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Biocatalytic protein-based materials for integration into energy devices

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Abstract: There is a current need to fabricate new biobased functional materials. Bottom-up approaches to assemble simple molecular units have shown promise for biomaterial fabrication due to their tunability and versatility to incorporate functionalities. Here, we demonstrate the fabrication of catalytic protein thin films by the entrapment of catalase into protein films composed of a scaffolding protein. Extensive structural, and functional characterization of the films evidences the structural integrity, order, stability, catalytic activity, and reusability of the biocatalytic materials. Finally, we couple these functional biomaterials with piezoelectric discs to fabricate a second generation of bio-inorganic generators. These devices are capable to produce electricity from renewable fuels through catalase-driven gas production that mechanically stimulate the piezoelectric material.

Introduction

Large efforts are currently being made in the field of fabrication of new functional biomaterials and their integration into technological devices.[1–4] A variety of bioinspired strategies have recently emerged to create biomaterials with tailored functionalities, improved properties and potential uses[1,5–7]. These strategies include the bottom-up approaches for the generation of supramolecular biomaterials,[2] with particular efforts devoted to achieve protein-based materials due to the amazing structural and functional diversity of these biomolecules.[5–9]

Of particular interest to the field of active biomaterials is the fabrication of catalytic materials in which active enzymes are immobilized.[10] Several immobilization methods have been used up to date, including adsorption,[11] covalent linkage,[12] affinity immobilization,[13] entrapment,[14] encapsulation,[15] or enzyme cross-linking.[16] Among them, the simple entrapment offers easy fabrication and handling and endows high stability to the immobilized enzymes.[17] Regarding the support, using natural materials as scaffold provides advantages as biocompatibility, chemical versatility, and biodegradability that organic or inorganic support materials do not offer. In this regard, proteins play a key role since they fulfill all these characteristics and their use for the fabrication of protein-based materials has been already demonstrated.[9,18] However, the robust fabrication of functional protein-based materials films that preserve both structural integrity and functionality of the entrapped enzymes remains a challenge in

biomaterials research. In fact, few examples of enzymes immobilized on protein-based materials have been successfully reported up to now. [19]

Several scaffolding proteins can be used for the fabrication of biomaterials. However, engineering proteins provide clear advantages in terms of tunability, reproducibility, scalability and rational design of the final properties of the material.[3] Modular building blocks with simple interactions allow for better control of the final assemblies through the implementation of bottom-up approaches. Engineering repeat proteins present these inherent features that make them suitable candidates to be used as scaffolding proteins.[20,21] In particular, tetratricopeptide proteins (TPR) have proven as exceptional scaffolds since consensus TPR (CTPR) proteins form ordered films, due to well characterized side-to-side and head-to-tail interactions.[21] The basic CTPR unit consists of 34 amino acids with a helix-turn-helix motif. CTPR proteins have a modular structure in which the repeats can be combined in tandem to form highly stable CTPR proteins that display a right-handed superhelical structure, with eight repeats per one full turn of the superhelix.[22,23] These features make these repeat protein scaffolds ideal building blocks for numerous applications,[24,25] as previously demonstrated through the fabrication of functional protein-based materials.[26,27] Similarly, we hypothesize that materials based on CTPR proteins will be applicable for the ordered entrapment and immobilization of enzymes toward the generation of novel heterogeneous biocatalysts readily integrated into devices.

Catalase (CAT) is an excellent candidate to be entrapped into protein-based materials since is extensively used for technological applications where biocatalyst robustness is often demanded. CAT catalyzes the hydrogen peroxide disproportionation releasing water and molecular oxygen as innocuous products.[28] Such catalytic reaction makes this enzyme industrially relevant for the development of biosensors,[18] preventing food oxidation,[29] removing hydrogen peroxide from residual water,[30] and intensification of oxidase-driven biotransformations.[31,32] In a pioneering attempt to expand the CAT applications, our group has recently exploited CAT in solution to produce bubbling upon H₂O₂ disproportionation reaction as a source of mechanical energy then harvested by coupled piezoelectric materials to generate electricity.[11] Since the described system integrated a soluble CAT that formed bubbles within a reaction chamber, the reaction mixture, and consequently the enzyme, must be removed once the fuel (H₂O₂) is consumed and maximal energy is produced. Moreover, the soluble enzyme normally becomes inactive after one operational cycle after being exposed to the high concentration of H₂O₂ required for the energy generation process. Hence, the current bio-inorganic generator is limited to only one cycle of energy generation, which precludes the reusability and the continuous operation of the system. In this emerging field, enzymes could play a key role since they are able to catalyze reactions whose by-product may be used as source of mechanical energy. However, the coupling of enzymatic systems and nanogenerators would require automatic, independent and reusable systems so as to minimize costs and enhance the efficiency. For the aforementioned reasons, the physical immobilization of CAT on the surface of the piezoelectric material would make the enzyme more robust and recyclable, overcoming the major limitations of the current system for energy harvesting and ease the automation and integration of this bio-inorganic generator into more complex

