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Electron Transfer Properties of Dual Self-Assembled Architectures Based on Specific Recognition and Electrostatic Driving Forces: Its Application To Control Substrate Inhibition in Horseradish Peroxidase-Based Sensors

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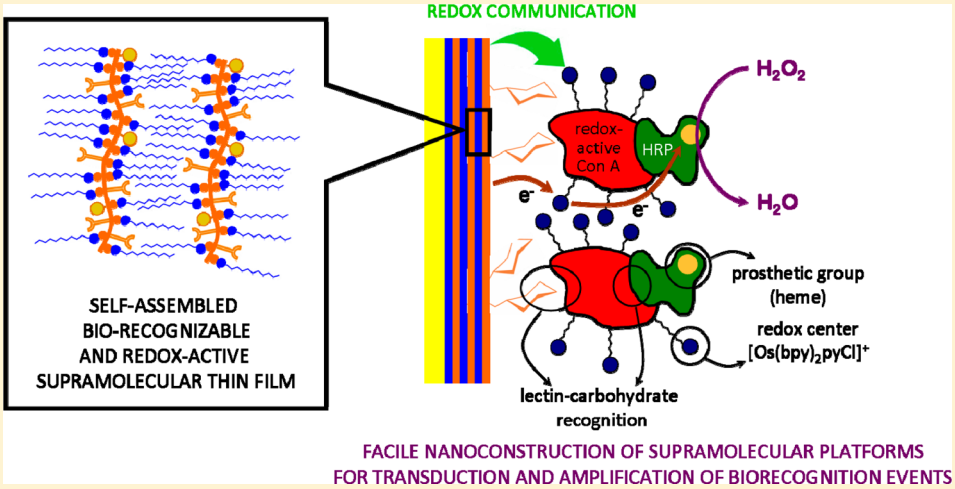

ABSTRACT: This work describes the synergistic combination of ionic self-assembly and recognition-directed assembly with the aim of creating highly functional bioelectrochemical interfaces compatible with the supramolecular design of a wide variety of biosensing platforms. A recently synthesized glycopolyelectrolyte constituted of polyallylamine bearing redox-active osmium complexes and glycosidic residues (lactose) is used to create a self-assembled structure with sodium dodecylsulfate. In turn, this supramolecular thin films bearing redox-active and biorecognizable carbohydrate units enable the facile assembly of functional lectins as well as the subsequent docking and "wiring" of glycoenzymes, like horseradish peroxidase (HRP) (an elusive enzyme to immobilize via noncovalent interactions). The assembly of this system was followed by quartz crystal microbalance and grazing-incidence small-angle X-ray scattering (GISAXS) studies confirming that spin-coated ionically self-assembled films exhibit mesostructured architectures according to the formation of self-organized lamellar structures. In-depth characterization of the electrocatalytic properties of the biosupramacromolecular assemblies confirmed the ability of this kind of interfacial architecture to efficiently mediate electron transfer processes between the glycoenzyme and the electrode surface. For instance, our experimental electrochemical evidence clearly shows that tailor-made interfacial configurations of the ionic self-assemblies can prevent the inhibition of the glycoenzyme (typically observed in HRP) leading to bioelectrocatalytic currents up to 0.1 mA cm^{-2}. The presence of carbohydrate moieties in the ionic domains promotes the biorecognition-driven assembly of lectins adding a new dimension to the capabilities of ionic self-assembly.

Polyelectrolyte−surfactant complexes are stable systems formed by charged polymeric chains (polyelectrolytes) and oppositely charged small amphiphilic molecules (surfactants). Such complexes combine in unique ways the properties of polyelectrolyte with those of low molecular weight amphiphiles.[1−5] The polyelectrolyte components can provide, for instance, mechanical strength and thermal stability, while the surfactants retain their tendency to assemble in layered structures.[6,7] They are able to form stable colloidal suspensions in water,[8] and they can be completely dissolved in organic solvents[6,9] allowing its handling and obtaining a very stable structure when it is dried. Recently, we have shown the ability

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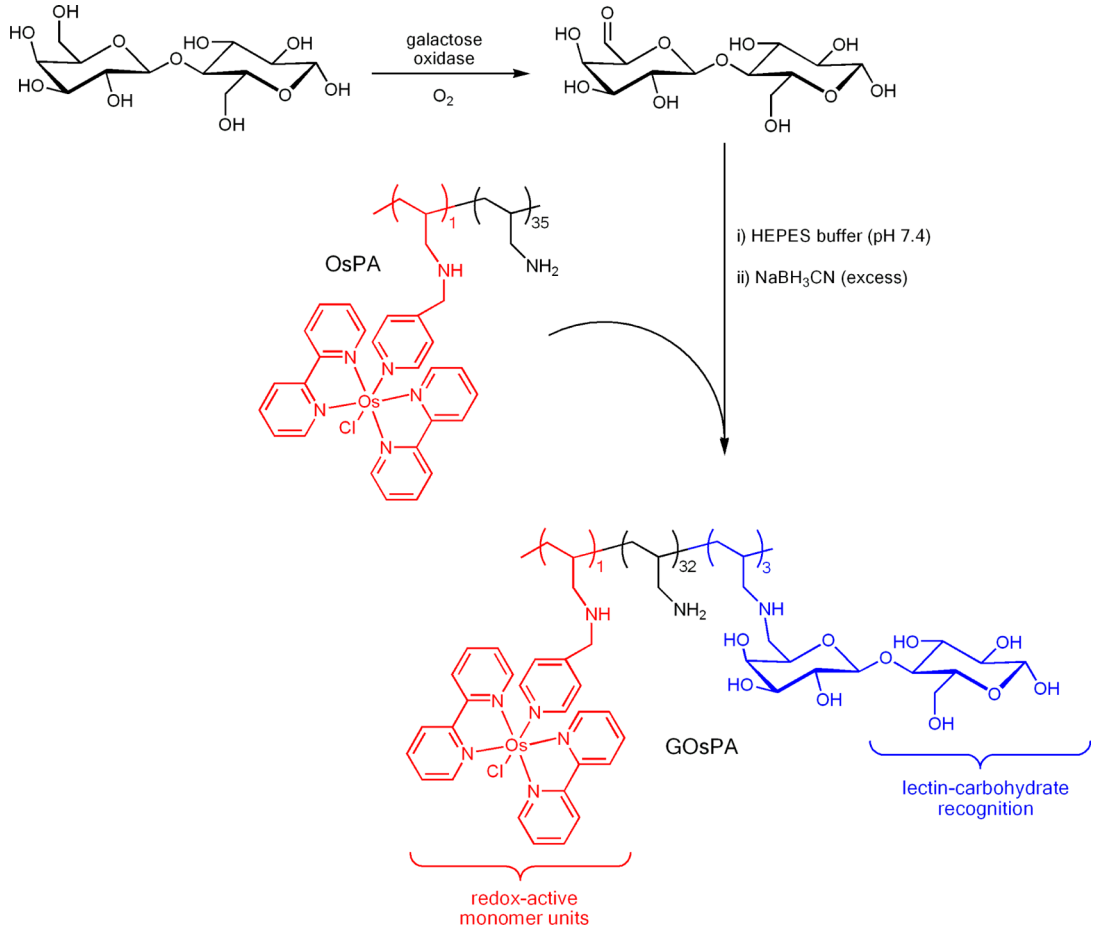

