Functionalization of Gold Surfaces for Specific and Reversible Attachment of a Fused β-Galactosidase and Choline-Receptor Protein

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Abstract: A method that allows the specific immobilization of proteins onto a gold electrode has been developed. Mixed self-assembled monolayers of thiol chains functionalized with choline and hydroxyl groups have been synthesized step-by-step over a template of thiocarboxylic acid adsorbed onto gold. Choline-functionalized monolayers displayed affinity for a chimera protein made by the fusion of the β-galactosidase (β-Gal) from *Escherichia coli* and the choline-binding domain of the (acetylmuramoyl)-L-alanine amidase (C-LYTA) from *Streptococcus pneumoniae.* This chimera maintains both the hydrolase activity and the affinity for choline, respectively, of its parent proteins. The binding of the protein to the tailored interface was specific and could be inhibited either by soluble choline or by saturating the monolayer choline groups with the C-LYTA fragment. Using an ^{35}S-labeled chimera, saturation coverage was found under optimized binding conditions. The activity of the immobilized chimera was determined with *(p*-aminophenyl)-β-D-galactopyranoside, a synthetic substrate of β-galactosidase. The product of the enzymatic reaction, *p*-aminophenol, was detected electrochemically using the functionalized gold surface with bound chimera protein as a working electrode in a conventional electrochemical cell. Gold electrodes covered with chimera protein were very stable and gave fast and reproducible electrochemical response to the addition of β-Gal substrate in a conventional flow injection analysis system.

Introduction

A challenging goal in biotechnology, molecular electronics, and biosensors is the immobilization on surfaces of proteins while retaining their full activity and stability. Most of the commonly used methods, e.g., nonspecific adsorption, physical entrapment in polymeric gels, chemical cross-linking, or covalent attachment to an insoluble support,[1] are difficult to control and usually yield randomly bound proteins. On the contrary, an ideal immobilization would produce saturation coverage of reversibly and specifically bound proteins. Reversibility allows the substitution of inactivated proteins by active ones. Specificity permits the selective immobilization of a given protein from a complex mixture. Under adequate circumstances, specific binding would result in oriented immobilization of the proteins.[2−6]

Molecular recognition properties of biological systems have been used for specific binding of proteins to gold surfaces covered with a self-assembled monolayer (SAM) of thio compounds.[7] Spinke *et al.*[8] demonstrated the possibility of using SAMs of biotinylated alkanethiols for binding streptavidin molecules. Later this biotin−avidin system was used as a template to direct the binding of monobiotinylated Fab fragments of monoclonal antibodies.[9] Katz *et al.*[10a] bound pyrroloquinoline quinone, the coenzyme of the quinoprotein glucose dehydrogenase, to a SAM of cysteine. Incubation of this functionalized SAM with glucose dehydrogenase apoprotein resulted in reconstituted active enzyme. A similar strategy has been used for reconstitution of Fe- and Zn-myoglobin over a SAM of thiol derivatives of metalloporphyrins.[10b] In a recent work by Whitesides and co-workers[11] carbonic anhydrase was specifically bound to SAMs of alkanethiols terminated in benzenesulfonamide, a ligand for which it displays high affinity.[12]

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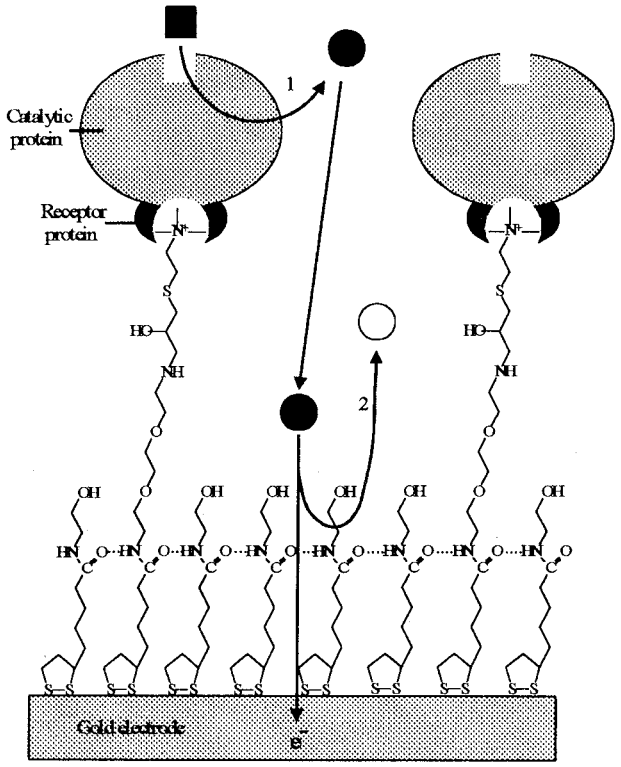

Figure 1. Schematic representation of the enzyme electrode showing the hydrolysis of the substrate by the enzyme (reaction 1) and further oxidation of the reaction product (reaction 2): (■) PAPG; (●) PAP; (○) quinonimine (not to scale; molecular details not accurate).

In all the above studies the specific binding motif was unique to the protein under study. A more general method would require, firstly, a protein with a unique and specific binding site and, secondly, a well-defined, functionalized substrate surface. It seemed to us that the conjunction of SAM technology with the potential of molecular genetic techniques to produce chimeric proteins by the fusion of two parental genes[13] could yield a general method for immobilization of enzymes through specific binding sites: the enzyme would be fused to the binding domain of a biological receptor, and consequently the topology of the affinity site would be identical in the entire population of chimeric proteins. The surface would be a tailored SAM functionalized with a ligand chosen by its affinity for the receptor.

In the present work we have explored this approach using β-galactosidase (β-Gal) from *Escherichia coli* fused to the choline-binding domain of (acetylmuramoyl)-L-alanine amidase (C-LYTA) from *Streptococcus pneumoniae*,[14] this chimera protein retaining both the hydrolase activity and the affinity for choline of its parent proteins.[14] We have covalently bound an analog of choline to the end of functionalized thiol chains forming SAMs on gold electrodes. The enzyme-modified gold itself was used as a working electrode to monitor the rate of hydrolysis of (*p*-aminophenyl)-β-D-galactopyranoside (PAPG) catalyzed by the immobilized enzyme, as depicted in Figure 1. We have investigated the specificity, efficiency, and binding reversibility of the fused protein to choline-functionalized SAMs as well as the stability of the modified electrodes.

Experimental Section

Electrode Preparation. Polycrystalline gold wire electrodes were polished with γ-alumina (0.05 μm, Buehler), washed, sonicated for 5 min in doubly distilled water, treated with H_2SO_4 (98%) at 100−120 °C for 1.5 h, and then rinsed in doubly distilled water. The voltammogram in 0.1 M H_2SO_4 at this stage was characteristic of a clean polycrystalline gold surface.[15] The area of the electrode was determined from chronocoulometric Anson plots with 1.1 mM ferricyanide in 0.1 M KCl, pH 3, using a value of 7.6×10^{-6} cm^2 s^{-1} for the diffusion coefficient of ferricyanide.[16] Typical effective areas were 0.30 cm^2 (geometrical area 0.26 cm^2). The electrode was rinsed with water and dipped in a 1 mM solution of 6,8-dithioctic acid (Aldrich) in 2:1 ethanol/water for 24 h to allow the formation of a monolayer of dithioctic acid (SAM-TOA). The modified electrodes were washed with 2:1 ethanol/water, dried in air, and subjected immediately to carboxylic activation. Gold electrodes modified with 4,4′-dithiodibutyric acid (SAM-TBA), thiocholine (ICN Biochemicals, TCh), or TBA-TCh mixtures of different molar ratios were prepared in a similar way.

