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Facile Glycoenzyme Wiring to Electrode Supports by Redox-Active Biosupramolecular Glue

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The integration of redox glycoproteins on conductive supports plays a pivotal role in the successful design of bioelectronic platforms, including, among others, biosensors or biofuel cells.[1] This is strongly related to the ability to control the interfacial architecture and the quality of the association between the enzyme and the electrode surface. This aspect is particularly important for amperometric biosensors by virtue of the complex interplay between the reactions that give rise to the electronic signal and the assemblage of the biorecognition elements onto the solid support.[2] Apart from retaining the biological activity of the enzyme, the immobilisation procedure must guarantee the accessibility of its active site to the target analyte and other molecules involved in the biorecognition event. It is known that many immobilization methodologies can induce conformational changes in the enzyme, which could be accompanied by a significant loss of enzymatic activity.[3] Within this framework, recognition-directed biosupramolecular assembly emerged as an interesting and attractive alternative due to its simplicity and versatility, without introducing chemical modifications to the enzyme. This non-covalent approach, also known as bioaffinity layering, is based on the remarkable selectivity of the interaction between the constituting building blocks.[4] In the case of glycoenzymes, it has been demonstrated that this methodology allows the rapid immobilization of considerable amounts of protein on surfaces modified with lectins.[5] Concanavalin A (Con A), the best studied of the lectins, is extracted from easily available, inexpensive seeds by simple steps; for example, up to 3 g of Con A can be isolated from 100 g of jack-bean (*Canavalia ensiformis*) flour.[6] The protein exists as a tetramer with a molecular mass of 104 KDa at neutral pH.[7] 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.[8] In the case of bioaffinity layering, the glycoenzyme is supramolecularly conjugated to the tetravalent Con A through its carbohydrate moiety. 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.[4,5] These properties allow Con A to act as a bioaffinity bridge between a sugar-modified surface and the glycoprotein. However, important limitations arise when the bioaffinity layering approach is intended to be implemented in bioelectronic devices. Electrical communication between the glycoenzyme and the electrode is not feasible because the non-electroactive lectin acts as an insulating biorecognizable spacer, not only inhibiting the shuttling of electrons across the interfacial architecture but also demanding the use of leachable diffusional redox mediators in solution to overcome this limitation.[9] Within this framework, the incorporation of predesigned redox centers into the Con A molecules could open up the possibility of creating biorecognizable platforms to enable the spontaneous assembly and electrical wiring of glycoenzymes onto electrodes. The redox-tagged Con A layer would act not only as a "biosupramolecular glue" to facilitate the robust and easy attachment of the enzyme without affecting its catalytic activity but also as an

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electron-conducting phase to enable the electrical communication between the prosthetic groups of the enzymes and the electrode support.[10] Recent works by Lisdat and co-workers illustrate the potential of self-assembling all-protein interfacial architectures that incorporate intrinsically electroactive biomolecules to create biomimetic signal chains.[11] These authors demonstrated the creation and remarkable functional features of electrostatic bioassemblies constituted of cytochrome *c* as the redox mediator and sulfite oxidase or bilirubin oxidase as the sensing elements. In both cases, the electrostatic interactions between the constituting elements was the driving force for building up the biomolecular film. Along these lines, we propose a novel strategy for bi-protein recognition-driven self-assembly to create efficient enzymatic electrodes. Notably, in spite of the increasing interest in exploiting the biorecognition properties of Con A at electrochemical interfaces,[12] the incorporation of redox centers into the lectin periphery and its application to the construction of biomimetic signal chains or chemoresponsive bioelectronic architectures has not been reported so far. Our approach is based on the use of redox-active Con A as a bifunctional versatile building block to direct the assembly of HRP layers on the electrode surface and also to communicate the prosthetic group of the enzyme to the electrode surface.

