^{1/2}$ (year)		E_a (kJ/mol)
	37 °C	4 °C	37 °C	4 °C	
PBS	4.2	0.29	0.05	0.76	28
glycerol 50%	3.3	0.26[a]	0.07	0.85[a]	29
StabilSelect	1.4	0.14	0.16	1.6	48
StabilGuard	0.46	0.04	0.53	2.1	50

[a] Stored at −20 °C.

by gel electrophoresis at the beginning and end of the study, obtaining the same electrophoretic pattern in both cases, which indicates no dissociation.

Figure 6 compares the residual enzymatic activity in real time obtained at 4 °C in StabilGuard buffer with values predicted taking into consideration the results of the accelerated stability studies, calculated according to eq 4. The real time data was recorded after 222 days, and there is excellent agreement of predicted and experimental values over this time interval. After this time, the remaining points represent a prediction of the stability trend based on the experimental accelerated stability data, which, in turn, fits very well with the theoretically retained activity calculated from the first-order kinetic law (eq 1) using the measured value of k_T at 4 °C. In addition, from the k_{37}/k_4 ratio, it is possible to calculate the equivalence between the activities measured in

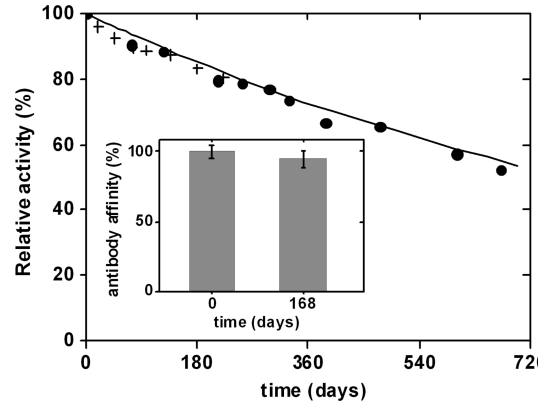

Figure 6. Plot of residual activity values obtained in real time at 4 °C (+), extrapolated from 37 to 4 °C according to eq 4 (•), and predicted at 4 °C from eq 1 (—). Inset: Variation of conjugate affinity for 10 ng/mL CEA with time after storage at 4 °C in StabilGuard Select buffer.

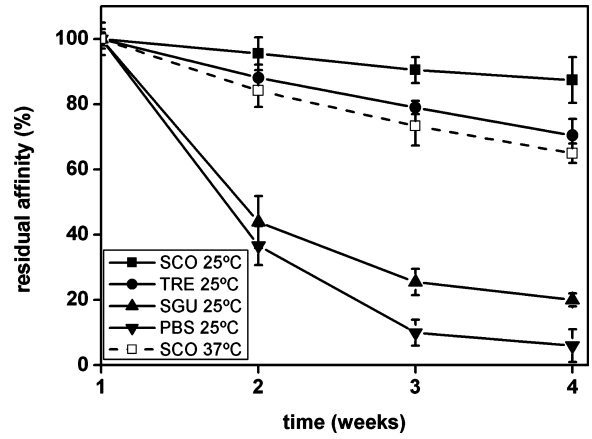

Figure 7. Residual antibody affinity of coated electrodes stored in different media. Legend: PBS, 0.1 M sodium phosphate + 0.15 M NaCl (pH 7.2); TRE, 1% trehalose; SGU, StabilGuard Biomolecule Stabilizer; SCO, StabilCoat Stabilizer.

real time and in the accelerated study. In StabilGuard buffer, $k_{37}/k_4 = 11.5$, which means that 1 month of storage at 37 °C represents approximately 1 year at 4 °C. We are currently evaluating other conjugates with the above-described protocol, which should be particularly suitable for the evaluation of stability of in-house prepared noncommercial conjugates.