Figure 1. Reactions involved in the preparation of the redox-active glycopolyelectrolyte (GOsPA). The first step involves the oxidation of lactose in the presence of oxygen and galactose oxidase. Then, the oxidized lactose is covalently grafted to the OsPA backbone in the presence of borocyanohydride.

of one of these complexes, polyallylamine-dodecyl sulfate, to form stable interfaces on different types of surfaces: graphite, gold, and silicon with a mesoscopic order.[10] The system has been modified in different ways by introducing ferrocyanide, carbon nanotubes[10] or by modifying the polyelectrolyte with an osmium complex.[11] All these modifications show very interesting electron transfer properties for different applications; however, always the main forces maintaining the system assembled were electrostatic and van der Waals forces. Even though these types of interactions have an important effect in the assembling of biological systems, sometimes they are not enough to maintain the structural integrity. In these cases other types of noncovalent interactions are needed; as an example of an alternative method of biosupramolecular assemblies, lectins have shown to be very efficient for the immobilization of glycoenzyme through a soft noncovalent approach, i.e. bioaffinity layering.[12−15] Concanavalin A (Con A), the best studied among lectins, is extracted from easily available, inexpensive seeds by simple steps. The protein exists as a tetramer with a molecular mass of 104 KDa at neutral pH. Each Con A monomer contains one calcium ion binding site, one transition metal binding site, and one carbohydrate binding site (specific to α-D-mannose and α-D-glucose), also referred to as the combining site. In the case of the bioaffinity layering the glycoenzyme is supramolecularly conjugated to the tetravalent Con A through its carbohydrate moiety.[16−24] The carbohydrate regions in the glycoenzyme are generally located in areas that are not involved in the enzyme activity, and therefore they can retain their biological function even when their carbohydrate regions are conjugated on the lectin layer. These properties allow Con A to act as a bioaffinity bridge between a sugar-modified surface and a glycoprotein.

In order to improve versatility and stability to the polyallylamine-dodecylsulfate complex, we have recently synthesized a polyallylamine derivative containing an osmium complex and a glycosidic moiety (lactose) in order to generate a material able to self-assemble through two different mechanisms, ionic and biorecognition (Figure 1). Preliminary results[25] have shown its ability to the construction of biomimetic signal chains using a redox-active Con A and HRP.

In this work we explore the different configurations that this new glycopolyelectrolyte is able to build when self-assembled systems of different thickness are used to incorporate Con A and HRP, representing an alternative to the layer-by-layer construction method. Quartz crystal microbalance and GISAXS experiments were carried out to understand the structure of the modified surface; while electrochemical experiments with Con A or redox-active Con A combined with HRP show the ability of this new system to govern electron transfer process in biomolecular systems, exemplified here by the control of HRP inhibition.

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EXPERIMENTAL SECTION

Reagents and Materials. Sodium dodecyl sulfate (SDS), poly(allylamine) (PA, MW: 65000), concanavalin A (Con A, *Canavalia ensiformis* from jack bean), horseradish peroxidase (HRP, Type VI), lactose, galactose oxidase (GO, from *Dactylium dendroides*), and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) were purchased from Sigma Aldrich. The redox polymer $Os(bpy)_2ClpyNH$poly(allylamine) (OsPA) was synthetized as previously reported.[26] The synthesis of redox-active Con A (Os-Con A) was achieved by labeling Con A with $[Os(bpy)_2Clpy]^+$ by using a 336 Da MW polyethylene glycol (PEG) bifunctional spacer containing amino and carboxylate terminal groups, following a procedure described in the literature.[27] Briefly, $Os(bpy)_2ClpyCH_2NH$-PEG-COOH (20 mg, 0.02 mmol), NHS (15 mg, 0.13 mmol), and EDC (31 mg, 0.16 mmol) in 0.5 mL of dry DMF were heated with stirring at 80 °C under argon atmosphere for 1 h. After cooling the mixture to room temperature, portions of this solution were added to a 10 mg/mL Con A solution in phosphate buffer (0.1 M, pH 8.5). The mixture was left to react at room temperature overnight under smooth stirring. To remove unreacted osmium, the solution of modified Con A was purified using a series of two Hitrap desalting columns (GE Healthcare, 5 mL each) with phosphate buffer (25 mM, pH 7.4) at an elution rate of 3 mL/min. The product was then dialyzed at 4 °C for 24 h against Milli-Q water using a 3500 MWCO membrane and lyophilized. The Os/Con A ratio was determined by MALDI-TOF spectrometry. A ratio of 12 redox probes per protein molecule was obtained. All other reagents were analytical grade. The synthesis of the glycopolyelectrolyte containing redox active moieties was previously described.[25] Figure 1 shows the synthesis, final structure, and stoichiometry of the polyelectrolyte.

Synthesis of the Supramolecular Material GOsPA-DS. 400 μL of SDS 1% in Milli-Q water was added to 200 μL of GOsPA (0.2 mM). The mixture immediately generated a precipitate (GOsPA+DS), which was easily separated by centrifugation. The precipitate was dissolved in 500 μL of DMSO and sonicated for 15 min to facilitate complete dissolution of the solid.

