A Versatile Approach for the Assembly of Highly Tunable Biocatalytic Thin Films

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The deposition of protein thin films on (in)organic surfaces is a key approach to incorporate new functionalities into these materials for a broad number of applications. However, most of the current methods used for the controlled assembly of such biomolecules and eventual film formation are limiting since entail either the chemical modification of the proteins, which leads sometimes to impaired materials, or the sequential layer-by-layer deposition of charged macromolecules. In this work, a facile bioinspired method for the versatile fabrication of robust catalytic films is developed. The herein shown approach involves the one-pot synthesis of porous enzyme nanogels decorated with imidazole motifs. These nanogels act as small nanoreactors that are further self-assembled into continuous catalytic nanocoatings through metal-imidazole coordination. An extensive structural, chemical, and functional characterization of the biocatalytic coatings evidences the integrity, stability in organic solvents and high temperatures, and the reusability of the deposits. Moreover, the thickness and metal composition of the nanocoatings can be tuned on demand. Finally, interesting applications for the bioinspired films are demonstrated, i.e., the fabrication of a glucose electrochemical biosensor and the completion of tandem enzymatic reactions, which suggest a broad applicability potential of this methodology.

1. Introduction

The design of new robust functional biomaterials has garnered a great deal of attention in the last years. Aiming at progressing in many applications such as miniaturizing devices, sensing, catalysis, luminescence, diagnosis, or biomedicine, new methods to control the arrangement and deposition of biomolecules in confined locations are being developed.[1–3]

Nevertheless, the utilization of biomolecules, i.e., proteins, to impart functionality to inorganic and/or organic materials and afford highly efficient functional devices presents a number of challenges in the research of functional biomaterials.[4–6] The main limitations predominantly arise from intermolecular aggregation, surface-induced denaturation, steric hindrance of active sites, and lack of dynamical freedom imposed by solid state.[7–9] The deposition of continuous protein thin films seems to be a good strategy that fulfill those needs.[10,11] With particular emphasis on biocatalytic coatings, the fabrication method should guarantee high enzyme loads, low substrate/product flow transport limitations, and improve the lifetime and stability of the biomolecule.[12] Currently, reported methods for the fabrication of functional biofilms are based on the utilization of a relatively limited range of naturally self-assembling proteins, layer-by-layer deposition approaches, and the adsorption of the proteins to amphiphilic copolymers.[13–17] However, these approaches usually require of the covalent crosslinking of the components in order to avoid the disaggregation of the film in water and at broad range of pH.[18] Yet, the uncontrolled covalent crosslinking might be especially damaging in the formation of functional protein films. The protein's amino acids can be altered and severe substrate diffusion issues might be caused within the film, resulting in impaired biomaterials.[19] Therefore, an alternative sequence-independent methodology that allows the fabrication of functional protein films would vastly expand the toolkit for creating biomaterials.

In this regard, the bioinspired self-assembly of hierarchically structured peptide or protein films is an attractive approach.[20–23] In nature, the metal-driven crosslinking of specific peptidic building blocks leads to complex hierarchical structures across many lengths, as it happens in mussel byssus or worm jaws.[24–26] Furthermore, metal-directed protein self-assembly (MDPSA) methodology is inspired by the affinity of distinct residue side chains such as histidines, cysteines, lysines, and asparagines toward metallic cations (mainly Ni, Cu, Co, and Zn). MDPSA allocates such key residues on the surface of the protein as anchoring points.[27–29] Hence, metal ions are used as inorganic bridges that not only guide the assembly of the proteins into hierarchical architectures, but also might
bring along their intrinsic reactivity (Lewis acidity, redox reactivity) that in this way can be integrated into the system. Yet, requirements of computational and molecular biology tools make MDPSA a complex and lengthy process for protein film formation, thus alternative and more versatile approaches for metal driven protein assembly are sought for.