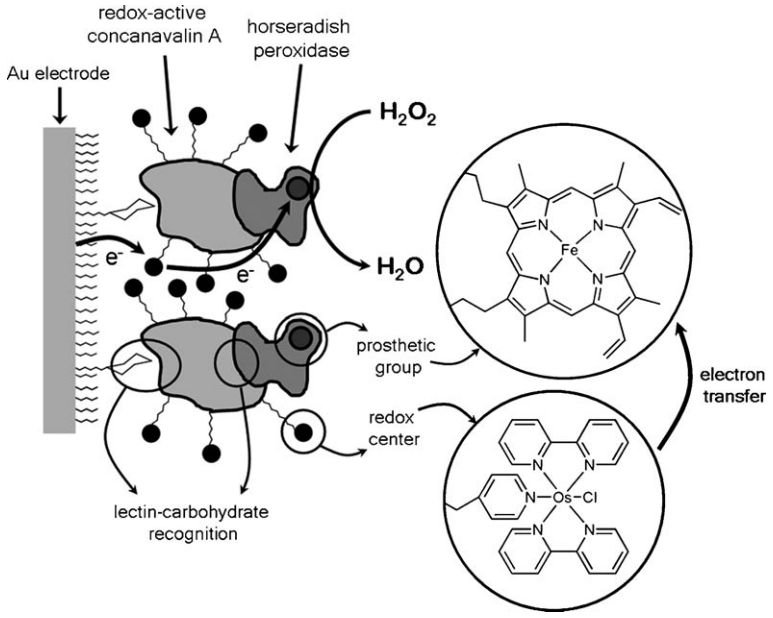

Figure 1. Schematic of the interfacial supramolecular bioconjugate spontaneously assembled by molecular recognition processes, showing the building blocks participating in the generation of the bioelectronic signal in the presence of hydrogen peroxide.

Con A was labeled with $[Os(bpy)_2Clpy]^+$ by using a 336 Da MW polyethylene glycol (PEG) spacer. The choice of this linker relies on its ability to expose the redox centers to the surrounding solution to facilitate their connection to the enzyme and to inhibit unspecific interaction with biological milieu owing to its hydrophilic character. The incorporation of 12 redox labels to the proteic matrix was monitored by MALDI-TOF-MS. A monolayer of $([Os(bpy)_2Clpy]^+$-$PEG)_{12}$-Con A (Os-Con A) was first assembled onto a mannosylated Au electrode.[13] This was followed by the recognition-directed assembly of HRP on the redox-tagged lectin layer (Figure 1). The stable biding of the native HRP to the interfacial architecture is originated from lectin-carbohydrate interactions between the glycoenzyme and the Con A protein.[4,5]

We estimated the mass coverages of Os-Con A and HRP subsequently assembled on the mannosylated gold surface by monitoring the immobilization process by using surface plasmon resonance (SPR) spectroscopy. The surface coverage of Os-Con A ($\Gamma_{\text{Os-Con A}}$) was 1.73 pmol cm^{-2}. This value is in good agreement with spectrophotometric measurements on native Con A immobilized on quartz crystal by Anzai et al.,[14] who estimated a value of 1.6 pmol cm^{-2} for the Con A monolayer coverage. In a similar vein, the assembly of HRP on the Os-Con A monolayer led to a glycoenzyme surface coverage value (Γ_{HRP}) equal to 0.59 pmol cm^{-2}.

Although direct electron transfer (DET) is possible between an electrode and a peroxidase that catalyses the reduction of hydrogen peroxide, this is generally a slow process on conventional electrode materials. An appropriate electron donor, such as $[Os(bpy)_2Clpy]^+$, can mediate the electron transfer between the peroxidase and the electrode and, therefore, such a mediator is expected to greatly improve the H_2O_2 responsiveness of the interfacial architecture. It is worth noting that the redox tags themselves have no catalytic activity for micromolar hydrogen peroxide concentrations in the relevant potential range. As such, the only role of $[Os(bpy)_2Clpy]^+$ centers is to "wire" the HRP to the gold electrode. Our strategy that employs the Os-Con A bifunctional building block provides an effective alternative for connecting glycoproteins to electrodes in systems in which DET is not efficient. In fact, only some haem proteins, mainly cytochrome *c*, have so far been reported to present efficient DET,[15] so the Os-Con A approach offers a comprehensive alternative to the design of glycoprotein bioelectronic arrays.