Construction of Modified Electrodes. The construction of the molecular assemblies was achieved by using silicon coated with 15 nm of Ti, 20 nm of Pd, and 200 nm of gold by evaporation. The first step of the substrate modification was the application of a uniform layer of GOsPA+DS by spin coating. Afterward, the electrode was left at room temperature for 1 h to allow complete evaporation of the solvent. Then, it was rinsed with Milli-Q water and dried with N_2. The incorporation of the subsequent building blocks was achieved through a series of sequential steps. Protein building blocks such as Con A, Os-Con A, and HRP were incorporated by incubating the modified electrode for 1 h in 1 μM solutions in 0.05 M HEPES buffer (pH 7.4), containing 0.5 mM $CaCl_2$ and 0.5 mM $MnCl_2$. The same buffer was used to rinse the electrode after each assembling step. To immobilize another layer of GOsPA onto the protein-modified surface, the electrode was incubated for 1 h in 0.2 mM GOsPA solution in 0.05 M HEPES buffer (pH 7.4). Afterward, the electrode was rinsed with the same buffer. All steps were carried out at room temperature (ca. 22 °C).

Electrochemical Measurements. Cyclic voltammetry experiments were carried out using a purpose-built potentiostat (TEQ-02) using a three-electrode Teflon electrochemical cell equipped with a platinum mesh counter electrode and an Ag/AgCl reference electrode. Unless otherwise stated, all electrochemical experiments were performed at room temperature (ca. 22 °C) in a 0.05 M HEPES, 0.1 M KNO_3 buffer solution at pH 7.4.

Quartz Crystal Microbalance Monitoring (QCM). QCM experiments were carried out at 22 °C in a 5 MHz QCM200 (Standford Research Systems) coupled to a flow injection system. All experiments were performed at a flow rate of 50 μL min^{-1}. The variation in the resistive parameter of the Butterworth−Van Dyke electrical equivalent circuit that represents the composite quartz crystal resonator loaded with the film, ΔR, in all cases is negligible compared to the inductive quartz impedance component, ΔX_L. Therefore, the films behave as acoustically thin in the gravimetric regime, and the mass uptake was calculated in each case with the Sauerbrey equation.[28]

Surface Profilometry. Profilometry measurements were carried out in a Dektak 150 Stylus Surface Profiler.

Grazing-Incidence Small-Angle X-ray Scattering (GISAXS). GISAXS measurements were performed at the D10A-XRD2 line of Laboratório Nacional de Luz Síncrotron (LNLS, Campinas, Brazil). A monochromatic beam of 7709 eV (λ=1.608 Å) was used to perform the experiments. Si(100) wafers were used as support substrates and were pretreated before coating. They were rinsed with acetone and dried with argon before coating. Thin polymer films were prepared by spin coating.

RESULTS

Glycopolyelectrolyte-SDS Complex Structure. Recently, we have synthesized a redox polyelectrolyte capable of presenting two different types of interactions/functional features for the construction of self-assembled systems. The strategy used in that work was the introduction of glucose moieties to the redox polyelectrolyte allowing the subsequent binding of lectin-type proteins. To carry out this task lactose was chosen. This carbohydrate is a dimer composed of galactose and glucose through a β-glycosidic 1−4 bond.

The resulting glycopolyelectrolyte is soluble in water and upon mixing with a SDS solution yields a precipitate, a result already observed with OsPA;[11] however, in this case, the supramolecular complex is not soluble in methanol and dissolves very slowly with DMF, while DMSO easily dissolves the precipitate. Apparently, the introduction of the carbohydrate moieties produces changes in its solubility in organic solvents.

The solution of glycopolyelectrolyte−surfactant complex can be easily manipulated and applied onto gold or silicon surfaces, producing a stable layer after solvent evaporation. Previous SAXS studies[25] have shown the presence of a narrow scattering peak corresponding to the long period of *ca.* 3.7 nm. This result is consistent with the formation of lamellar mesostructures in the supramacromolecular assembly. In this work, complementary GISAXS studies on these films show a bright region (highest intensity) in the direction q_z (for $q_y \rightarrow 0$) and the presence of an appreciable intensity halo (Figure 2). This suggests that while there are lamellar domains oriented parallel to the substrate there is a strong contribution of multioriented lamellar domains (randomly oriented small domains), thus the modification of $-NH_2$ monomer units with lactose and osmium-bypiridine redox centers may introduce some structural disorder in the supramolecular system. This assertion relies

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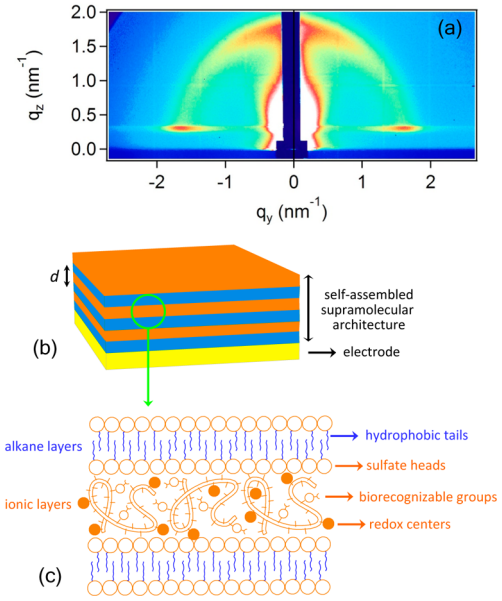

Figure 2. (a) GISAXS pattern corresponding to the GOsPA-DS complex. (b) Schematic of the redox-active glypolyelectrolyte–surfactant lamellar assembly (d = lamellar spacing). (c) Schematic of the different layers constituting the lamellar assembly: ionic layers correspond (orange) to the polyelectrolyte and dodecyl sulfate head groups, whereas the alkane layers (blue) correspond to interdigitated hydrophobic tails (dodecyl groups).

on the fact that similar GISAXS studies performed on PA+DS assemblies only revealed a very weak halo arising from the contribution of multioriented lamellar domains.[10]