The design of polymers that self-assemble through multiple intermolecular interactions such as hydrogen bonding, hydrophobic interactions, \( \pi-\pi \) stacking, metal-ligand coordination, and host–guest interactions is a growing field in the last years.\(^\text{[35–38]} \) Additionally, the biomimetic assembly of imidazole bearing polymers has attracted interest due to their potential as catalyst, their use as polymer electrolytes or metal cation adsorbents.\(^\text{[32–34]} \) Moreover, the combination of polymers with enzymes has been demonstrated to be beneficial to increase the stability of the biomolecules under harsh operational conditions.\(^\text{[15–38]} \)

In this work, we seek to develop a novel and easy method for the fabrication of robust and versatile biocatalytically active films deposited on inorganic surfaces. For that, we decorate the surface of enzymes with coordination ligands, i.e., imidazole, using random copolymers. Thus, a thin polymeric network wraps the enzyme and renders individual and small nanocontainers with conserved functionality as ready-to-assemble nanoreactors. Only under presence of metal cations such as Cu(II), Zn(II), Co(II), or Ni(II), those nanoreactors are spontaneously self-assembled in a layered fashion to render highly stable biocatalytic films, without the requirement of further chemical modifications. As far as we know, this is the first example of catalytic films built through metal-coordination to entrapping enzymes. Moreover, the herein demonstrated applications in the field of sensing and biocatalysis showcase the enormous potential of these functional films.

2. Results and Discussion

2.1. Fabrication Procedure of the Functional Films

2.1.1. Synthesis of Ready-To-Assemble Enzyme Nanoreactors

The herein proposed biocatalytic nanoreactors are polymeric nanogels filled with glucose oxidase (GOx) enzyme (here referred to as imGOx) (Scheme 1i). These nanogels are synthesized in situ following a one-step protocol developed in our lab and described in detail in the Experimental Section. The use of vinyl imidazole (VIm) and hydroxylacylamide (HEAA) comonomers for the synthesis of a poly(HEAA-co-VIm) nanogel wrapping the enzymes is intended to promote the assembly of the nanogels through metal-imidazole coordination and the interaction with hydrophilic surfaces, respectively. Importantly, the protein sequence of the enzyme was not chemically modified. Moreover, polymer growth was optimized and limited to the synthesis of small-sized nanocontainers in order to diminish the introduction of noncatalytic components to the film and eventual mass transport issues.

The characterization of imGOx nanoreactors by Fourier-transform infrared spectroscopy (FTIR), dynamic light scattering (DLS), and atomic force microscopy (AFM) confirmed the entrapment of glucose oxidase enzyme within \( \approx 15 \) nm diameter size nanogels (Figure S1, Supporting Information).

2.1.2. Self-Assembly of the Polymeric Nanoreactors

Transparent metal–organic protein films were prepared in a two-step protocol. First, the addition of Zn(II) cations to imGOx solution (Scheme 1ii) led to the metal-directed assembly of those into larger metal–organic clusters \( \approx 800 \) nm (Experimental Section, Figure S2, Supporting Information). Thereafter, pre-assembled clusters are drop-casted on a hydrophilic surface such as glass or silicon substrate for their assembly into supramolecular architectures (Scheme 1iii,iv). Films fabricated following this protocol, from imidazole-bearing polyhydroxylated nanogels, were continuous and uniform. Nevertheless, the assembly of hydroxyl-free enzyme nanocojunctions, synthesized by the replacement of HEAA by acrylamide (AA) as propagating comonomer, rendered films with “coffee ring” effect as depicted in Figure 1a. Therefore, it seems that hanging hydroxyl groups of HEAA containing nanoreactors boost the nanogel–nanogel and nanogel–substrate interactions and thus are necessary for the homogeneous spatial distribution of the nanoreactors within the films. This effect has been already reported for other systems.\(^\text{[39,40]} \) We also observed that the pre-assembly with metal cations is needed for the fabrication of robust and homogenous films (see the Experimental Section). Moreover, the supramolecular assembly of the nanoreactors was particularly effective with \( \approx 100\% \) of the enzyme.