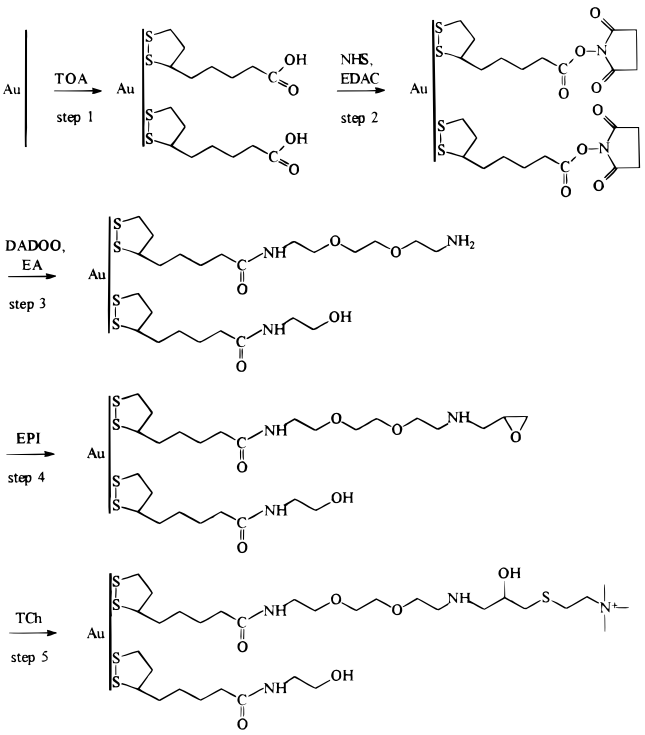

Figure 2. Sequence of chemical steps used in the synthesis of the choline-functionalized monolayer shown in Figure 1.

Synthesis of Choline-Modified SAM-Thioacids. The choline-modified electrodes were prepared following the scheme in Figure 2 for SAM-TOA. (1) Activation of carboxylic acid groups of the monolayer: The SAM-TOA electrode was immersed for 3 h in a dioxane solution of 0.1 M *N*-hydroxysuccinimide (Sigma; NHS) containing 0.1 M 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide (Sigma; EDAC) which catalyzed the esterification[17] and rinsed three times with dioxane and dried in air. (2) Coupling of hydrophilic spacer: Amidation of NHS-esters of SAM-TOA was effected overnight in undiluted 1,8-diamino-3,6-dioxaoctane (Merck, DADOO), after which the unreacted diamine was removed by washing with ethanol followed by dipping in 0.1 M KOH (SAM-TOA-DADOO). Alternatively, mixtures of DADOO and ethanolamine (Aldrich; EA) were used to prepare electrodes in which the DADOO spacers were diluted with shorter hydroxy-ending molecules (SAM-TOA-[DADOO-EA]). (3) Activation of terminal amino groups of SAM-TOA-DADOO: The unprotonated amino end groups were activated by dipping the electrode in undiluted 1-chloro-2,3-epoxypropane (epichlorhydrin, Merck) for 3 h (SAM-TOA-DADOO-Epi). Unreacted epichlorohydrin was eliminated by washing with 2:1 ethanol/water. (4) Coupling of thiocholine to activated SAM-TOA-DADOO-Epi: The terminal epoxy groups

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generated in the previous step were reacted for 18 h with a 10 mM solution of thiocholine in 50 mM phosphate buffer, pH 8, followed by washing with the same buffer (SAM-TOA-DADOO-Epi-ThC).

For control purposes *N*-methyl-*N*′-(aminopropyl)-4,4′-bipyridinium (MAPV)[18] was used instead of DADOO at step 2, and 2-[*N*-(methylferrocenyl)amino]ethanethiol or *N*-(methylferrocenyl)cystamine (Aldrich, CA) was used in step 4 instead of thiocholine. All electrode preparations were run in duplicate.

Detection of SAMs. After each step of SAM synthesis, a control electrode was used for cyclic voltammetry in 0.5 M KOH supporting electrolyte, under nitrogen, in the potential range 0 to −1.1 V. The presence of a reductive desorption wave at −1.0 V was taken as evidence for the existence of a SAM.[19]

Preparation of the Choline-Binding Domain/β-Galactosidase Chimera. The fusion protein of β-Gal and C-LYTA ("chimera protein" in what follows) was produced and purified as reported previously.[14] C-LYTA is a polypeptide of 16 kDa.[20] β-Gal is a tetramer of 465 kDa.[21] The molecular mass of the chimera protein is 529 kDa. Chimera protein labeled with ^{35}S in methionine residues was produced using the strain of *E. coli* W3140 [*met-B1*] transformed with plasmid pEG4012.[22] The radioactivity of the chimera protein was 2850 cpm/μg protein.

Enzymatic Activity. The galactosidase activity of the soluble chimera protein was determined spectrophotometrically with (*o*-nitrophenyl)-β-D-galactopyranoside (Sigma; ONPG) as substrate.[23] Alternatively, the activity was measured with (*p*-aminophenyl)-β-D-galactopyranoside (Sigma; PAPG). In the latter case the course of the hydrolysis reaction was followed by cyclic voltammetry (CV) of the reaction product *p*-aminophenol,[24] a good method for the analysis of the activity of the bound enzyme since the gold wire served both as enzyme support and as an indicator of catalytic activity. Since Mg^{2+} and Na^+ ions are necessary for β-galactosidase enzymatic function,[25] 7 mM magnesium chloride was added to the pH 7.3, 50 mM sodium phosphate buffer used as supporting electrolyte. The electrochemical response of PAP on a gold electrode covered with SAM-TOA-[DADOO-EA, 1:8]-Epi-TCh was very stable, the CV remaining unchanged during more than 100 cycles at 100 mV s^{-1}. The slope of a plot of the current density of the PAP oxidation peak, j^a, *vs* PAP concentration was 46 μA μmol^{-1} cm^{-3} with SAM-TOA-[DADOO-EA, 1:8]-Epi-TCh as working electrode, from which the activity of the enzyme could be estimated from the peak intensity measured after 10 min of incubation of the enzyme with PAPG. The electrochemical turnover of PAP did not affect the catalytic activity of the bound enzyme, since the measured activity did not vary after a period of incubation in supporting electrolyte containing 1mM PAP during which 100 CVs at 100 mV s^{-1} were run. From the dependence of the activity on PAPG concentration, the following parameters of chimera protein were determined: $K_m = 0.4$ mM, $k_{cat} = 86$ s^{-1}. Routine analyses of the activity of the enzymatic electrode were carried out with 4 mM PAPG.

Binding of Chimera Protein to the Functionalized SAM. From preliminary experiments we found that nonspecific adsorption of chimera protein could be minimized by incubation of the modified electrode for 10 min in a solution of lactoalbumin (0.5 mg mL^{-1}) prior to addition of the fused protein, followed by washing with 50 mM sodium phosphate buffer, pH 7.3, for 10 min. This pretreatment was applied to all electrodes. Pretreated electrodes were incubated with the solution of chimera protein in 50 mM sodium phosphate buffer, 0.1 M KCl, pH 7.3, containing 7 mM $MgCl_2$. Electrodes incubated with enzyme preparations were washed with this buffer and placed in the electrochemical cell. From binding experiments at different concentrations of chimera protein in the solution of incubation, a minimum concentration of protein (0.1 mg/mL) yielding maximum activity was selected.