Self-Assembly of Con A onto GOsPA-Modified Electrodes. As was reported in previous works,[10,11] one of the main advantages of this type of supramolecular material relies on the fact that they can be applied practically in any surface without requiring previous modification steps; for example, surface preconditioning with thiol or silane derivatives is not necessary. In our experiments gold surfaces were modified with a solution of the complex by spin coating yielding a very stable material. In order to study the ability of this modified surface to recognize lectins, it was exposed to a 1 μM Con A solution in 50 mM HEPES buffer solution. Crystal quartz microbalance was used to follow the adsorption of Con A; Figure 3 shows the decrease in resonant frequency that can be attributed to a change of mass due to the adsorption of Con

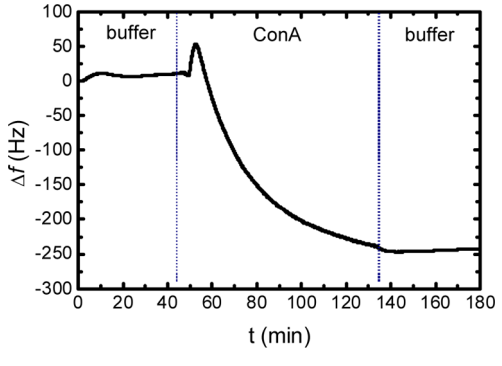

Figure 3. QCM frequency change due to the flow of 1 μM Con A solution onto a gold electrode modified with GOsPA-DS and then rinsed with the flow of buffer solution (50 mM HEPES, pH 7.4).

A. The initial exposure to the solution of Con A led to a rapid decrease in frequency, followed by a slight and steady decline. Then, the system was rinsed with buffer solution, and the frequency remains constant, indicating that Con A is firmly anchored in the polymer. This frequency change corresponds to a change in mass of 4.4 μg cm^{-2}, which involves protein and water. It is worthwhile to mention that similar experiments performed with OsPA+DS-modified electrodes show no adsorption neither of Con A nor HRP, verifying the role of the glucose moiety as anchor for the recognition-directed assembly of Con A.

Self-Assembled Construction of Modified Electrodes: Au/GOsPA+DS/Con A/HRP. Recently, Con A modified with osmium complex moieties (Os-Con A)[15,27] was presented in self-assembled systems in order to successfully mediate the electron transfer process between a gold surface and HRP. In order to build such systems gold electrodes were modified with cystamine followed by the covalent binding of α-D-mannopyranosylphenyl isothiocyanate; in this way the gold surface was modified with mannose moieties onto which the Os-Con A could be assembled. Furthermore, the same biorecognition process involving the mannose residues of HRP directed the immobilization of the enzyme. For this system, an efficient electron transfer process could be observed between gold and HRP. Based on this knowledge, our first question regarding the GOsPA-DS/Con A system was whether the combination of the electroactive glycopolyelectrolyte with Con A might lead to a supramolecular structure capable not only of incorporating HRP but also conducting an efficient electron transfer process between the electrode and the enzyme. To clear up this question we functionalized a gold electrode with GOsPA+DS and Con A, and then HRP was immobilized through a recognition-driven process. One interesting feature of this system is the fact that cyclic voltammograms carried out before and after of the proteins incorporation are practically equal, suggesting that the close proximity between redox centers is not influenced by the introduction of ConA and HRP in the film.

HRP incorporation was also evaluated by QCM producing a frequency shift equivalent to 0.38 μg cm^{-2}, and no changes in frequency were observed when the system was rinsed with a buffer solution, showing that the glycoprotein is also firmly anchored. Even though HRP was introduced in the system, there was no connection between its prosthetic group and the redox centers in the polymer as revealed by the cyclic voltammogram in the presence of 500 μM of H_2O_2 (Figure 4, red trace). However, by adding a small amount of a mediator in solution it was possible to observe the evolution of the catalytic wave, thus confirming the presence of HRP within the assembly. Control experiments with Au/GOsPA+DS as precursor layer for the immobilization of the enzyme evidenced no catalytic response, indicating that HRP cannot be directly bound to the glycopolyelectrolyte. Therefore, the enzyme is immobilized on the Au/GOsPA-DS/Con A-modified electrode through the carbohydrate-lectin interaction; however, Con A acts as an insulator hindering the electron transfer between the redox centers in the glycopolyelectrolyte and the catalytic domain of HRP. It is worthwhile to mention here that efficient redox communication between osmium containing polyelectrolytes and HRP has been already demonstrated in hydrogels.[29]

Au/GOsPA+DS/Os-Con A/HRP. To avoid the insulating effect of Con A, we built up a supramolecular system incorporating a redox-active Con A (Os-Con A) as a

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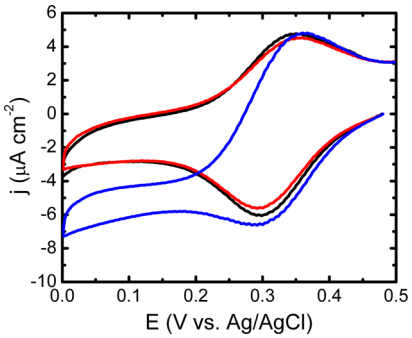

Figure 4. Cyclic voltammograms for Au/GOsPA-DS/Con A/HRP in 50 mM HEPES buffer (pH 7.4, 0.1 M KNO_3) in the absence (black) and in the presence of 500 μM H_2O_2 (red) and in the presence of 500 μM H_2O_2 + 20 μM $[Os(bpy)_2pyCl]^+$ (blue). Scan rate: 10 mV s^{-1}.

bifunctional building block to direct the assembly of HRP on the redox glycopolyelectrolyte complex and also to connect its prosthetic group to the electrode surface.[27] Os-Con A refers to Con A containing 12 $Os(bpy)_2ClpyCH_2-$ moieties bound to the protein through a bifunctional amino-PEG-acid spacer. The location of these redox centers in the protein periphery is not involved in the recognition process of the carbohydrate; therefore, Os-Con A and Con A display very similar carbohydrate recognition properties. The gold electrodes were first modified by spin coating of GOsPA-DS solutions, followed by immersion of the modified electrode into the corresponding protein solutions. As the spin coating process allows producing layers of different thicknesses, we also investigated the influence of thin and thick layers on the bioelectrochemical signal generation through the supramolecular assembly (Figure 5).