Scheme 1. Scheme of the synthesis procedure of biocatalytic nanocoatings. Protein film formation procedure entails i) the synthesis of GOX nanoreactors, imGOx, in which the protein is embedded into a poly(HEAA-co-VIm) nanogel wrapping the enzymes is intended to promote the assembly of the nanogels through metal-imidazole coordination and the interaction with hydrophilic surfaces, respectively. Importantly, the protein sequence of the enzyme was not chemically modified.
immobilized into larger metal–organic nanoclusters, with a protein content of \( \approx 30\% \) (w/w, measured in dry solid) (Table S1, Supporting Information).

The use of other metal cations for the assembly of the nanoconjugates and subsequent film formation was evaluated in this study. As shown in Figure 1b, @Cu, @Co, and @Ni directed assembly yielded light-transparent, thin, and homogeneous layers similar to those fabricated with @Zn. Different degree of noncovalent crosslinking provided by distinct metal cations might affect the flexibility and brittleness of the films, leading to the occurrence of cracks in some of the samples. Certainly, other metal cations tested with lower affinity toward imidazole ligand, i.e., Pd\(^{2+}\) and Cr\(^{3+}\), showed grainy, rough, and noncontinuous deposits (Figure 1c and Figure S3, Supporting Information). As described below, a deep characterization was performed in order to ascertain the structure and chemical composition of the films.

2.2. Structural and Chemical Characterization of the Films

2.2.1. Thickness and Morphology

Zinc containing films were chosen for a deep structural and chemical characterization. The homogeneity and thickness of the films was controlled by the adjustment of the concentration of deposited metal–organic clusters. The deposition of 2 \( \mu \)L of those at 0.75 mg mL\(^{-1}\) yielded wavy, continuous, and homogeneous surface made of Zn-assembled clusters with a thickness of \( \approx \)600 nm as revealed by environmental scanning electron microscopy (ESEM) (Figure 2a,b). However, deposition at higher concentration (\( \geq 2\) mg mL\(^{-1}\), 2 \( \mu \)L) led to the formation of cracks and subsequent peeling off of the dry film from the surface (Figure 2c), and yielded noncontinuous films at lower concentrations (\( \leq 0.1\) mg mL\(^{-1}\), 2 \( \mu \)L) (Figure 2d). Likewise, the thickness of the metal–organic films could be modulated from hundred nanometers to over 4 \( \mu \)m (Figure S4, Supporting Information).

Details of the nanostructure of the films were elucidated by AFM imaging (Figure 2e). AFM images showed homogeneous grainy surfaces, with measured root mean square (RMS) roughness values between 6.4 and 12.2 nm, for all divalent metal cation used for film formation (Figure S5, Supporting Information). Furthermore, interconnected nanoparticles forming a hierarchical network were likewise identified by ESEM (Figure 2f). We attributed such structures to individual entities of imGOx nanogels (Figure S1B, Supporting Information). Moreover, a deep insight into the side face of a fragmented film suggested self-organization of the nanoreactors into layered structures (Figure 2g and inset figure). Therefore, the above results suggest a homogeneous and higher order assembly of the metal–organic clusters on the substrate.

2.2.2. Compositional Characterization of the Films

Film composition and the effect of film formation on the molecular structure of the protein were evaluated using a range of spectroscopic techniques. The UV–vis spectrum of dry films showed a clear maximum peak at 450 nm, typical of the flavin adenine dinucleotide (FAD) cofactor of glucose oxidase enzyme (Figure 3a). Furthermore, circular dichroism (CD) experiments on dry hybrid films showed the characteristic minima at 222 and 208 nm typical of \( \alpha \)-helical structure (Figure 3b). We detected a minimal perturbation of the secondary structure of GOx enzyme after being assembled and, therefore, significant distortion effects on the protein were discarded. In addition, the conservation of the secondary structure of GOx enzyme was confirmed by attenuated total reflectance-FTIR (ATR-FTIR) spectroscopy (Figure 3c).[41] We observed no effect neither on the position nor on the symmetrical shape of the Amide I infrared peak (settled at 1636 cm\(^{-1}\)) of GOx after the assembly. The presence of new peaks in the infrared spectrum of the film could be assigned to the vibrational modes of imidazole (Figure S1c, Supporting Information).