devices. Although enzyme immobilization is often considered as one of the most effective methods to increase the biocatalyst robustness, the preservation of the enzymatic activity into the materials during the immobilization process is still a challenge.[33] This limitation arises mainly from loss of catalytic activity due to enzymes undergoing denaturing conformational changes, intermolecular aggregation, steric hindrance of the active sites, lack of dynamical freedom, and mass transport restriction of reactants imposed by the structure of the solid materials.[34] In addition, depending on the immobilization methodology the enzyme might be leached to the reaction media resulting in activity loss of the immobilized biocatalyst. Hence, the immobilization of enzymes on solid materials shall be optimized according to their final use, seeking for protocols that maintain the activity and increase the stability relative to the enzyme in solution.

To advance in the fabrication of novel bio-inorganic generators based on CAT, this paper describes the development of a novel protein-based biomaterial that can be readily coupled with piezoelectric materials to increase the operational life span of the energy harvesting process. This increased life span has been possible because the protein environment of the CTPR scaffolding protein forming the biomaterial stabilized the entrapped CAT as demonstrated by thermal inactivation studies. The solid self-standing biomaterial with catalase activity was casted into a piezoelectric disc and proven to produce electricity from H₂O₂. Although the entrapped CAT presents lower apparent catalytic efficiency than the enzyme in solution, the proximity of the bubbling enzyme to the piezoelectric surface improves the energy outcome at large operational volumes compare with the system using the enzyme in solution.

Results and Discussion

Protein-based biomaterial fabrication and characterization

Here we report the fabrication of a bioactive material based on the self-assembly of CTPR proteins which irreversibly entraps CAT as functional unit and its casting on piezoelectric materials to give rise the new generation of energy harvesting devices based on the previously reported ones (Figure 1A).[35] The formation of this active biomaterial relies on the self-assembling properties of CTPR and its ability to form self-standing ordered protein thin films. As previously described by our group,[21] the protein thin film fabrication requires 3% (w/v) protein concentration of a CTPR10 variant (a consensus CTPR protein with 10 identical repeats) to ensure the appropriate packing and handling of the resulting film. The biocatalytic protein film was prepared under these conditions by homogeneously mixing the CTPR10 and CAT in the selected ratios, and then casting the film through two different strategies: using spin-coating in a piece of quartz, or using drop casting method over a hydrophobic material (see experimental section for further details) (Figure 1A,1B). The resulting self-standing protein films quantitatively entrapped CAT and led to a stable, flexible, easy to handle and transparent material (Figure 1B). The homogeneity and surface uniformity of the material is evident from the SEM image (Figure 1C), and the SEM cross-section image revealed a thickness of approximately 20 μ m for these fabrication conditions (Figure 1C, Figure S1). Expectedly, the functionalized protein film showed a peak at around 400 nm

that corresponds to the maximum absorption of heme group of the catalase, which provides the film a light green color (Figure 1D). The CD spectrum of the protein film entrapping CAT shows the characteristic signal of the alpha-helical structure of the scaffolding protein that agrees with results previously observed by our group where the secondary structure of the CTPR10 is preserved.[21] CD data are only revealing the secondary structure of CTPR since the protein stoichiometry in the film is 1:100 CAT:CTPR, thus being the contribution of the CAT to the CD spectrum negligible (Figure 1E).

It is well-known that CTPR proteins present intrinsic properties to form nanostructured ordered films since they macroscopically align by head-to-tail and side-to-side interactions.[22,36] However, the mobility and order of entrapped enzymes in the biomaterial is unknown. To shine light on these two parameters, fluorescence polarization studies were performed by firstly labeling the amine groups of CAT with the fluorescent dye rhodamine. Through fluorescence microscopy, we demonstrated that entrapped CAT is homogeneously distributed across the protein film (Figure 2A). Moreover, we showed by confocal microscopy that the CAT is also homogeneously distributed throughout the film thickness (Figure 2B, Figure S2, S3). To show the tumbling freedom and potential order of the CAT in the biomaterial (Table 1), fluorescence anisotropy was measured, observing that catalase-rhodamine conjugate in solution showed a higher anisotropy than free rhodamine as expected from its larger mass. Interestingly, rhodamine-labeled CAT entrapped into the solid protein film presented a large anisotropy value, which indicates that the confined enzyme is significantly less mobile than its in solution counterpart. Next, the organization of CAT within the protein film was determined by measuring the fluorescence of the rhodamine-labelled enzyme with plane polarized light. The fluorescence intensity changed when the excitation and emission polarizers were move from horizontal to vertical. These changes indicate that labeled CAT entrapped into the protein film exhibits a directional macroscopic order within the material; that order is imposed by the anisotropic arrangement of the CTPR scaffolding protein (Figure 2B).