In the case of the thin layer configuration, a thickness of 110 nm was measured using a Dektak 150 Stylus Surface Profiler, and Figure 6 shows the behavior of Au/GOsPA-DS/Os-Con A/HRP in the absence of hydrogen peroxide; a charge of 8.5

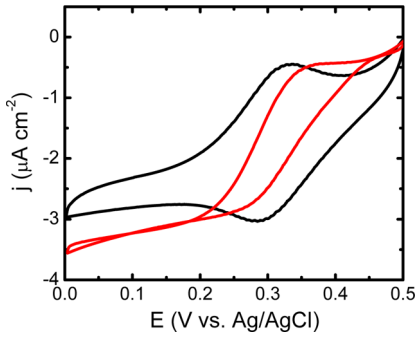

Figure 6. Thin layer system. Cyclic voltammograms for Au/GOsPA +DS/Os-Con A/HRP in 50 mM HEPES buffer (pH 7.4, 0.1 M KNO_3) in the absence (black) and in the presence of 250 μM H_2O_2 (red). Scan rate: 10 mV s^{-1}.

μC cm^{-2} is observed. After the addition of hydrogen peroxide to a final concentration of 250 μM the typical behavior corresponding to an electrochemical catalytic mechanism is observed, even though the backward voltammogram displays a small hysteresis. This behavior is related to a relatively slow electron transfer process between the osmium centers and the heme site of the glycoenzyme.

In order to determine whether this response can be enhanced, a new layer of the redox glycopolyelectrolyte was assembled on the HRP layer (Au/GOsPA-DS/Os-Con A/HRP/GOsPA electrode). Figure 7 shows the behavior of this system in the absence and in the presence of hydrogen peroxide. The introduction of this new top layer produces a slight increase in the osmium charge. When hydrogen peroxide is added to the same final concentration than before, the

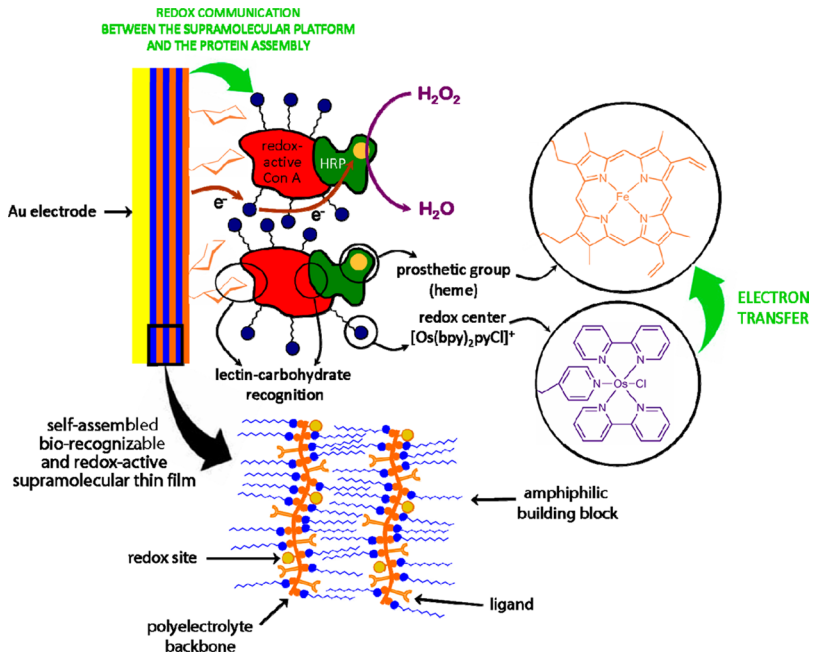

Figure 5. Simplified schematic of the H_2O_2-responsive interfacial supramolecular architecture. Redox-active Con A and HRP are spontaneously assembled via molecular recognition processes onto the redox-active glycopolyelectrolyte−surfactant supramolecular thin film. The figure displays the constituting building blocks participating in the generation of the biolectrochemical signal in the presence of hydrogen peroxide as well as a simplified view of their organization in the interfacial architecture.

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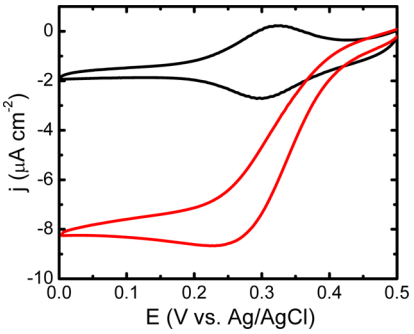
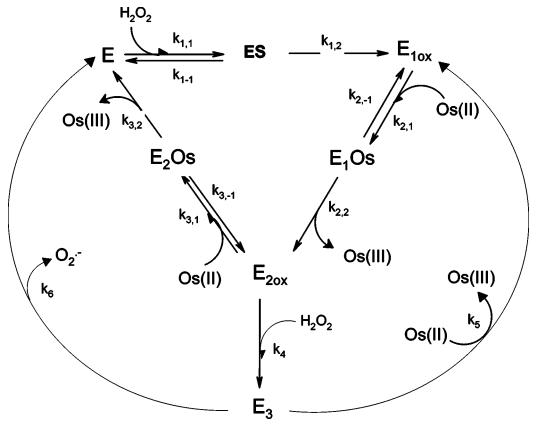


Figure 7. Cyclic voltammograms for Au/GOsPA-DS/Os-Con A/HRP/GOsPA in 50 mM HEPES buffer (pH 7.4, 0.1 M KNO_3) in the absence (black) and in the presence (red) of 250 μM H_2O_2. Scan rate: 10 mV s^{-1}.

maximum current rises to 8.3 μA cm^{-2} (Figure 7, red trace), against 3.5 μA cm^{-2} of the previous experiment (Figure 6, red trace). The evidence clearly alludes to an improved electron transfer process by the addition of a new layer of the redox glycopolyelectrolyte. The forces involved in the adsorption of GOsPA can be mainly attributed to the presence of remaining sugar binding sites in the Os-Con A, improving the catalytic current almost three times. A similar experiment carried out with native Con A does not show any catalytic response, suggesting that the incorporation of the polyelectrolyte improves the connection between the osmium moieties present in the modified Con A and the polyectrolyte-surfactant complex. These results illustrate the advantages that *"soft"* biorecognition assembly process can introduce in the construction of molecular devices as well as demonstrate that the presence of percolation effects can lead to more efficient redox connectivity.