The metal component of the hybrid films was also checked. Energy-dispersive X-ray spectroscopy (EDX) confirmed the...
presence of metal within the film (Figure 3d and Figure S6, Supporting Information). Further compositional information was enlightened by X-ray photoelectron spectroscopy (XPS) (Figure 3e and Figure S7, Supporting Information). A Zn content of 2.9% (at%) was determined (Table S2, Supporting Information). Interestingly, the peaks of Zn 2p with the binding energy of 1022 and 1045 eV detected in our films (Table S3, Supporting Information) fit with those reported in the literature for zeolitic imidazolate frameworks (ZIF-8 type MOFs), suggesting similar Zn-imidazole coordination.[42] Therefore, we can conclude that the compositional characterization of the films evidences metal-coordination driven assembly.

2.3. Evaluation of the Catalytic Response of the Films

2.3.1. Effect of the Thickness and Metal Composition on the Functionality of the Film

The catalytic response of the films was tested. Glucose oxidation ability was measured in situ using the glucose/ABTS/HRP assay after deposition of the films onto the wells of a 96 well plate as shown in Figure 4a. The deposition of thin films, which are transparent to the visible light, allowed the in situ absorbance readout using chromogenic substrates (Figure S8, Supporting Information). Dry films were washed 3 times with water prior to carry out catalytic measurements (see details in the Experimental Section). Interestingly, we observed a lag-time of 200–400 s before monitoring the highest oxidation rates. This effect relied on the amount of deposited enzyme and thus film thickness. The thicker film deposited, the longer retardation of the activity was observed (Figure S9, Supporting Information). Therefore, a diffusion issue related to the flow of substrates throughout the deposit is evidenced. Hence, the catalytic response of GOx@Zn films could be increased from $3.2 \pm 0.6$ to $35.7 \pm 1.5$ U by decreasing the concentration of deposited metal–organic clusters from 3 to 0.2 mg mL$^{-1}$, respectively (Figure 4b). Catalysis with noncontinuous films (0.1 mg mL$^{-1}$ deposited) showed the best performances with low reproducibility, with 73 $\pm$ 33 U. Furthermore, we observed significant differences in glucose oxidation rates when compared films fabricated with distinct metal cations (Figures S10 and S11, Supporting Information). We calculated the apparent Michaelis constant ($K_m$) kinetic parameter to examine the effect of metal cations on the ability of GOx to
bind glucose. Interestingly, $K_m$ values were significantly lower than that calculated for free GOx (from $1.0 \pm 0.1$ to $3.0 \pm 0.6 \times 10^{-3}$ M vs $18.0 \pm 1.7 \times 10^{-3}$ M measured for hybrid films and free enzyme, respectively) (Figure S12 and Table S4, Supporting Information). Yet, differences in $V_{\text{max}}$ (12.5 $\pm$ 0.2, 8.2 $\pm$ 0.3, 4.0 $\pm$ 0.2, and 1.1 $\pm$ 0.1 $\mu$mol min$^{-1}$ mg$^{-1}$ measured for @Zn, @Co, @Ni, and @Cu films, respectively) might be explained by impediment issues of the substrate to the catalytic site of GOx. These results indicate that the degree of crosslinking and compactness of the film can be modulated by the selection of the appropriate divalent metal cation for film formation.