The ordered self-assemble material as described above is soluble in aqueous media since it is formed mainly due to the non-covalent interactions between CTPR proteins, which are too weak to maintain the integrity of the macrostructure. For operational purposes the disassembly of the film and the lixiviation of the entrapped protein need to be minimized. Therefore, the biomaterial was cross-linked using glutaraldehyde at 1% for 24 h by means of vapor diffusion method to result in a material stable in water. Upon cross-linking the biomaterial was immersed into a buffer solution and showed no lixiviation of the protein in the medium, keeping more than 95% of protein upon 7 days of immersion, which indicates an effective crosslinking (Figure 3A). Moreover, the mild crosslinking reaction did not affect the structural properties of the protein as it is shown in the dichroism spectra, thus the material is expected to also preserve the activity after the reaction (Figure 3B).

Figure 1. Catalytic protein-based biomaterial composed of CAT and scaffolding protein (CTPR). **A.** Schematic representation of the generation of the protein-based catalytic biomaterials and their coupling with piezoelectric discs to fabricate a second generation of bio-inorganic generators. **B.** Optical microscopy image of the protein thin film. **C.** Scanning electron microscopy (SEM) image of the protein thin film showing the cross section and thickness of the film and the surface in the inset. **D.** Absorbance spectra of protein films of scaffolding protein (dashed red line) and the CAT containing thin films (solid black line). The inset shows a zoom in in the region of the absorption band of heme group. **E.** Circular dichroism spectra of nanostructured CTPR protein film (dashed red line) and the thin film with CAT entrapped (solid black line).

Figure 2. Fluorescence characterization. A. Overlay of the bright field image and the confocal fluorescence image of the biomaterial generated using rhodamine labeled CAT. B. Intensity of fluorescence depending on the polarizing direction.

Table 1. Fluorescence anisotropy measurements of CAT in solution and in solid film with the standard error of the mean.

Sample	Anisotropy
Rh control	0.052 ± 0.006
CAT-Rh solution	0.166 ± 0.003
CAT-Rh film	0.819 ± 0.001

Activity and stability of CAT entrapped into the CTPR protein film

The functional properties (activity and stability) of CAT immobilized on the protein-based material described above were determined through an indirect colorimetric assay coupling peroxidase to titrate the concentration of H₂O₂ upon the CAT action.

Figure 3. Crosslinking of the CAT-biomaterial. A. Percentage of protein retained in the cross-linked material after incubation in solution. B. Circular dichroism spectrum of biomaterial before (dashed red line) and after the crosslinking reaction (solid black line).