The description of the electrochemical mediated catalysis and inhibition processes exhibited by HRP was thoroughly studied by Savéant and co-workers,[30] and a complete catalytic cycle is presented in Scheme 1. It is well established that as the

Scheme 1. HRP Catalytic Cycle



concentration of H_2O_2 increases, the formation of oxy-peroxidase (E_3 in Scheme 1) becomes more important and a decrease in the catalytic current is observed. This process can be moderated whether a greater amount of osmium complex is present to regenerate the enzyme (see step 5 in Scheme 1), a fact that can be evidenced when a second layer of GOsPA is introduced. Comparing the catalytic responses of both systems at increasing concentrations of substrate (Figure 8) it is evident that the saturation of the enzyme occurs at a higher

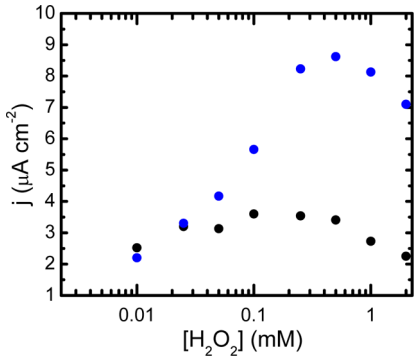


Figure 8. Variation in the electrocatalytic current density as a function of the concentration of H_2O_2 for the two thin layer systems presented. Au/GOsPA-DS/Os-Con A/HRP (black) and Au/GOsPA-DS/Os-Con A/HRP/GOsPA (blue).

concentration when a new layer of GOsPA is added, suggesting that new osmium sites are able to regenerate the enzyme deferring its inhibition to higher substrate concentrations.

This experimental observation is in agreement with previous results reported by Pallarola et al. for layer-by-layer Os-Con A/HRP self-assembled systems.[15] To evaluate the possibility of increasing the current density, gold electrodes were modified with a thicker layer of GOsPA-DS, assuming that a higher amount of Os-Con A and HRP can be assembled. In this case a layer of 820 nm was generated. The density of electroactive Os sites connected to the gold electrode in the thick film configuration increases more than 10 times, yielding a charge density of 111 μC cm^{-2} (Figure 9). It is worth mentioning that

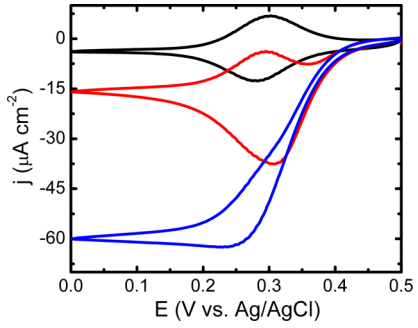


Figure 9. Thick layer system. Cyclic voltammograms for Au/GOsPA-DS/Os-Con A/HRP in 50 mM HEPES buffer (pH 7.4, 0.1 M KNO_3) in the absence (black) and in the presence of 250 μM (red) and 1000 μM H_2O_2 (blue). Scan rate: 10 mV s^{-1}.

even thick films of GOsPA-DS display a remarkable quasi-reversible electrochemical behavior, expressing a notable connectivity between redox centers that allows a fast intrafilm electron transfer process. Sequential assembly of Os-Con A and HRP layers achieves the completion of the system. In the absence of H_2O_2, recognition-driven assembly of Os-Con A practically does not produce significant changes in the voltammogram, and its contribution to the total charge density of osmium centers is negligible. By adding the substrate a catalytic wave evolves. In the presence of 250 μM H_2O_2 an important increase in the cathodic current (reaching a peak current of −40 μA cm^{-2}) was detected (Figure 9). Comparing

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both films, it can be observed that the amount of electrically connected osmium increases 13 times and the catalytic current increases 11 times for the Au/GOsPA-DS/Os-Con A/HRP configuration. However, when the concentration is increased to 1000 μM the electrochemical response increases, in contrast to the behavior observed in the thin film configuration, where at this concentration an evident inhibition process is observed. This behavior is different than that observed by Pallarola et al.[15] and Savéant et al.[31] where the inhibition of HRP by H_2O_2 is observed at 200 μM and 100 μM, respectively. These results suggest that in the present configuration the osmium:HRP ratio is higher than that used in previous reports, and as a consequence this fact minimizes the formation of oxiperoxidase.

The assembly of a new GOsPA layer does not produce significant changes in the catalytic behavior already exhibited by the electrode. However, subsequent assembly of a new Os-Con A/HRP bilayer presents interesting changes. Figure 10 shows

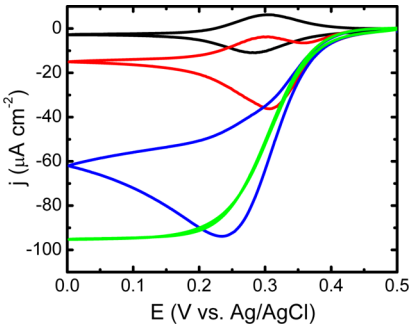

Figure 10. Thick layer system. Cyclic voltammograms for Au/GOsPA-DS/Os-Con A/HRP/GOsPA/Os-Con A/HRP in 50 mM HEPES buffer (pH 7.4, 0.1 M KNO_3) in the absence (black) and in the presence of 250 μM (red), 1000 μM (blue), and 2000 μM H_2O_2 (green). Scan rate: 10 mV s^{-1}.

the behavior of this system in the absence and in the presence of H_2O_2. In the absence of H_2O_2 there is practically no increase in the current density due to the addition of new osmium centers; however, upon exploring the catalytic response two interesting features come to light: (i) the current density for 1000 μM H_2O_2 solutions reaches −97 μA cm^{-2}, and (ii) the voltammetric features reveal that the local consumption of the substrate is important provided that the signal acquires the shape of a peak current instead of a plateau. In fact, the typical plateau shape for EC' mechanism is reached at a concentration of 2 mM. It can be observed that the inhibition process is shifted again to a higher concentration. Figure 11 presents the catalytic current at different concentrations for two systems built from an initial thick layer of GOsPA-DS: It is worth pointing out that for both systems the response is practically the same at low concentrations. This can be related to the fact that at these concentrations diffusional control prevails, as it was previously observed by Savéant and collaborators,[31] whereas at higher concentrations the maximum current that can be obtained depends on the number of osmium centers and the amount of peroxidase present in the system.