2.3.2. Recyclability of the Films as Heterogeneous Catalysts

Of particular importance is the integrity and the stability of responsive inorganic–organic hybrid films as promising materials for biotechnological applications.[6] Hence, deposited films were subjected to several long reaction cycles, i.e., 5 cycles of 30 min each. GOx@Zn films lost 15% of the activity upon the first reaction cycle and the catalytic performance was thereafter conserved in the rest of the cycles (Figure S13, Supporting Information). Similar experiments were undertaken with @Cu, @Co, and @Ni assembled films with noticeable differences in the robustness and recyclability of deposited material. From one side, Cu(II) containing films, despite being the less active materials, were found as the most robust films with the 95% of the activity conserved. On the contrary, Co(II) films continuously lost their performance in each cycle (only 55% of the activity remained after 5 cycles). These results might be related with the different chelating strength shown by metal cations with imidazole and denoted by their stability constant ($K_{\text{MMIm}}$). Indeed, highest $K_{\text{MMIm}}$ are reported by Cu-imidazole interactions, followed by Ni, Zn, and Co.[43] Therefore, it seems that most of the nanoreactors are tightly assembled among themselves and firmly bound to the substrate, which generate a fairly robust and recyclable responsive film.

2.3.3. Robustness of the Films

Next, we tested the solubility and stability of the films in organic solvents such as dimethyl sulfoxide (DMSO), isopropanol (iProp), and acetonitrile (ACN). Films were immersed for 1 h in 100% (v/v) solutions of organic solvents, washed and glucose oxidation ability tested as aforementioned (Figure S14A, Supporting Information). Films showed high robustness in water, iProp, and ACN. However, the catalytic performance was decreased after the incubation in DMSO. We demonstrate that this is due to the partial inactivation of the GOx enzyme (Figure S14B, Supporting Information). Plus, same films were thereafter used for catalysis in such organic solvents. Surprisingly, the glucose oxidation rate in iProp and ACN was enhanced by a factor of $\times 370$ and $\times 50$, respectively,
compared to their activity in water (Figure 4c). Moreover, the thermostability profile of the films demonstrated a minimal activity loss (conserved performance of 85%) at high temperatures, while free enzyme only kept the 8% of the initial activity under the same conditions (Figure S15, Supporting Information). Both the high performance in organic solvents and under high temperatures are relevant for the biotechnological use of the films as heterogeneous biocatalysts. Moreover, additional applications of the films are explored, as described below.

2.4. Applications of the Biocatalytic Films

2.4.1. Fabrication of an Electrochemical Biosensor

Motivated by the ease fabrication of the films with high catalytic rates and robustness, we decided to fabricate a glucose biosensor based on a three-system gold electrode as an attractive technological application of our films. Compared to other methods described in the literature, in which layered structures are built by the sequential addition of charged polymers and
proteins,[16,44] our approach allows the one-step deposition into layers using simple drop-casting. Hence, GOx@Zn films were homogenously deposited on the working electrode, washed, and cyclic voltammograms (CV) acquired for a range of glucose concentrations in PBS buffer (Figure S16, Supporting Information). CV results suggested a direct electron transfer system between the film and the electrode.[48] Remarkably, preliminary studies evidenced that modified gold electrodes can be used for the electrochemical detection of glucose in the 0.1–1.5 × 10⁻³ M range with a linear response (Figure 4d).

2.4.2. Design and Fabrication of Tandem Nanoreactors

Finally, we tested this method for the controlled colocalization of enzymes for tandem catalysis. As example, we checked the colocalization of β-glucosidase(βGluc)/GOx duplex for the hydrolysis of cellobiose and subsequent oxidation of glucose tandem reaction (Figure 4e). For that, we synthesized β-Gluc nanoreactors and corresponding Co(II) clusters (βGluc@Co) (Figure S17, Supporting Information). Moreover, we designed different relative arrangement of enzyme layers (referred to as “configuration” in the following) for the fabrication of bi-enzymatic films. First configuration (named GOx/βGluc) consisted in a first layer of GOx@Zn film plus a second β-glucosidase film (βGluc@Co) on top. ESEM/EDAX imaging maps demonstrated that the enzymes were compartmentalized in two spatially resolved layers (Figure 4f). Also, we fabricated the reverse configuration, which leaves the GOx enzyme on top (named βGluc/GOx). As a second approach, we deposited a mix of βGluc@Co and GOx@Zn nanogels at different ratios (25%, 50%, and 75% of βGluc) for the fabrication of enzymatically heterogeneous layers (Figure S18, Supporting Information). Interestingly, different reaction rates were retrieved for each of the configurations (Figure S19, Supporting Information). As depicted in Figure 4g, βGluc/GOx configuration, with GOx on top, showed an enhancement of the activity of 25% compared to GOx/βGluc and 50% βGluc heterogeneous film. This effect is due to the compartmentalization of enzymes.[36,47]