We used cyclic voltammetry to study the bioelectrocatalytic properties of the supramolecular assemblies of HRP formed on the redox-tagged Con A-modified Au electrodes. Figure 2a describes the linear-sweep voltammograms of the Au/Os-Con A/HRP interfacial architecture from 0.5 to 0.0 V (vs. Ag/AgCl) in the absence and in the presence of 78 μM

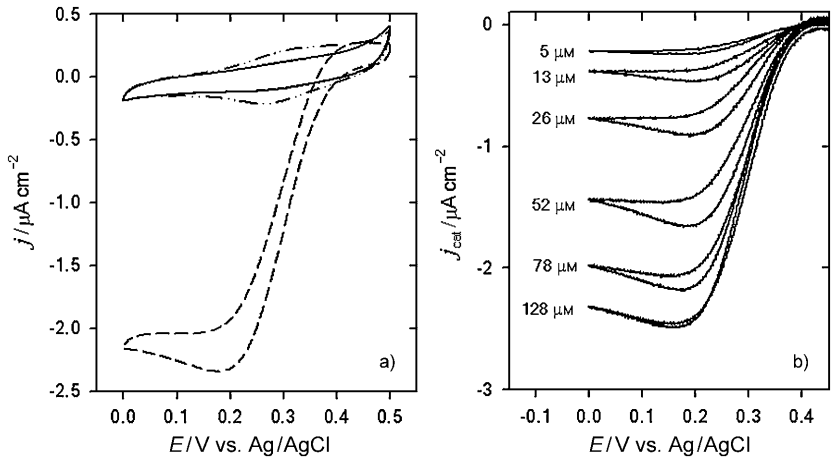

Figure 2. a) Cyclic voltammograms describing the electrochemical response of $(Con\ A)_1(HRP)_1$-modified electrodes in the presence of 78 μM H_2O_2 (——) and $(Os\text{-}Con\ A)_1(HRP)_1$-modified electrodes in the absence of H_2O_2 (–•–•) and in the presence of 78 μM H_2O_2 (-----). b) Cyclic voltammograms describing the bioelectrocatalytic behaviour of $(Con\ A)_1(HRP)_1$ assemblies in the presence of increasing amounts of substrate; scan rate: 10 mV s.$^{-1}$

Then, we used cyclic voltammetry to characterize the bioelectrocatalytic behaviour of the $(Os\text{-}ConA)_2(HRP)_2$ multilayer. The experimental protocols and control experiments were similar to those previously employed in the $(Os\text{-}ConA)_1(HRP)_1$ assemblies.

The well-defined voltammetric response indicates that the HRP confined within the assembly is "wired" to the Au electrode and, thus, the electron transfer between the heme site of the glycoenzyme and the redox labels is also feasible in the multilayered assembly (Figure 3). It is also evident that the electrocatalytic currents are higher than those measured in the $(Os\text{-}ConA)_1(HRP)_1$ assembly due to the increased amount of HRP and Os-ConA (Table 1). In both assemblies, $(Os\text{-}ConA)_1(HRP)_1$ and $(Os\text{-}Con\ A)_2(HRP)_2$, the catalytic current increases upon raising the substrate concentration. Then, upon further increase of the H_2O_2 concentration, the catalytic current gradually decreases, which indicates the progressive formation of the inactivated form of HRP (oxyperoxidase), similarly to that previously observed by Saveant and co-workers.[17] The crossover of the catalytic activity takes place at around 0.1 mM regardless of the number of HRP layers incorporated in the assembly; however, the maximum electrocatalytic current measured in the $(Os\text{-}ConA)_2(HRP)_2$ layer is four times higher than that measured in the $(Os\text{-}ConA)_1(HRP)_1$ assembly (Figure 4a). In addition, the calibration plots describing the correlation between the electrocatalytic current and the H_2O_2 concentration in the "fully active regime", that is, $[H_2O_2] < 0.1$ mM, for the $(Os\text{-}ConA)_1(HRP)_1$ and $(Os\text{-}ConA)_2(HRP)_2$ structures displayed sensitivity values corresponding to 28 and 94 nA cm^{-2} μM^{-1}, respectively. This suggests that upon increasing the total surface coverage of HRP from 0.59 to 0.82 pmol cm^{-2} ($\approx$40% increase) the sensitivity increased three times.