DISCUSSION AND CONCLUSIONS

The incorporation of lactose moieties in a redox polyelectrolyte and its subsequent complexation with dodecyl sulfate produces a supramolecular material that is amenable to the formation of robust, biorecognizable redox-active thin films compatible with

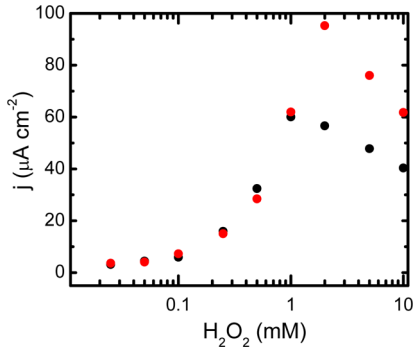

Figure 11. Variation in the electrocatalytic current density as a function of the concentration of H_2O_2 for two systems presenting a "thick layer configuration": Au/GOsPA-DS/Os-Con A/HRP (black) and Au/GOsPA-DS/Os-Con A/HRP/GOsPA/Os-Con A/HRP (red).

different substrates (in this work gold and silicon). Equally important is the excellent dimensional stability displayed by these assemblies provided that no evidence of delamination was observed over a wide range of electrolyte and electrochemical conditions. GISAXS studies show that the system also retains its mesostructural characteristics which correspond to a lamellar organization parallel to the substrate (with additional contributions from multioriented lamellar domains) in close resemblance to osmium-free systems.[10]

This glycosidic-functionalized redox polyelectrolyte exhibit a new functionality, which is the ability to undergo carbohydrate-recognition assembly of lectins, exemplified by Con A, thus promoting the lectin-mediated assembly of glycoproteins such as HRP. A striking feature of this supramolecular nanocomposite is the fact that the electron transfer process within the electron-conducting film is not affected by the assembly of Con A, evidencing that the redox centers are able to find alternative pathways for the electrochemical signal propagation. However, it can be observed that despite the high loading of osmium centers along the film the electron transfer process between HRP and the electrode is not facilitated. This indicates that the incorporation of HRP in the film is highly dependent on the population of preassembled Con A, which acts as an efficient insulating biorecognizable barrier between the redox polyelectrolyte and the catalytic domain of HRP. In this particular case electron transport is a functional limitation that can be easily overcome by using Con A modified with redox-active osmium complexes.

Film formation on the electrode surface can be readily done through spin-coating which allows for the facile control of film thickness via variation of the deposition solution and/or spin-coating conditions, and therefore routine fabrication of these supramolecular thin films is easily achievable. Our experimental studies were focused on two different configurations: thin and thick supramolecular films. In both cases the incorporation of Os-Con A and HRP successfully took place through a recognition-driven assembly process. For the Au/GOsPA-DS/Os-Con A/HRP thin film system, substrate inhibition occurs at the same concentration as for the analogue layer-by-layer (LbL) self-assembled system.[15] However, the maximum catalytic current observed in this work is almost twice the current measured in a typical LbL film, 3.5 vs 2 μA cm^{-2}. When an additional layer of GOsPA was added atop the film, an important increase in current was detected. This fact indicates a layered structure where sugar-binding sites in the Os-Con A are still available to incorporate and interact with a new

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glycopolyelectrolyte layer and facilitate the communication between the heme group of the HRP and the electroactive sites. In the presence of H_2O_2 the catalysis observed in both configurations under low concentration conditions (less than 25 μM) does not exhibit major differences. This indicates that the kinetics of the redox process is mainly governed by substrate diffusion, a phenomenon already observed by Savéant and co-workers in their study on the electron transfer between electrodes and HRP mediated by osmium complexes.[31] At higher substrate concentrations the catalytic current increases, reaching the maximum value (9 μA cm^{-2}) at 250 μM H_2O_2. In this case the current is 4 times higher than that obtained with a single Os-Con A/HRP bilayer assembled on a gold electrode. This enhancement of current activity can be attributed to a more efficient connection between the catalytic site of the enzyme and the conducting support. In a similar vein, the inhibitory effect of H_2O_2 is evidenced at a higher concentration (500 μM) compared to other systems,[15,31] which can be attributed to a higher density of redox centers in close proximity to the HRP active site and hence preventing the formation of oxiperoxidase.

In the case of GOsPA-DS thick films the shape of the voltammograms at low concentrations of H_2O_2 (less than 100 μM) clearly evidenced that a significant amount of the Os centers are not involved in the electrochemical signal chain. The catalytic response of the biointerface exhibited not only a mixed behavior under these conditions, showing an increase in the cathodic current due to the presence of H_2O_2, but also an oxidation peak in the reverse scan, corresponding to the population of the redox centers that do not participate in the catalytic cycle of HRP. On the other hand, when H_2O_2 concentration increases, no inhibition process is observed up to a concentration of 2 mM. This is attributable to the high concentration of osmium centers surrounding the peroxidase, which in turn minimize the formation of oxiperoxidase.

This work has shown that it is possible to modify a polyelectrolyte with a redox center as well as with glycosidic residues to favor biomolecular recognition, in this case a lectin. The presented system can be applied on almost any surface and shows a great stability. This novel polyelectrolyte, combined with a redox mediator-conjugated Con A, has proved to be capable of immobilizing HRP by a soft method. Another interesting feature of the results presented in this work is the possibility of controlling the optimal H_2O_2-responsiveness range by tuning the thickness of the redox-active glycopolyelectrolyte film.

We consider that the results presented here provide a novel contribution, yet simple to integrate glycoproteins into conducting supports, which we believe will have a positive impact in the field of bioelectronic device development. A direct implication of these results is the integration of complex biological entities on bioelectronic interfaces such as microorganisms as part of sensors or fuel cells,[32] which has attracted considerable interest in the scientific community in recent years. The cell walls of bacteria are composed of carbohydrates recognized by lectins that could be linked to the redox glycopolyelectrolyte thus allowing the electrical connection of bacteria to the surface of an electrode. On the other hand, high local concentrations of these lectins on the surface of the electrode would generate electronic tongues for the recognition of these microorganisms at very low concentrations.[33] These explorations are currently underway in our laboratory. Finally, there is renewed interest in the use of neurons as biosensor elements because they use electrical signals to process information.[34,35] In many cases, the silicon-neuron junction is performed using Con A as an adhesive between cell and substrate.[36] In this context, the combination of our glycopolyelectrolyte−surfactant complex, able to adhere to almost any surface, with Os-Con A could introduce a new variable for electrical communication between the cell and the transistor by introducing new perspectives in the development of biosensors.