Electrochemical Cell and Instruments. All the electrochemical experiments were carried out at 20 °C in an electrochemical cell with an electrolyte volume of 2 mL. A platinum auxiliary electrode and an Ag/AgCl, 3 M NaCl, reference electrode connected to the cell by a salt bridge filled with the electrolyte solution were used, as well as a BAS analyzer, model CV-50W. Diffusion coefficients for PAP through SAM modified gold electrodes were measured by chronocoulometry at 25 °C in 50 mM phosphate buffer, pH 7.3, 0.1 M KCl.

Experiment with the ^{35}S-Labeled Chimera Protein. Electrodes covered with SAM functionalized with choline residues were incubated in a solution containing 0.1 mg mL^{-1} of ^{35}S-labeled chimera protein in 50 mM phosphate buffer, 1 M KCl, pH 7.3, for 10 min. After washing with the same buffer solution for 15 min, the adsorbed protein was heated at 100 °C for 10 min in 0.3 mL of a 2% sodium dodecyl sulfate (Sigma, SDS) solution containing 0.1 M dithiothreitol in 60 mM Tris−HCl buffer, pH 7, after which less than 5% of the total radioactivity remained on the gold wire. The SDS solution was added to scintillation vials containing 3 mL of Ready Safe (Beckman) scintillation liquid.

Synthesis of 2-[*N*-(methylferrocenyl)amino]ethanethiol. The compound Fc-CH_2-NH-CH_2-CH_2-SH (FcRSH) was synthesized by condensation of ferrocenecarboxaldehyde (Aldrich) with a 5× excess of cystamine in a 1:1 water/ethanol mixture followed by reduction of the resulting Schiff base with $NaBH_4$. Ferrocene derivatives were extracted with chloroform, the solvent was allowed to evaporate, and the slurry was dissolved in a small volume (1.5 mL) of chloroform and purified on a silica gel (30−70 mesh ASTM, Merck) column equilibrated with chloroform. The elution was made with chloroform/ethanol mixtures of decreasing hydrophobicity. Red-colored fractions were analyzed for thiol content with 5,5′-dithiobis(2-nitrobenzoic acid) (Ellman's reagent, Sigma). Fractions eluted with 9:1 chloroform/ethanol that contained SH groups and produced a single spot in thin layer chromatography (DC-Alufolien Kiselgel GO F_{254}, Merk) were pooled. The solvent was evaporated under vacuum, and the product was stored under nitrogen. ^1H NMR spectrum ($CDCl_3$): 1.26 ppm (1H, s) 1.95 ppm (1H, s) 2.94 ppm (2H, t) 2.96 ppm (2H, t) 3.53 ppm (2H, s) 4.15 ppm (9H, m). Anal. ($FeC_{13}H_{17}NS$) Calcd: C, 56.74; H, 6.23; N, 5.09; S, 11.65. Found: C, 57.19; H, 5.80; N, 4.73; S, 11.25. Mass spectrometry: calcd, *m/z* 275.043; found, *m/z* 275.1.

X-ray Photoelectron Spectroscopy (XPS). XPS spectra were obtained on a Fisons ESCALAB 200R spectrometer equipped with a 120 W Mg Kd X-ray source ($h\nu = 1253.6$ eV). Pressure in the analysis chamber was maintained below 2×10^{-9} Torr. An acceptance angle of 45° and take-off angle of 45° were employed. Spectra were recorded with a 20 eV pass energy, 0.1 eV spot, with acquisition time of 30 s per scan. Monolayers were formed on 1 cm^2 gold square plates in the same way as for the gold wires. Elemental composition was determined from the integrated peak intensities after background subtraction and using the following atomic sensitivity factors (ASF): 0.35, 0.205, 0.40, and 0.63 for S, C, N, and O, respectively.[26]

Results

Thiocholine SAM. We attempted to chemisorb thiocholine onto gold by incubating the gold support with a 1 mM solution of this compound, but this did not yield a thiocholine SAM, since (i) the electrochemical response to charged probes, $Fe(CN)_6^{3-}$ and $Ru(NH_3)_6^{3+}$, was similar to those observed with bare gold electrodes (not shown), (ii) the reductive desorption wave typical of thio compounds adsorbed on gold[19] was not observed (not shown), and (iii) sulfur and nitrogen were undetectable by XPS (Table S1 available in the Supporting Information). This suggests that SAMs of thiocholine are

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extremely unstable, probably due to electrostatic repulsions among the charged groups. The stability of monolayers with charged quaternary ammonium groups has been studied, both theoretically and experimentally, by Doblhofer *et al.*[36] who concluded that the ionic group has a strong destabilizing effect upon immersion in solvents of high dielectric constant. Therefore, different preparation procedures of monolayers of thiocholine were attempted by diluting this compound with neutral or anionic thiols of similar chain length: (a) Thiocholine/1-butanethiol (1:8). This SAM was also unstable since after 24 h the reductive desorption wave due to adsorbed thiols could not be detected (not shown). (b) Thiocholine/4,4′-dithiodibutyric acid (1:1 and 1:4) SAMs were very stable, their CVs in 0.5 M KOH showing a large desorption wave at −1.0 V (not shown) typical of monolayers of thio compounds adsorbed on gold.[19] From the peak charge and assuming a value of 1 electron per sulfur atom, a surface coverage by sulfur atoms of $(8.1 \pm 0.5) \times 10^{-10}$ mol cm^{-2} ($n = 3$) and $(7.6 \pm 0.5) \times 10^{-10}$ mol cm^{-2} ($n = 3$), respectively, was estimated. Their atomic compositions as determined by XPS were the expected ones (Table S1; see below for further comments on the XPS results).

Freshly prepared gold electrodes with the above-mentioned thiocholine mixtures were incubated in solutions of chimera protein, and after washing, their hydrolytic activity toward PAPG was tested electrochemically. In neither case was bound chimera protein detected (not shown). These results suggest that the acces of the C-LYTAs binding pocket to the thiocholine molecules on the monolayer was sterically prevented, in agreement with the results of Spinke *et al.*,[8] who demonstrated that binding of avidin to biotin on monolayers required the inclusion of a spacer in the biotinylated thiol. To accomplish the inclusion of such a spacer in cholinethiol chains, we have synthesized step-by-step SAMs of thiocholine starting with a stable monolayer of thioacids.