H_2O_2. The voltammetric response eloquently illustrates the H_2O_2-responsiveness of the biosupramolecular enzymatic electrode. Moreover, control experiments performed on analogous supramolecular assemblies that contained native (non-electroactive) Con A revealed no electrocatalytic activity (Figure 2a, ——). This experimental evidence corroborates that the $Os^{II/III}$-modified Con A layer serves not only as a bioaffinity platform to anchor the peroxidase but also as a conducting phase to electrically connect its heme centers to the Au electrodes. Subtraction of background currents from as-obtained voltammograms generated plots that describe the actual bioelectrocatalytic current (j_{cat}) as a function of potential. Figure 2b shows the increasing bioelectrocatalytic response of the Au/Os-Con A/HRP assembly in the presence of increasing amounts of substrate.

So far, we have demonstrated that the redox-tagged Con A is capable of acting as a biorecognizable electron-conducting architecture that enables the immobilization and electrical communication between the enzyme and the electrode. Specific carbohydrate–lectin interactions between the glycoenzyme and the lectin have proven very efficient in assembling the interfacial architecture. Uncomplexed sugar residues available on the outermost surface of the Os-Con A-HRP assembly would enable the formation of a second layer of Os-Con A.[16] Thus, an alternate deposition of Os-Con A and HRP would result in the formation of a multilayered film, as demonstrated by Anzai et al.[14] Along these lines, the nanoconstruction of HRP-containing multilayered thin films is particularly attractive owing to the precise control over the loading of the enzyme and the functional features emerging from its catalytic activity. The sequential deposition of redox-tagged Con A and HRP layers led to the modification of mannosylated Au electrodes with $(Os\text{-}ConA)_2(HRP)_2$ assemblies. The surface coverage of the proteins incorporated during the sequential assembly was estimated by SPR (Table 1).

Table 1. Surface coverages of $([Os(bpy)_2Clpy]^+\text{-}PEG)_{12}$-Con A ($\Gamma_{Os\text{-}Con\ A}$) and horseradish peroxidase (Γ_{HRP}), as determined by surface plasmon resonance (SPR) spectroscopy.[a]

Assembly $(Os\text{-}Con\ A)_n(HRP)_n$	$\Gamma^n_{Os\text{-}Con\ A} \times 10^{12}$ [mol cm^{-2}]	$\Gamma^n_{HRP} \times 10^{12}$ [mol cm^{-2}]	$\Gamma^T_{Os\text{-}Con\ A} \times 10^{12}$ [mol cm^{-2}]	$\Gamma^T_{HRP} \times 10^{12}$ [mol cm^{-2}]
$n=1$	1.73	0.59	1.73	0.59
$n=2$	0.47	0.23	2.20	0.82

[a] Superindexes n and T denote the protein incorporated in the n layer and the total amount of protein assembled on the electrode surface, respectively.