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Notes

The authors declare no competing financial interest.

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REFERENCES

(1) Faul, C. F. J.; Antonietti, M. *Adv. Mater.* **2003**, *15*, 673.
(2) Ikkala, O.; Ten Brinke, G. *Science* **2002**, *295*, 2407.
(3) Thünemann, A. F. *Prog. Polym. Sci. (Oxford)* **2002**, *27*, 1473.
(4) Perico, A.; Ciferri, A. *Chem.—Eur. J.* **2009**, *15*, 6312.
(5) Gröhn, F. *Macromol. Chem. Phys.* **2008**, *209*, 2291.
(6) Thünemann, A. F.; Müller, M.; Dautzenberg, H.; Joanny, J. F.; Löwen, H. *Adv. Polym. Sci.* **2004**, *166*, 113.
(7) Zhou, S.; Burger, C.; Chu, B. *J. Phys. Chem. B* **2004**, *108*, 10819.
(8) Thünemann, A. F.; General, S. *J. Controlled Release* **2001**, *75*, 237.
(9) Antonietti, M.; Conrad, J.; Thünemann, A. *Macromolecules* **1994**, *27*, 6007.
(10) Cortez, M. L.; Ceolín, M.; Azzaroni, O.; Battaglini, F. *Anal. Chem.* **2011**, *83*, 8011.
(11) Cortez, M. L.; González, G. A.; Battaglini, F. *Electroanalysis* **2011**, *23*, 156.
(12) Kobayashi, Y.; Hoshi, T.; Anzai, J. I. *Chem. Pharm. Bull.* **2001**, *49*, 755.
(13) Azzaroni, O.; Álvarez, M.; Abou-Kandil, A. I.; Yameen, B.; Knoll, W. *Adv. Funct. Mater.* **2008**, *18*, 3487.
(14) Mir, M.; Álvarez, M.; Azzaroni, O.; Tiefenauer, L.; Knoll, W. *Anal. Chem.* **2008**, *80*, 6554.
(15) Pallarola, D.; Queralto, N.; Knoll, W.; Azzaroni, O.; Battaglini, F. *Chem.—Eur. J.* **2010**, *16*, 13970.
(16) Deacon, A.; Gleichmann, T.; Kalb, A. J.; Price, H.; Raftery, J.; Bradbrook, G.; Yariv, J.; Helliwell, J. R. *J. Chem. Soc., Faraday Trans.* **1997**, *93*, 4305.
(17) Edelman, G. M.; Cunningham, B. A.; Reeke, G. N., Jr.; Becker, J. W.; Waxdal, M. J.; Wang, J. L. *Proc. Natl. Acad. Sci. U.S.A.* **1972**, *69*, 2580.
(18) Arrondo, J. L. R.; Young, N. M.; Mantsch, H. H. *BBA, Biochim. Biophys. Acta, Protein Struct. Mol. Enzymol.* **1988**, *952*, 261.
(19) Alter, G. M.; Pandolfino, E. R.; Christie, D. J.; Magnuson, J. A. *Biochemistry* **1977**, *16*, 4034.

(20) Becker, J. W.; Reeke, G. N., Jr.; Cunningham, B. A.; Edelman, G. M. *Nature* **1976**, *259*, 406.
(21) Kalb, A. J.; Levitzki, A. *Biochem. J.* **1968**, *109*, 669.
(22) Derewenda, Z.; Yariv, J.; Helliwell, J. R.; Kalb, A. J.; Dodson, E. J.; Papiz, M. Z.; Wan, T.; Campbell, J. *EMBO J.* **1989**, *8*, 2189.
(23) Reeke, G. N., Jr.; Becker, J. W.; Edelman, G. M. *J. Biol. Chem.* **1975**, *250*, 1525.
(24) Pallarola, D.; Queralto, N.; Battaglini, F.; Azzaroni, O. *Phys. Chem. Chem. Phys.* **2010**, *12*, 8071.
(25) Cortez, M. L.; Pallarola, D.; Ceolín, M.; Azzaroni, O.; Battaglini, F. *Chem. Commun.* **2012**, *48*, 10868.
(26) Danilowicz, C.; Corton, E.; Battaglini, F. *J. Electroanal. Chem.* **1998**, *445*, 89.
(27) Pallarola, D.; Queralto, N.; Knoll, W.; Ceolin, M.; Azzaroni, O.; Battaglini, F. *Langmuir* **2010**, *26*, 13684.
(28) Flexer, V.; Forzani, E. S.; Calvo, E. J.; Ludueña, S. J.; Pietrasanta, L. I. *Anal. Chem.* **2006**, *78*, 399.
(29) Cortón, E.; Battaglini, F. *J. Electroanal. Chem.* **2001**, *511*, 8.
(30) Savéant, J. M. *Elements of Molecular and Biomolecular Electrochemistry*; Wiley: New Jersey, USA, 2006.
(31) Dequaire, M.; Limoges, B.; Moiroux, J.; Savéant, J. M. *J. Am. Chem. Soc.* **2002**, *124*, 240.
(32) Coman, V.; Gustavsson, T.; Finkelsteinas, A.; Von Wachenfeldt, C.; Hägerhäll, C.; Gorton, L. *J. Am. Chem. Soc.* **2009**, *131*, 16171.
(33) Ertl, P.; Mikkelsen, S. R. *Anal. Chem.* **2001**, *73*, 4241.
(34) Reska, A.; Gasteier, P.; Schulte, P.; Moeller, M.; Offenhäusser, A.; Groll, J. *Adv. Mater.* **2008**, *20*, 2751.
(35) Fromherz, P. *Solid-State Electron.* **2008**, *52*, 1364.
(36) Schätzthauer, R. *Eur. J. Neurosci.* **1998**, *10*, 1956.