The confinement of βGluc in the bottom layer forces the cellobiosehydrolysis product, i.e., glucose, to pass through a layer of GOx before it reaches the outer media, which enhances the cascade reaction rate.

3. Conclusions

In conclusion, we have developed an extremely simple bioinspired method for the confinement and arrangement of enzyme nanoreactors into layered structures for the fabrication of robust biocatalytic coatings by simple drop-casting, avoiding layer-by-layer sequential and slow depositions, and covalent crosslinking. This method does not involve chemical derivatization of neither the solid surface nor the protein. The synthesis of the nanoreactors has been optimized to minimize the polymeric shell and therefore reduce mass transfer issues within the biocatalytic coating. Moreover, the surface of the nanoreactors has been tuned to allow an efficient metal-driven assembly of those into supramolecular structures. Furthermore, as a versatile method, the thickness of the immobilized film can be altered simply by adjusting the concentration of the deposited solution. Moreover, the biocatalytic response and film integrity and compactness relied upon the thickness of the film and the metal cation used for the assembly. Interestingly, we demonstrated that the nanocoatings are stable in aqueous environment and in presence of organic solvents. Therefore, compared with other methods in which the enzymes are immobilized on the surface of the film, the embedment of those into a porous film increases the protein loading and protects the enzyme from denaturation under harsh conditions. Finally, we demonstrate that the confined nanoreactors within the films prompted the stability of those in some organic solvents and at high temperatures. This feature, plus the easy (co-)localization of enzymes onto surfaces and arranged layers, suggests these biocatalytic coatings as extremely good candidates to be used as heterogeneous catalysis in diverse technological applications.

4. Experimental Section

Materials: Purchased reagents are described in the Supporting Information.

Instrumentation—Attenuated Total Reflectance Fourier Transform Infrared Spectroscopy: The FTIR spectra were measured with a PerkinElmer Frontier spectrometer equipped with an ATR sampling stage. All spectra were measured with 20 scans from 600 to 4000 cm⁻¹ at 4 cm⁻¹ resolution. Each sample was measured three times, and the results were averaged.

Instrumentation—Dynamic Light Scattering: DLS measurements were performed on a Malvern Zetasizer Nano ZS. Proteins and polymer conjugates were prepared at 0.02 mg mL⁻¹ in 30 × 10⁻³ M phosphate buffer and filtered through 0.22 μm cutoff membranes. Experiments were performed at 22 °C and 13 readouts were taken in three independent measurements for each sample.

Instrumentation—Circular Dichroism: Circular dichroism measurements were carried out to determine the secondary structure of the GOx enzyme, in solution and in solid thin films. The GOx and the encapsulated GOx in solution were measured using a sandwich quartz cuvette (0.1 mm path length) at 1.58 × 10⁻⁶ M GOx concentration. The solid thin film was deposited on a quartz slide of 1 × 1 mm² by spin-coating using a Laurell Technologies corporation Model WS-400BP-6NPP/LITE, at 1000 rpm for 10 min with an acceleration of 3000 rpm s⁻¹. All the CD spectra were acquired using a jasco J-815 spectropolarimeter at 0.1 nm increments, 10 nm min⁻¹ scan speed, and 32 s integration time over a wavelength range of 190–260 nm. Each spectrum represented an accumulation of 5 scans.