Thioacid SAM. The formation of stable SAMs of long-chain thioacids on gold is well documented.[27] While commercially available short-length thioacids have also been used to form SAMs on gold substrates,[28] they are not as well characterized. Therefore, the presence of TBA- and TOA-SAMs, before and after each derivatization step, was investigated as well as their electrochemical response to charged probes. The CV of Figure 3a corresponds to a gold electrode covered with SAM-TOA and shows a large desorption wave at −1.0 V of thio compounds adsorbed on gold,[19] with a charge corresponding to a surface coverage by sulfur atoms of $(7.1 \pm 0.6) \times 10^{-10}$ mol cm^{-2} ($n = 8$). A similar determination of the SAM-TBA (Figure 3b) gives a surface coverage of $(7.5 \pm 0.5) \times 10^{-10}$ mol cm^{-2} ($n = 8$). Both values are in reasonable agreement with a published monolayer thiolate surface coverage of 7.6×10^{-10} mol cm^{-2} on Au(111).[19]

The TBA-SAM showed, as expected, that selectivity toward charged probes depends on the degree of ionization of the carboxylic groups (Figure 4a). At pH 5.5 the electrochemical responses of $Fe(CN)_6^{3-}$ and $Ru(NH_3)_6^{3+}$ on TBA-SAM electrodes were identical to those observed with bare gold electrodes (not shown). The marked dependence with pH of the electrochemical response of $Fe(CN)_6^{3-}$ in the pH range 5−12 is in agreement with the fact that the pK_a of thioacids increases after the formation of the monolayer.[30] While the faradaic response was not completely suppressed at alkaline pH, the electrochemical process became more irreversible (Figure 4a). In contrast, complete suppression of the response of ferricyanide was obtained at pH 5.5 with the TOA-SAM (Figure 4b), in agreement with a previous report on TOA monolayers.[28b] The different permselectivity toward ferricyanide displayed by TBA- and TOA-SAMs could be due to the known tendency of very short chain alkanethiolates to form disordered structures.[29]

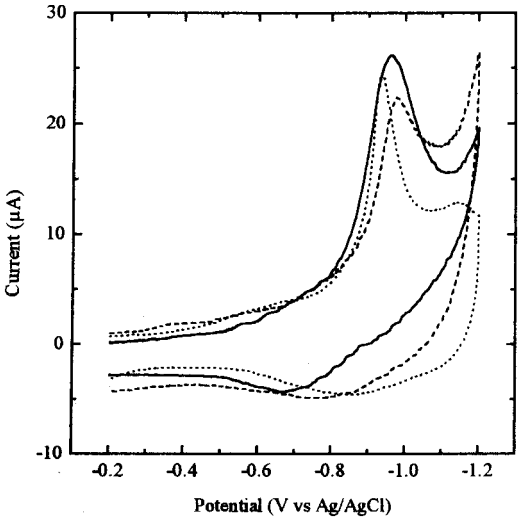

Figure 3. Cyclic voltammograms of modified electrodes in 0.5 M KOH: (a) SAM-TOA (solid line); (b) SAM-TBA (dotted line); (c) SAM-TOA-[DADOO-EA,1:8]-Epi-TCh (dashed line). The scan rate was 0.1 V s^{-1}. The starting potential was −0.2 V.

Stepwise Construction of the Choline-Functionalized SAM. The first step of our SAM modification scheme was the activation of the carboxy terminal groups in the monolayer by esterification with *N*-hydroxysuccinimide, a well-established method for binding primary amine groups to organic acids by amidation.[17] Since the reactive species is the un-ionized carboxylic group after rinsing with deionized water, the surface carboxy groups must be protonated, after which the reaction goes with a high yield. It was run in dioxane, since the intermediate succinimide esters are very stable in the absence of nucleophiles.

To check the efficiency of the amidation reaction of the TBA monolayer, the activated COOH groups were reacted with 3-aminopropyl methyl viologen (MAPV) and the resulting electrode was analyzed by CV. We checked that bare electrodes showed no electrochemical peaks after incubation with MAPV followed by rinsing with water. For the modified electrode the nearly symmetric CV in Figure 5 is suggestive of species immobilized on a monolayer:[32] the peak current densities, j^a and j^c, had the same values and varied linearly with the scan rate, and the peak-to-peak separation, ΔE_p, was small, 27 mV. However, the width of the peak at half-height was 120 mV, larger than the theoretical value of 90.6/*n* mV ($n = 1$) expected for an ideally reversible process, perhaps due to repulsive interactions between charged viologen. The redox potential of the one-electron reduction of immobilized viologen was significantly less negative than the reduction potential of viologen in solution, a shift which has been previously observed for

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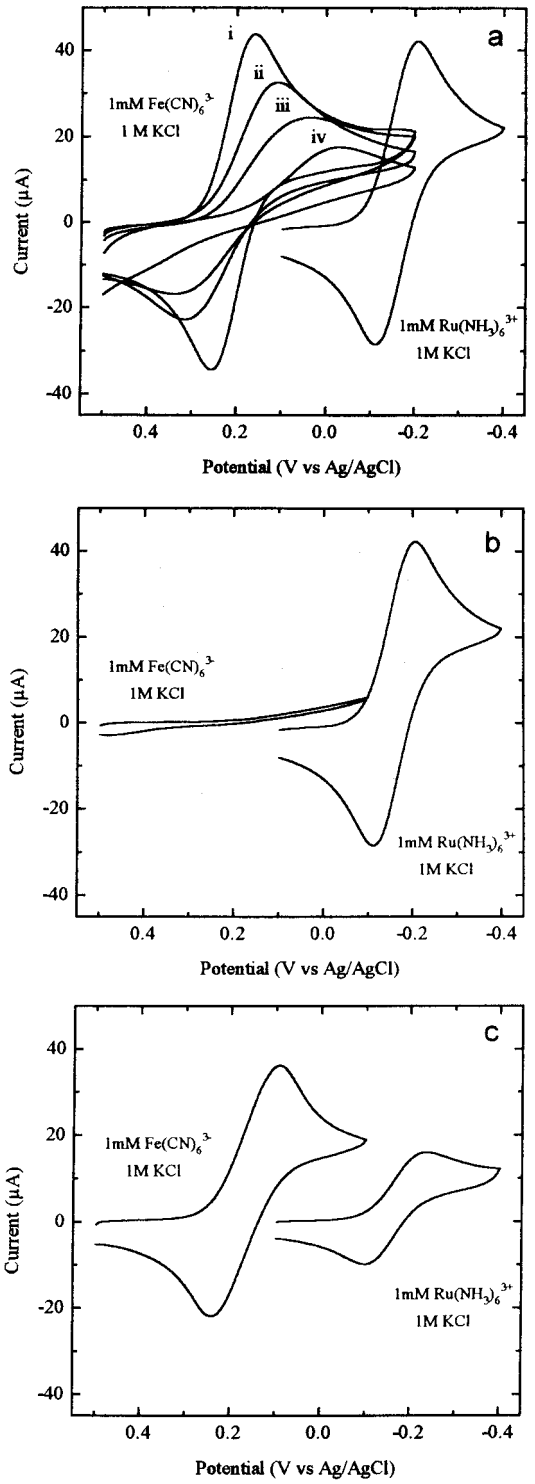

Figure 4. Cyclic voltammograms of 1 mM $Fe(CN)_6^{3-}$ and 1 mM $Ru(NH_3)_6^{3+}$, 1 M KCl, on gold electrodes covered with (a) SAM-TBA, (b) SAM-TOA, and (c) SAM-TOA-[DADOO-EA, 1:8]-Epi-TCh. The pH of the supporting electrolyte was 5.5 except for $Fe(CN)_6^{3-}$ in (a), which was (i) 5.0, (ii) 7.9, (iii) 8.6, and (iv) 11.7. The scan rate was 0.1 V s^{-1}.