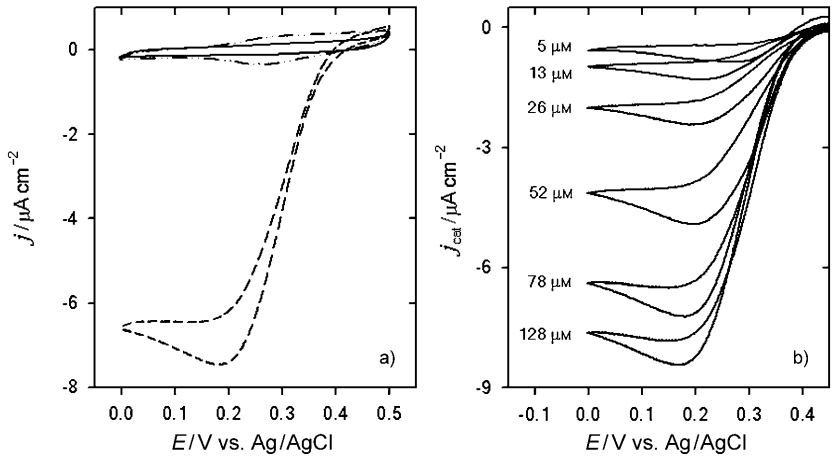

Figure 3. a) Cyclic voltammograms describing the electrochemical response of $(Con\ A)_2(HRP)_2$-modified electrodes in the presence of 78 μM H_2O_2 (——) and $(Os\text{-}Con\ A)_2(HRP)_2$-modified electrodes in the absence of H_2O_2 (–•–•) and in the presence of 78 μM H_2O_2 (-----). b) Cyclic voltammograms describing the bioelectrocatalytic behaviour of $(Con\ A)_2(HRP)_2$ assemblies in the presence of increasing amounts of substrate; scan rate: 10 mV s.$^{-1}$

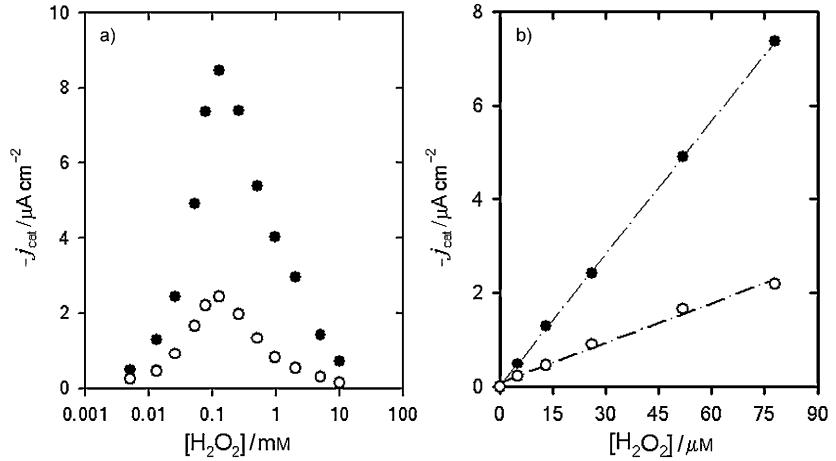

Figure 4. a) Variation in the electrocatalytic current density as a function of the concentration of H_2O_2 for $(Os\text{-}Con\ A)_1(HRP)_1$ (○) and $(Os\text{-}Con\ A)_2(HRP)_2$ (●). b) H_2O_2 calibration curves for $(Os\text{-}Con\ A)_1(HRP)_1$ (○) and $(Os\text{-}Con\ A)_2(HRP)_2$ (●).

To address this point, we need to refer to the intrinsic electroactivity of the supramolecular bioconjugate. The electrochemical charge involved in the voltammetric response of the bioconjugates in the absence of H_2O_2 provides a quantitative estimation of the density of electroactive sites connected to the electrode. In the case of $(Os\text{-}Con\ A)_1(HRP)_1$, this values corresponds to 0.63 μC cm^{-2}, which is equivalent to 3.9×10^{12} centers cm^{-2} (or 6.5 pmol cm^{-2}) connected to the Au electrode. From the SPR measurements we can estimate the actual density of Os sites provided by knowing the average number of redox centers per protein ($n=12$) and the surface coverage of the redox-tagged Con A (1.04×10^{12} molec cm^{-2}). This value corresponds to 1.25×10^{13} centers cm^{-2} (or 21 pmol cm^{-2}) By comparing information derived from cyclic voltammetry and SPR we can infer that only 33% of Os centers are connected to the electrode support in the $(Os\text{-}ConA)_1(HRP)_1$ assembly. Conversely, a similar comparison performed on the $(Os\text{-}ConA)_2(HRP)_2$ revealed that 75% of the redox sites are connected to the electrode.[18] These results strongly suggest the presence of percolation effects within the protein assembly that lead to a more efficient connectivity upon increasing the number of layers into the interfacial architecture.[19] The redox mediators peripherally attached to Con A may exchange electrons through either an intramolecular or an intermolecular process acting in the latter as a conventional diffusing mediator with direct access to the heme prosthetic group.[20] Then they conduct electrons through the supramolecular bioconjugate by self-exchange of electrons or holes between rapidly reduced and rapidly oxidized redox functions tethered to the lectin layer. Incorporating a second (Os-Con A)(HRP) bilayer not only increases the population of enzyme on the electrode surface but also improves the electrical connectivity between the redox mediators owing to the emergence of percolation effects. This explains the strong enhancement of the bioelectrocatalytic behaviour upon increasing the number of protein layers in the supramolecular assembly.