Instrumentation—Atomic Force Microscopy Experiments: Glass substrates were cleaned by immersing them in a piranha solution (7:3 mixture of 96% H₂SO₄ and 30% H₂O₂) at 80 °C for ~30 min. After that the substrates were rinsed with Millipore water in an ultrasonic bath for 30 min and blow dried with a nitrogen stream. 100 μL of GOx@imNG dispersions were spin-coated onto the substrates with an acceleration rate of 300 rps to a final speed of 2000 rps. The topography of the nanogels and films was characterized with an AFM 5500, Agilent Technologies/Keysight Technologies in AC mode using a silicon tip with a force constant of 6 N m⁻¹ and resonant frequency of ~150 kHz. Height, phase, and amplitude images were recorded simultaneously. AFM images were taken at several positions to be sure that they were representative for the sample surface. All the data were processed with Gwyddion 2.31 program.

Instrumentation—Environmental Scanning Electron Microscopy and EDX Mapping: Morphological characterization and EDX analysis were done with an environmental scanning electron microscope (FEI, Quanta 250 FEG) and an Octane Elect EDS (AMETEK) detector at 5.0 kV
Films were deposited in each well with a 0.375 mg mL\(^{-1}\) concentration were determined with a NanoDrop\textsuperscript{TM} spectrophotometer by measuring the absorbance at 450 nm. The GOx concentration before and after the assembly. Native protein deposition that was removed from solution after washing step. Assays. As sample control, imGOx sample (without the pre-assembly step) was drop-casted under same conditions yielding a nonuniform deposit that was removed from solution after washing step.

**Methods—** Synthesis of Protein Nanogels: Imidazole-decorated GOx nanogels (imGOx) were synthesized following a protocol based on the conditions described before.\(^{[48]}\) GOx enzyme (20 × 10\(^{-6}\) µL) was mixed with either AA or HEAA (AA or HEAA/GOx 600:1, n/n), 10\(^{-3}\) M N,N\textemdash methylendisacrylamide (MBAAm, MBAAm/GOx 400:1, n/n), vinyl imidazole (VIm/GOx 1000:1, n/n), and ammonium persulfate (APS/protein 400:1, n/n). Sucrose (5%, w/v) and DMSO (10% v/v, 33 × 10\(^{-3}\) M) were added. This mixture was deoxygenated by bubbling N\(_2\) through the solutions for 45 min. Polymerization reaction started upon addition of tetramethylethylenediamine (TEMED, APS/TEMED 2:1, w/w). The reaction was kept under N\(_2\) and shaken at room temperature for 2 h. GOx nanogels were dialyzed against PBS buffer to remove low-molecular mass reagents and passed through a Sephadex G-75 column in order to remove nonencapsulated enzymes and protein-free polymer hydrogels. Imidazole nanogels were further purified through Ni-NTA agarose affinity columns.

**Methods—** Synthesis of Metal–Organic Enzyme Clusters: Protein nanogel solution (2 mg mL\(^{-1}\), 1 mL) in Tris buffer (3 × 10\(^{-3}\) M, pH 7.0) was mixed with CuSO\(_4\), Co(NO\(_3\))\(_2\), Ni(NO\(_3\))\(_2\), or Zn(NO\(_3\))\(_2\) (1.5 × 10\(^{-3}\) M) and incubated for overnight to fabricate @Cu, @Co, @Ni, and @Zn-MDEAs. Samples were thereafter centrifuged (8000 rpm, 3 min) and suspended in 75 µL of water. A range of dilutions (from 3 mg mL\(^{-1}\) to 0.09 mg mL\(^{-1}\)) of this solution are used for metal–organic film formation.

**Methods—** Deposition of Metal–Organic Protein Films: Aforementioned dilutions were drop-casted (2 µL) on silicon and glass substrates for the in situ assembly at room temperature. Dry films were washed with water to remove unbound material and used for the characterization or activity assays. As sample control, imGOx sample (without the pre-assembly step) was drop-casted under same conditions yielding a nonuniform deposit that was removed from solution after washing step.