viologens in other systems.[33] A surface concentration of (3.6 $\pm$ 0.1) $\times$ 10^{-10} mol cm^{-2} ($n = 6$) for the immobilized viologens was determined from the charge of the CV peaks, assuming a one-electron reduction.[34] For SAMs of viologenthiols on gold, saturation coverage values of (3.5−3.9) $\times$ 10^{-10} mol cm^{-2} have been reported.[35] Thus, the reaction of activated TBA-SAM with MAV produces high coverages which are near those expected for a close-packed monolayer of viologen with a roughly perpendicular orientation of the ring systems to the electrode surface.[35] However, as the cross-sectional area of the viologen group is twice that of the alkyl chains, due to steric hindrance only half of the carboxy groups in the monolayer might have been esterified with MAV, and therefore this reaction with TBA-SAMs cannot give information about the efficiency of the sterification step. On the contrary, TOA-SAM should not have this limitation since its chain density is half that of the TBA-SAM. From CVs obtained after viologen binding to TOA-SAMs a sulfur surface concentration of (2.5 $\pm$ 0.3) $\times$ 10^{-10} mol cm^{-2} ($n = 7$) was estimated, corresponding to a yield of condensation between COOH and NH_2 groups of about 70%. However, this yield could be improved by increasing the concentration of the amine groups and by eliminating from the reaction medium other nucleophiles, e.g., water molecules, that would compete with the amine groups for the activated carboxy groups. The results obtained with the coupling of viologens also indicate that the thioacid SAMs were not damaged by the activation of carboxy groups and further amidation. Upon scanning the potential to −1.0 V in 0.5 M KOH, the monolayer was desorbed since in repetitive scans the reduction current of viologen decreased in parallel with the reductive desorption wave (not shown), which confirms that viologen was bound to the gold electrode through the thiol chain. We can conclude that with this procedure activation of surfaces for amino compounds can be easily and efficiently achieved.

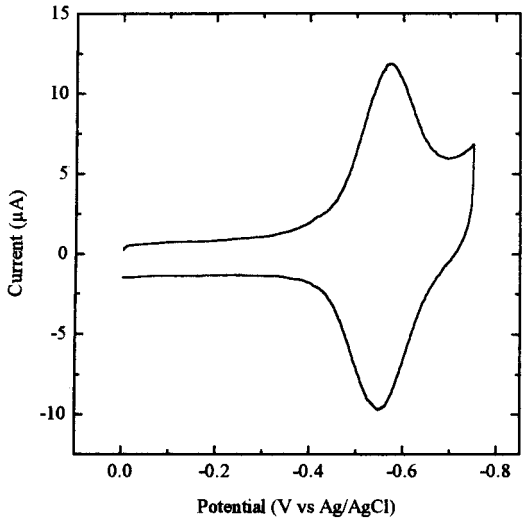

Figure 5. Cyclic voltammograms in 50 mM phosphate buffer, pH 9.0, 0.1 M KCl , of gold electrode covered with SAM-TBA-MAV. The scan rate was 0.1 V s^{-1}.

Attachment of a Diamine Spacer. It has been shown that optimum binding of avidin to biotin on monolayers requires hydrophilic spacers,[8] and consequently our next step was the coupling of the spacer DADOO. As mentioned above, *N*-hydroxysuccinimide esters of carboxylic acids prepared in anhydrous media are very stable, and ligands with free amine groups can be efficiently coupled through an amide linkage. In our case activated esters were reacted with undiluted DADOO in order to achieve a high concentration (6.8 M) of reactive unprotonated amine groups, so that an almost complete amidation of carboxy groups could be obtained. Cross-linking of

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a single DADOO molecule to two neighboring carboxy groups was hindered by the high concentration of added nucleophile.

In their work on the optimization of biotin-functionalized monolayers for avidin adsorption Spinke *et al.*[8] found that the best results were obtained by dilution of the biotinylated chain with shorter hydroxythiols. Moreover, in the construction of the thiocholine SAM charged quaternary ammonium groups will be introduced at the monolayer surface, and therefore choline groups on the surface should be diluted to minimize this destabilizing effect. Consequently, DADOO was diluted with variable amounts of ethanolamine (EA) for the coupling reaction to the activated carboxylic SAM. We have used different dilutions of DADOO with EA in order to study the influence of this dilution effect on the β-galactosidase binding (see below).

Further Derivatization of SAMs with Epichlorohydrin. ω-Halo-1,2-epoxyalkanes are versatile bifunctional reagents with two different electrophilic centers and whose alkylation reactions depend markedly on the solvent. In aprotic solvents these compounds react with nucleophiles by displacement of the halide ion to give the epoxyalkyl derivatives.[37] In the present work terminal amine groups of the spacer DADOO were activated by reaction with epychlorohydrin (step 3 in Figure 2). The use of undiluted epichlorohydrin in this step allows a high concentration of the electrophilic center (12.7 M), avoids protonation of the nucleophilic terminal amine groups, and minimizes the opening of oxyrane groups.

Covalent Linkage of Thiocholine. In protic solvents terminal epoxides are very reactive toward nucleophiles. Among the possible analogues of choline with functional groups suceptible to nucleophilic reaction with oxiranes, we chose thiocholine because thiol groups are more acidic, stronger nucleophiles than their homologous alcohols, e.g., choline. Thiocholine is largely ionized at pH 8, at which the oxirane groups are perfectly stable and react as shown in step 4 of Figure 2. Experiments of reductive desorption of SAM-TOA-[DADOO-EA, 1:8]-Epi-TCh indicate saturation coverage by a thio compound after the full synthesis (Figure 3c) with a peak charge corresponding to a sulfur atom concentration of 6.9×10^{-10} mol cm^{-2}. This monolayer was found to be permeable to a negatively charged electrochemical probe such as $Fe(CN_6)^{3-}$, while the peaks of $Ru(NH_3)^{3+}$ were greatly diminished and less reversible (Figure 4c). These results support the presence of thiocholine $-N(CH_3)_3^+$ terminal groups on the monolayer.

In order to check the effectiveness of the oxirane reaction with thiols, SAM-TOA-[DADOO-EA, 1:4]-Epi was reacted with 2-[*N*-(methylferrocenyl)amino]ethanethiol. After extensive washing of the electrode, ferrocene was detected by CV (Figure 6, solid line). As a control, electrodes modified with SAM-TOA-[DADOO-EA, 1:4] were reacted under the same conditions with the thiol derivative of ferrocene, in which case no CV peaks were observed (Figure 6, dashed line). The surface concentration of the immobilized ferrocene calculated from the charge of the cathodic CV peak was $(1.1 \pm 0.2) \times 10^{-10}$ mol cm^{-2} ($n = 5$) as compared with a 0.9×10^{-10} mol cm^{-2} concentration of reactive oxirane groups expected from the 1:4 DADOO−EA molar ratio used in the amidation step. After 3 months of storage in 2:1 ethanol/water the electrodes showed well-resolved voltammograms of ferrocene, although the peak intensity was reduced by half (not shown). This high stability is not unprecedented since it has also been observed for gold microelectrodes covered with ferrocenyl thiols.[38] Similar CVs were observed for SAM-TBA-[DADOO-EA, 1:8]-ferrocenyl thiol (not shown). These results indicate that the reaction of DADOO terminal amines with epichlorhydrin and subsequent attack by ferrocenyl thiol had a high yield. The absence of electrochemical response in the SAM-TOA or -TBA electrodes whose amine groups had not been activated with epichlorhydrin after incubation with ferrocenyl thiol allows us to conclude that ferrocenyl thiol molecules detected by CV were indeed covalently bound to the activated thiol chains and not directly adsorbed onto gold by displacement of a thiol chain on the monolayer. This conclusion could also be inferred from a similar control experiment in which a SAM-TBA-[EA] electrode did not show electrochemical response after incubation with a 1 mM solution of ferrocenyl thiol and further washing. CV peak intensities of SAM-TOA electrodes modified with ferrocene varied linearly with the scan rate up to 2 V/s. However, even at low scan rates the CV showed a peak splitting of 56 ± 15 mV at 100 mV/s. The electron transfer rate constant obtained using Laviron's model[39] was 3.8 ± 0.3 s^{-1} ($n = 4$), similar values having been reported for ferrocene groups immobilized in SAMs.[40] However, higher electron transfer (ET) rate constants have been observed[40a] with mixed monolayers of $FcCO_2(CH_2)_{11}$-SH and $CH_3(CH_2)_{11}SH$ ($\chi_{Fc} = 0.25$, similar to the ferrocene molar fraction expected in our above electrodes). This high ET rate was explained by the presence of defective Fc sites, e.g., at the domain boundaries, that can act as intermediates for electron transfer to the other Fc sites in the interior of the SAM domains.[40a] After exchange of some ferrocene-terminated thiols with unsubstituted alkanethiols, the standard rate constant fell to 1.8 s^{-1}.[40a] Our results with ferrocene-functionalized SAMs suggests that SAM-TOA-Fc layers are ordered and that the ferrocene heads are held away from the surface of the electrode, thereby slowing the electron transfer.