In conclusion, we have introduced a new method to construct electrically contacted glycoenzyme electrodes by using a redox-active Con A as bifunctional bio-hybrid building block for the association/integration of recognition elements on the electrode support. This interfacial architecture represents, to the best of our knowledge, the first example of a modular biosupramolecular approach leading to electron-conducting biorecognizable platforms capable of spontaneously assembling glycoenzymes and electrically connecting their redox-active prosthetic groups to the electrode surface. We reveal that the facile decoration of Con A, a protein obtained from easily available and inexpensive seeds, with redox centers enables the formation of a bioaffinity layer that can act as "biosupramolecular conducting glue" that leads to novel configurations for mediated electron transfer in enzymatic electrodes. The use of alternating

layers of Os-ConA to adsorb different layers of enzyme not only overcomes the low efficiency in direct electron transfer of protein multilayer architectures but also evidences an enhancement effect on connectivity owing to a percolation mechanism. The example given is remarkable not only for its simplicity but also for its versatility, which opens the way to other glycoprotein-based heterostructures that can now be rapidly integrated and connected to electrode supports via recognition-driven assembly. Ultimately, the heteroprotein configuration could be extended to the development of multi-analyte sensing bio-arrays. Furthermore, these results have even wider implications for the integration of complex biological entities on bioelectronic interfaces. On the one hand, the virtues of working with microbial cells as biological sensing materials in the fabrication of biosensors are increasingly recognized by the scientific community.[21] The bacterial cell walls are characterized by the presence of a peptidoglycan layer that could be biorecognized by the redox-active lectin, thus we envision an alternative biosupramolecular method to "wire" the bacterial cells to electrodes. On the other hand, there is renewed interest in exploiting neurons as biosensing devices because they use electrical signals for task-related information processing.[22,23] In most of cases, neuron-silicon junctions are created by attaching the cells to the substrate by using Con A as an adhesive.[24] In this context, the incorporation of electroactive centers into the Con A layer would introduce a new variable to modulate the electrical communication between the cells and the transistor. We consider that these results not only introduce a new, facile and straightforward route for fully integrating glycoproteins on electrode supports, but also herald interesting new possibilities in the science and technology of bioelectronic devices.

Experimental Section

Materials: Concanavalin A (Con A, extracted from the jack bean, *Canavalia ensiformis*), *N*-(3-dimethyl-aminopropyl)-*N'*-ethylcarbodiimide hydrochloride (EDC), *N*-hydroxysuccinimide (NHS), cystamine dihydrochoride (Cys), α-D-mannopyranosylphenyl isothiocyanate (Man), and horseradish peroxidase (HRP) (type VI; RZ = 3.1) were purchased from Sigma. FMOC-PEG-COOH was provided by Novabiochem. $[Os^{II}(bpy)_2ClpyCHO]PF_6$ (bpy = bipyridine, pyCHO = pyridine-4-aldehyde) was synthesized as previously described,[25]. Hydrogen peroxide (30%) was obtained from Carlo Erba and its concentration was determined by permanganate titration. All other reagents were analytical grade.