The possible effect of storage conditions on antibody affinity was also analyzed by ELISA. No significant changes in the affinity of the conjugate for CEA were observed after 5 months at 4 °C in StabilGuard buffer (Figure 6, inset). In addition, amperometric detection of 10 ng/mL CEA was carried out using a conjugate sample taken after 180 days of storage at 4 °C in StabilGuard buffer; no major decrease (<5%) was observed in the current values recorded when compared with a conjugate sample taken immediately after preparation.

Stability Studies of Coated Electrodes. Figure 7 shows the plots of the residual affinity of the coating anti-CEA in the different conditions after several weeks of storage. The best stabilizing agent was found to be StabilCoat Immunoassay Stabilizer, in which the coating components retained more than 90% of affinity at 25 °C and ~70% at 37 °C after 1 month of storage. The affinity as a function of the stabilizing agent was found to decrease in the order

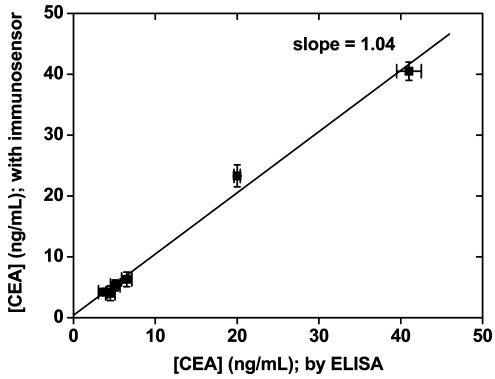

Figure 8. Correlation between CEA levels measured in real serum samples using the developed immunosensor and ELISA results.

of StabilCoat > trehalose > StabilGuard > PBS. The equivalence between the affinities measured in real time and in the accelerated study was calculated in a similar way as that used for HRP conjugate. In StabilCoat buffer, $k_{37}/k_{25} = 6.1$, which means that 1 month of storage at 37 °C represents ~6 months at 25 °C.

Real Sample Analysis. The developed immunosensor was then applied to the detection of CEA in real samples using serum samples from colon cancer patients. Samples were taken and divided into two aliquots, one for ELISA and one for immunosensor detection. As can be seen from Figure 8, the slope of the ELISA vs immunosensor plot was 1.04, indicating an excellent correlation between the CEA levels obtained by both methods. In addition, the total assay time is less than 10 min with the developed immunosensor, which represents a dramatic reduction in assay time when compared with commercial immunoassays, suggesting the possible applicability of the immunosensor to in situ use. The multiplexed detection of several markers in an array format is currently being explored.

CONCLUSIONS

In this paper, we have demonstrated the applicability of SAMs of a relatively unexplored dithiolated aromatic compound in the detection of proteins in a complex matrix such as human serum. An amperometric biosensor for the detection of CEA based on the immobilization of anti-CEA monoclonal antibody at these SAMs was developed with a detection limit of 0.2 ng/mL, a value below the commonly accepted concentration threshold used in clinical diagnosis. The prepared biosensor is also reproducible and highly stable. Real time and accelerated stability studies of the reporter antibody and coated electrodes under various storage conditions demonstrated that the enzymatic activity and antibody affinity is retained for long periods of time in commercial stabilizing buffers such as StabilGuard Biomolecule Stabilizer and StabilCoat Imunoassay Stabilizer, and a prediction of the stability trends was carried out using the kinetic and thermodynamic parameters obtained from the Arrhenius equation. The developed immunosensor was successfully applied to the detection of CEA in serum samples obtained from colon cancer patients, and the results show an excellent correlation with those obtained using a commercial ELISA kit. In our ongoing work, we are looking at the incorporation of the developed biosensor into a platform for multiplexed simultaneous detection of several breast cancer related biomarkers.

ACKNOWLEDGMENT

The authors thank the EU SmartHEALTH project (FP6-2004-IST-NMP-2-016817) for financial support and Fujirebio Diagnostics AB for providing the monoclonal antibodies. A.F. thanks Ministerio de Ciencia e Innovacion, Spain, for a "Ramón y Cajal" Research Professorship.

Received for review September 25, 2009. Accepted January 19, 2010.

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