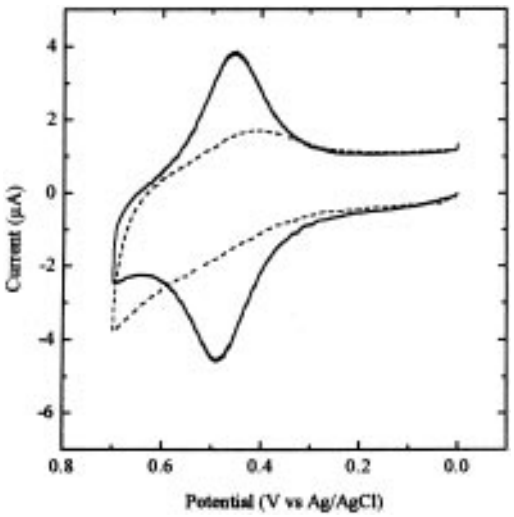

Figure 6. Cyclic voltammograms in 0.1 M $HClO_4$ of gold electrode covered with (a) SAM-TOA-[DADOO-EA, 1:8]-Epi-ferrocenyl thiol (solid line), (b) SAM-TOA-[DADOO-EA, 1:8] incubated for 20 h in 1 mM ferrocenyl thiol (dashed line). The scan rate was 0.1 V s^{-1}.

Completeness of the Reaction of Oxirane Groups with Thiocholine. Because epoxy groups are very reactive in aqueous media and could possibly bind proteins nonspecifically,[41] e.g., β-galactosidase, it was necessary to check for the presence of residual epoxides after the oxirane−TCh reaction. With this aim a SAM-TBA-[EA-DADOO, 1:8]-Epi-TCh elec-

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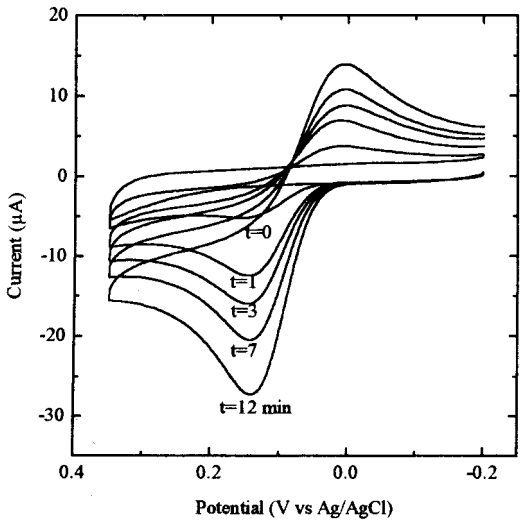

Figure 7. Catalytic activity of β-galactosidase from *E. coli* in 50 mM phosphate buffer, pH 7.3, 0.1 M KCl, with 4 mM PAPG as substrate, as determined by cyclic voltammetry at 0.1 V s^{-1} with a gold electrode modified with SAM-TOA-[DADOO-EA, 1:8]-Epi-TCh. Voltammograms were recorded at the indicated times after addition of the enzyme to the electrochemical cell.

trode was incubated for 1.5 h in a 1:1 water/ethanol solution of 1 mM ferrocenyl thiol. The absence of peaks in the CV after washing (not shown) indicates complete reaction of TCh with oxirane groups and that the substitution of the monolayer chain units by the ferrocenyl thiol was negligible, in agreement with the result of Figure 6.

XPS of SAMs. Chemical analysis by XPS of the monolayer at the different stages of synthesis was attempted. The results obtained for one of the SAMs under study (SAM-TBA) are shown in Table S1 (Supporting Information). In none of the spectra were elements other than S, C, N, and O detected. The experimental intensities normalized to the expected atomic carbon content were in reasonable agreement with the theoretical content of N and O. However, the concentration of S found for SAM-TBA, SAM[TBA-TCh], and SAM-TBA-DADOO was always about half of the theoretical one, in agreement with the XPS data reported for SAMs of long-chain alkanethiols[42] and thioalkanoic acids.[43] Since Bain *et al.*[42] have shown conclusively that the signal of sulfur atoms in contact with the gold substrate is much lower than expected due to attenuation of the photoelectrons by the interference of the monolayer with the photoelectrons, one can anticipate that the atomic sensitivity factor (ASF) for sulfur will depend on its position in the monolayer. This could explain the higher $S_{found}/S_{expected}$ ratio in our electrodes covered with SAM-TBA-DADOO-Epi-TCh (which should contain sulfur atoms with two different positions in the monolayer), as compared to the SAM-TBA (Table S1). These results are consistent with the layer structure expected from our synthesis.

Evidence for Specific Binding of Chimera Protein on the Electrode Surface Modified and Functionalized with Choline Residues. Figure 7 shows the CVs of a gold wire modified with SAM-TOA-[DADOO-EA, 1:8]-Epi-TCh taken at different times after PAPG addition to an electrochemical cell containing 0.1 unit of native *E. coli* β-galactosidase (obtained from commercial sources). As the enzymatic reaction proceeds PAP accumulates in the bulk and peak currents increase with time (Figure 7), which suggests that the CV can be used to follow the binding of chimera protein. For this purpose gold electrodes were incubated in protein solutions, washed with buffer, and inserted into an electrochemical cell to which was added PAPG at zero time. CVs were recorded after 12 min of reaction.