**Methods—** Measurement of Protein Immobilization Yields and Protein Content (Mass Fraction): The amount of protein that is incorporated to the nanohybrid-immobilized protein is calculated by measuring the GOx concentration before and after the assembly. Native protein concentrations were determined with a NanoDrop\textsuperscript{TM} spectrophotometer by measuring the absorbance at 450 nm using UV-transparent quartz cuvettes with a 1 cm path length (ε = 28 200 m\(^{-1}\) cm\(^{-1}\)). Protein mass fraction (%w) was measured by gravimetry. Metal–organic solutions (40 µL) of known protein content (2.0 to 2.5 mg mL\(^{-1}\)) were freeze-dried and weighted. %w was calculated as follows: %w = 100 × added protein (µg) / weighed dry solid (µg).

**Methods—** Kinetics Measurements of Films: The Michaelis–Menten constant (Km) and the V\(_{\text{max}}\) were calculated at 42 °C in 96-well plates. Films were deposited in each well with a 0.375 mg mL\(^{-1}\) concentration. A solution of D-glucose, 2,2\'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS, 0.1 × 10\(^{-3}\) µL), Horseradish peroxidase enzyme (HRP, 0.1 ng mL\(^{-1}\)) in a sodium phosphate buffer (30 × 10\(^{-3}\) M, pH 6.0) was added in every well, with a final volume of 200 µL. The glucose concentration utilized varied in a range from 0 to 100 × 10\(^{-3}\) M.

**Methods—** Glucose Oxidase Activity Measurement: Activity assays were performed at 37 °C in 96-well plates with 200 µL as final volume per well, unless otherwise mentioned in the text. A typical GOx activity measurement was performed using 7.5 µg of GOx, 20 × 10\(^{-3}\) M glucose in 30 × 10\(^{-3}\) M sodium phosphate pH 6.0 with 2,2\'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) 1 × 10\(^{-3}\) M and 0.1 ng mL\(^{-1}\) of HRP. Color development from oxidized ABTS was monitored in the spectrophotometer at 416 nm. A molar attenuation coefficient of 36 000 m\(^{-1}\) cm\(^{-1}\) was used for the calculations.

**Methods—** Catalysis in Solvents: The performance of glucose oxidase catalysis in presence of organic solvents was tested using isopropanol, acetone, and dimethyl sulfide. A 20 × 10\(^{-3}\) M glucose solution in such solvents (200 µL, 90% v/v) was added to the films (0.75 µg of protein). After 20 min at 37 °C, 2 µL of respective reaction solution were added to a HRP/ABTS solution (0.1 ng mL\(^{-1}\) of HRP and 1 × 10\(^{-3}\) M of ABTS in 30 × 10\(^{-3}\) M sodium phosphate pH 6.0). The activity was monitored spectrophotometrically at 416 nm. Reaction in water was taken as reference (100%).

**Methods—** Cascade Reaction Measurement: The activity of the 8Gluc/GOx tandem catalyst was measured using cellulose (20 × 10\(^{-3}\) M), ABTS (1 × 10\(^{-3}\) M), and HRP (0.1 ng mL\(^{-1}\)). The green color from oxidized ABTS meant the release of H\(_2\)O\(_2\) to the solution, which in turns, came from the hydrolysis of the cellulose by 8Gluc and subsequent oxidation of the glucose by GOx. Therefore, the overall reaction was monitored in the spectrophotometer at 416 nm. A molar attenuation coefficient of 36 000 m\(^{-1}\) cm\(^{-1}\) was used for the calculations.

**Supporting Information** Supporting Information is available from the Wiley Online Library or from the author.

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**Conflict of Interest**

The authors declare no conflict of interest.

**Keywords**

catalytic surfaces, enzyme films, protein-polymer nanogel, supramolecular assembly