Synthesis of redox-active Con A: $[Os(bpy)_2ClpyCHO]PF_6$ (0.06 mmol) in methanol (3 mL) was added to a solution of NH_2-PEG-COOH (0.07 mmol; obtained after removing the FMOC group in piperidine 20% in DMF) and *p*-toluenesulfonic acid (0.01 mmol) in methanol (2 mL). The mixture was stirred at 20 °C under argon for 20 h. Afterward, the mixture was treated with an excess of sodium borohydride (0.16 mmol), and stirred overnight at RT. NaOH (1 M, 2 mL) was added to quench the reaction, and the mixture was stirred for another 15 min and then extracted with CH_2Cl_2. The extract was washed with HCl (1 M saturated with NaCl), dried (MgSO4) and evaporated in vacuo. The dark brown solid was dissolved in CH_3CN (5 mL) and added to a large volume of diethyl ether. The osmium derivative is not readily soluble in diethyl ether and precipitated as a fine brown solid that was filtered, washed with cold diethyl ether and allowed to dry in air. The redox-active Con A derivative was obtained by following a procedure described in the literature.[26] Briefly, $Os(bpy)_2ClpyCH_2NH$-PEG-COOH (0.02 mmol), NHS (0.13 mmol) and EDC (0.16 mmol) in 0.5 mL of dry DMF were heated with stirring at 80 °C under argon for 1 h. After cooling the mixture to RT, this solution was added in a ×100 molar excess to a solution of Con A (10 mg mL^{-1}) in phosphate buffer (0.1 M, pH 8.5). The mixture was left to react at RT overnight under stirring. Unreacted osmium was removed from the solution of modified Con A by 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^{-1}. The product was then dialysed at 4 °C for 24 h against MilliQ water by using a 3500 MWCO membrane and lyophilized. The Os/Con A ratio was determined by MALDI-TOF spectrometry following a procedure described elsewhere.[27]

Construction of self-assembled layers: The construction of molecular assemblies was achieved by using a BK7 glass coated with 2 nm of chromium and 50 nm of gold by evaporation. The substrate was incubated overnight with a solution of cystamine dihydrochoride (5 mM) in ethanol, then the electrode was rinsed with ethanol and dried with N_2, followed by 2 h incubation in a solution of α-D-mannopyranosylphenyl isothiocyanate (10 μg mL^{-1}) in PBS buffer (0.05 M, pH 7.4).[13] Then the electrode was rinsed with PBS buffer and immersed for 1 h in a solution of Con A or Os-Con A (1 μM) in PBS buffer that contained $CaCl_2$ (0.5 mM) and $MnCl_2$ (0.5 mM). The same buffer was used to incorporate the HRP to the surface and to rinse the electrode after a Con A, Os-Con A or HRP assembling step. To immobilize the enzyme onto the Os-Con A-modified surface, the electrode was incubated for 1 h in a HRP solution (1 μM). All steps were carried out at RT (ca. 20 °C).

Surface plasmon resonance (SPR) spectroscopy: SPR detection was carried out in a homemade device by using the Kretschmann configuration. The SPR substrates were BK7 glass slides evaporation-coated with 2 nm of chromium and 50 nm of gold. To estimate the biomolecule coverage during the sequential recognition-directed assembly steps, the SPR signal at different angles was recorded prior to and after injection of the corresponding protein solution in the liquid cell. This was done to detect the shift of the minimum angle of reflectance due to the protein assembly on the surface. The SPR angle shifts were converted into mass uptakes by using the experimentally determined relationship, Γ (nanograms per square millimeter) = $\Delta\theta$ (degrees)/0.19. The sensitivity factor was obtained by procedures reported in the literature.[28]

Electrochemical measurements: Cyclic voltammetry experiments were performed by using a μAutolab potentiostat (Echo Chemie) equipped with a three-electrode cell and an Ag/AgCl reference electrode and platinum mesh counter electrode. All electrochemical experiments were carried out at room temperature (ca. 20 °C) in a Teflon cell designed to expose 0.18 cm^2 of the surface of the electrode to the solution. Electrochemical experiments were carried out in a 0.05 M KH_2PO_4/K_2HPO_4, 0.1 M KCl buffer solution at pH 7.4.

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