The specificity of the binding of chimera protein to choline-modified electrodes was tested as follows: PAP was detected after thiocholine electrode incubation with chimera protein, but no PAP was observed if native β-galactosidase was used instead (not shown). Moreover, dipping of a thiocholine electrode in a solution of C-LYTA protein, which has only the choline-binding domain, blocked the binding of chimera protein since PAP was not detected (not shown). Although both results demonstrate that the binding of chimera protein requires the receptor domain, they do not prove that this adsorption involves a specific, unique choline−receptor interaction. Results in Figure 8 answer this question: no PAPG hydrolysis was observed after thiocholine electrode incubation in chimera protein solution containing 1 M choline (Figure 8a), which suggests that dissolved choline molecules saturated the receptor sites on the chimera protein, leading to a lower affinity for the choline-modified SAM. On the contrary, the binding of chimera-protein in a KCl solution of the same ionic strength to the modified electrode (Figure 8b) indicates that the low binding observed in the presence of choline was not due to an electrostatic effect. The ability of an electrode to hydrolyze PAPG was lost after incubation in a 3 M choline solution (Figure 8c), but not after incubation in 3 M KCl solution (not shown) which indicates that soluble choline molecules remove the immobilized chimera protein by competition with the thiocholine residues on the SAM. Washing with buffer recovered the affinity of the electrode of Figure 8c for chimera protein (Figure 8d). No chimera protein binding was detected after incubation with SAM-TOA-[DADOO-EA, 1:8]-Epi electrodes reacted either with 2-mercaptoethylamine or 2-mercaptoethanol instead of thiocholine (not shown). PAP was also not detected with bare gold electrodes incubated either in β-galactosidase or in chimera protein solutions after 12 min of PAPG addition (not shown). All these results demonstrate that chimera protein binds *reversibly* and *specifically* to choline residues on the SAM-modified electrode through its choline-binding domain.

Dependence of the Protein Binding on the Composition of the Monolayer. We have studied the effect of choline residue concentration on the binding of the chimera protein by diluting DADOO with EA at the second step of monolayer construction. If we assume a similar reactivity of ethanolamine and DADOO toward hydroxysuccinimide esters of thiocarboxylic monolayer, then the molar fraction of thiocholine in the monolayer (X_B) corresponds to the molar fraction of DADOO present in the reaction with succinimide esters. Dilution of DADOO with the shorter hydrophylic chain, EA, produces a large increase of the bound chimera protein with a sharp maximum at $X_B = 0.03$ (Figure S1, Supporting Information). The same trend was observed with monolayers built up from thiobutyric or thioctic acids (Figure S1). Spinke *et al.*[8] observed a similar effect of surface-biotin dilution on the adsorption of avidin molecules and explained it as being due to steric hindrance in the undiluted biotin layer. A similar explanation could apply to our case. The lower value of X_B at which maximum chimera protein adsorption is observed would be due to the larger size of the chimera protein molecules compared to avidin.

Calculation of Protein Coverage. From binding experi-

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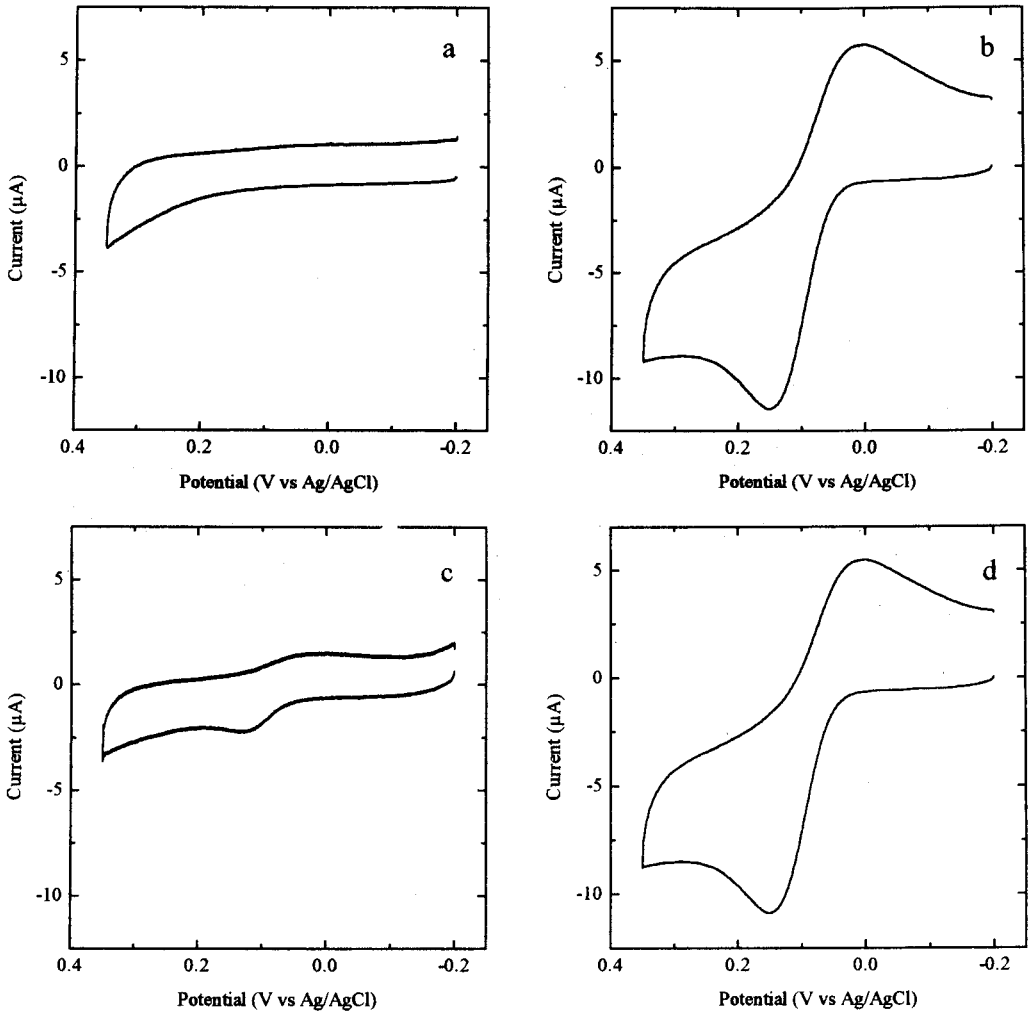

Figure 8. Enzymatic activity of SAM-TOA-[DADOO-EA, 1:8]-Epi-TCh electrodes after immersion in solutions of (a) C-LYTA-β-galactosidase chimera, 1 M choline, (b) C-LYTA-β-galactosidase chimera, 1 M KCl, (c) electrode used in (b), after 10 min of incubation in 1 M choline solution, and (d) electrode used in (c), after washing with 1 M KCl and subsequent incubation in a solution of C-LYTA-β-galactosidase chimera, 1 M KCl. After incubation the electrodes were washed in 50 mM phosphate buffer, 1 M KCl, pH 7.3, and placed in an electrochemical cell containing 50 mM phosphate buffer, pH 7.3, 0.1 M KCl. Cyclic voltammograms at 0.1 V s^{-1} were recorded 12 min after addition of 4 mM PAPG.

ments with ^{35}S-labeled chimera protein an average of 0.30 $\pm$ 0.03 μg of bound protein cm^{-2} was found, which corresponds to a chimera protein coverage of $(5.7 \pm 0.6) \times 10^{-13}$ mol cm^{-2}. The crystal structure of β-galactosidase at 3.5 Å resolution indicates that the protein is a tetramer with 222-point symmetry with dimensions of 17.5 $\times$ 13.5 $\times$ 9 nm along the respective 2-fold axes.[21] The projection of the largest face of the protein has a rhombus shape (Figure 9). The N-terminal residues of the subunits to which the C-LYTA polypeptides are incorporated in the chimera protein are located at the ends of the shortest diagonals of the rhomb faces,[21] and the distance between the nearest N-terminal neighbors is about 1.5 nm.[45] The hydrodynamic behavior of C-LYTA monomers suggests that these polypeptides are prolate ellipsoids of 1.5 $\times$ 3 nm.[46] Therefore, plausible dimensions along the symmetry axes of the chimera protein would be 17.5 $\times$ 19.5 $\times$ 9 nm. The following points suggest that the binding to the choline monolayer involves more than one C-LYTA binding domain: (i) the high stability of the SAM-chimera assembly (*vide infra)*; (ii) the high concentration of choline required for the desorption of the chimera protein (>0.6 M); (iii) the strong dependence of the binding on the dilution of choline in the SAM (Figure S1 in the Supporting Information). For steric reasons, of the four binding sites only two of them could be simultaneously bound to the choline SAM (Figure 9). In Figure 9 a binding mode that involves a nearest-neighbor pair of C-LYTAs with a projection area of 160 nm^2 (17.5 $\times$ 9 nm) is represented. Since lateral diffusion of firmly bound proteins is restricted, binding proceeds randomly and a maximum of 60% of the electrode can be covered with protein.[47] The effective area occupied by each molecule of chimera protein

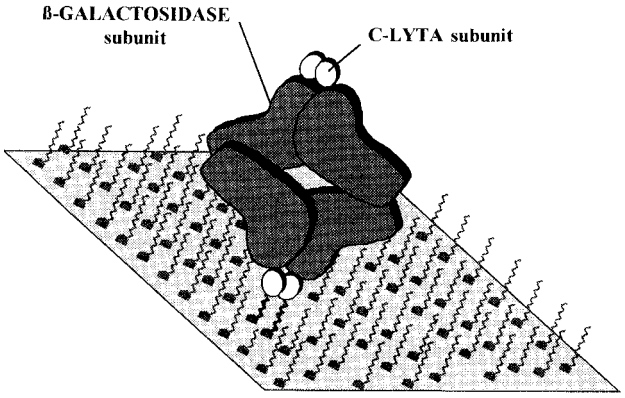

Figure 9. Schematic representation of the chimera protein binding to a choline-functionalized SAM of thioctic acid. For the sake of clarity, thioctic chains functionalized with ethanolamine have been omitted. (The sizes of the components were kept in proportion; other molecular details are not accurate.)

(45) Atomic coordinates of the refined protein model were taken from Brookhaven Protein Data Bank, codes 1BGL and 1BGR.

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should be *ca.* 285 nm^2 for the orientation considered above, and corresponds to a saturation coverage of 5.8×10^{-13} mol cm^{-2}, in good agreement with the coverage determined by ^{35}S labeling. On the contrary, for other possible binding orientations involving two opposite C-LYTAs with a projection area of 340 nm^2 (17.5 × 19.5 nm) the electrode would be covered, on average, with two monolayers of chimera protein. However, a patchy multilayer formation seems to be very unlikely for a highly hydrophylic protein, such as C-LYTA-β-Gal, at the conditions used during the binding step and further washing (50 mM phosphate buffer, pH 7.3, 0.1 M KCl). Also the absence of diffusional limitations of the substrate PAPG and of PAP (see below) argues against the formation of protein aggregates. These experimental results are in favor of a binding configuration as that given in Figure 9. Other authors have proven that specific interaction between proteins and uniform substrate surfaces results in oriented protein films.[6b,44] Therefore, it is reasonable to conclude that chimera protein molecules would be bound with nearly the same orientation to the electrode surface in those domains of the SAM-choline monolayer which were uniform at the molecular level.

Characterization of the Enzymatic Electrode. Chimera protein electrodes had a half-life of 10 days at 4 °C in 50 mM phosphate buffer, 7 mM Mg^{2+}. Stability was also tested by conventional flow injection analysis (FIA) with the enzymatic electrodes as working electrodes. The electrode was very stable during 6 h of continuous testing on flow (Figure S2, Supporting Information), with half-lives of 20 h. Electrodes that lost most of their activity after days of continuous use recovered almost 90% of their original activity after being dipped in fresh solutions of chimera protein, which suggests that the modified SAM was stable during the experiment, but that enzyme desorption occurred. This is in agreement with the results of the stability of SAMs in experiments with ferrocene-modified SAMs. In the FIA system the response of these modified electrodes to repetitive injections of substrate was highly reproducible, with a relative standard deviation of 2.5% for 40 consecutive determinations of a 4 mM PAPG solution (Figure S2). From the dependence of the peak current intensity on the PAPG concentration (not shown) an apparent value for K_m of 0.2 mM, close to that found for the soluble enzyme and the same substrate, was determined. This indicates that the immobilization of the chimera protein to the choline SAM does not create diffusional limitations since this would increase the observed K_m. In agreement with this the apparent diffusion coefficient (D_{app}) of PAP did not significantly change after chimera protein binding (D_{app} of PAP on the bare electrode was 8.7×10^{-6} cm^2 s^{-1}, while on a SAM-TOA-[DADOO-EA, 1:8]-Epi-TCh it was 7.8×10^{-6} cm^2 s^{-1}; a similar value, 7.7×10^{-6} cm^2 s^{-1}, was found after chimera protein binding to the latter electrode).

Conclusions

The results reported here support the *in situ* step-by-step synthetic strategy used to prepare functionalized SAMs of thiols[48] as an alternative to the well-established method of coadsorption of a mixture of alkanethiols with the desired functionalities.[4d,8,49,50] The main advantage of solid-phase synthetic chemistry is that it can be easily performed in laboratories with little experience in organic synthesis, avoiding costly steps of reactive side group protection and separation of the product from reagents and byproducts. The proposed synthetic route produces highly stable functionalized SAMs capable of binding large macromolecules. The remarkable stability may be due to the formation of a network of hydrogen bonds between neighboring amides generated after the amidation step of the thioalkanoic acid monolayer, since Tam-Chang *et al.*[50] have reported experimental results on the stabilizing role of internal amides in SAMs. This is in agreement with earlier results of Ringsdorf and co-workers,[44a] who found that phase transition of biotin lipids capable of hydrogen bond formation with neighboring chains took place at lower surface pressures and that the biotin lipids have a smaller molecular area in the solid condensed state than a homologous biotin lipid without the internal amide. Their interpretation of these results as well as of the much higher melting point found for amide-containing biotin lipids was that the formation of hydrogen bonds leads to a better packing of the monolayer.[44a]

Our results confirm the previous conclusions obtained by Knoll and co-workers[8] about the requirements for optimal binding of protein through molecular recognition: (i) a substrate of hydroxy thiols; (ii) the inclusion of a spacer on the thiol chains that carry the specific ligand; (iii) an adequate proportion of the two types of thiol chains.

The combination of SAMs and genetic engineering constitutes a powerful tool for immobilizing proteins on functionalized surfaces, this tool having potential use in biosensors and other bioelectronic devices. The proposed system is conceptually simple and could be easily extended to other well-known receptor−ligand systems such as avidin−biotin.

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Supporting Information Available: Table S1, with the atomic composition of the monolayers investigated in the present work as determined by XPS, Figure S1, which illustrates the dependence of the protein binding on the composition of the monolayer, and Figure S2, which shows the recording of repetitive determinations of PAPG on a conventional FIA setup (3 pages). See any current masthead page for ordering and Internet access instructions.

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