The enzymatic activity was assayed at room temperature for the biomaterial and the enzymatic solution in the same conditions. It is worth mentioning that swelling of film was observed upon immersion into the aqueous solution, a 0.5 mg film absorbed approximately 1 μ l of water. The apparent specific activity of the entrapped CAT was 0.08 U/mg-1 compared to the 6.7 U/mg-1 observed for the enzyme in solution under the same experimental conditions. Moreover, the activity for different film thickness was evaluated keeping the CTPR:CAT ratio constant. These results showed that the total catalase activity is maximized when using the standard thickness of 20 μ m, thicker films showed lower activity and significantly lower specific activity (Figure S4). Additionally, the apparent kinetic parameters (K_M and V_{max}) of the immobilized enzyme were determined through a Michaelis-Menten fit (Figure S5) and compared to the values calculated for the soluble enzyme under the same conditions. Table 2 shows that the apparent K_M and k_{cat} for the entrapped CAT towards H_2O_2 were 3.6-fold higher and 370 times lower than for the soluble enzyme, which resulted in that the confinement and cross-linked of CAT within the protein biofilm decreased the catalytic efficiency 1519 times. According to the specific activity and apparent kinetic parameters of entrapped CAT, the crowded environment of the protein film may limit the conformational flexibility of entrapped CAT to perform catalysis. Moreover, the higher apparent K_M values of entrapped CAT suggest mass transport issues that may hamper the diffusion of substrates towards the enzyme active sites. These issues can be related to 1) the enzyme entrapment in the compact protein film that makes it less accessible to the medium;[37] and/or 2) the bubbles generated on the surface of the material by the oxygen released during the reaction that hamper the substrate diffusion.[38] These bubbles grow over the surface and due to the hydrophilic nature of the protein material, the bubbles stay at the film-solution interphase, thus hampering the diffusion of the substrate properly. In addition, this change in activity could be partially attributed to the loss of catalytic activity due to the immobilization or/and the crosslinking processes. Noteworthy, the time-course curves show a lag-time of around 400-500 sec when the CAT is entrapped, while that lag is not appreciated in the time-course of the soluble enzyme (Figure S6, S7). Mass transfer issues are also supported by loading experiments where specific activity of entrapped enzymes linearly increases with the reduction of CTPR:CAT ratio (Figure S8). Total apparent activities of films with different ratios are similar, but the specific activity of lower ratio is dramatically reduced. It seems that under high load conditions, the substrate molecules can not reach all the entrapped enzyme molecules due to transport limitation rather than conformational changes related to the entrapment or crosslinking. A population of inaccessible enzyme molecules might explain the low catalytic efficiency observed for the entrapped CAT. Lower CAT loadings ameliorate this effect since a larger enzyme population actively participates in the H_2O_2 disproportionation because of the improved substrate accessibility. These diffusional restrictions have also been observed when CAT is randomly aggregated and irreversibly cross-linked in presence of a feeder protein through cross-linked enzyme aggregate (CLEA) technology.[39] Unlike the entrapment into thin protein films, CLEAs of CAT have not proven the order of the enzyme within the solid material as demonstrated in this work using scaffolding proteins.

Table 2. Kinetic parameters of CAT in solution and in the solid protein thin film. * Apparent parameters.

Sample	K_M / mM	V_{max} / mM s^{-1}	k_{cat} / s^{-1}	k_{cat}/K_M / $s^{-1}mM^{-1}$
CAT in solution	84.02 ± 31.46	10.21 ± 2.74	$2.55 \times 10^6 \pm 0.68 \times 10^6$	$3.04 \times 10^4 \pm 1.40 \times 10^4$
CAT in film	301.89 ± 175.12	0.24 ± 0.10	6000 ± 2500	19.87 ± 14.19

In spite of exhibiting low catalytic efficiency, the entrapped CAT is still active enough for further applications, illustrating the potential and biocompatibility of this simple immobilization strategy. To assess the robustness of the functional biomaterial, we first tested the thermal stability of the entrapped CAT in comparison with the soluble enzyme. Figure 4 shows that the immobilized CAT was significantly more thermostable than its soluble counterpart since it retained approximately 100% of its initial activity after 3 hours incubation at 50°C while the soluble enzyme lost 70% of its initial activity under the same inactivation conditions, which represents a stabilization factor of 3.5. Accordingly to other immobilization protocols, CAT could be stabilized by entrapment CTPR-protein thin films demonstrating its potential as heterogeneous biocatalyst able to be reused for several operational cycles.[40]

Figure 4. Enzymatic thermal stability of CAT in solid thin film (black) and in solution (red).

Energy output measurements

Recently, our group described the production of electric energy from chemical energy by using bioinorganic generators.[35] Such devices are able to convert the chemical energy stored in renewable molecules (as carbohydrates, amino acids, alcohols, etc) into mechanical energy (in form of gas bubbles) which is further harvested by a piezoelectric material based on PZT disc (Lead zirconate titanate) and transduced into open circuit voltages that are finally transformed into an energy using an oscilloscope. This concept

has proven using soluble catalase that disproportionate hydrogen peroxide into oxygen and water, forming oxygen bubbles that trigger a mechanical stimulus further harvested by the piezoelectric material. The drawback of the reported system is its disposal character that avoids the enzyme reutilization after one energy generation cycle. To make these systems reusable, protein films entrapping CAT were casted at the surface of the piezoelectric surface coated with a nanometric inorganic layer with the aim at fabricating bioinorganic generators that can be repeatedly used for the continuous production of electric energy to achieve a more sustainable process.

Hence, we entrapped soluble catalase into a CTPR film that is subsequently cross-linked with glutaraldehyde as above described. Primarily, we assessed the effect of coating of the piezoelectric surface with CTPR protein layer on the energy outcome. Using the soluble enzyme as catalyst, we observed that the energy generated was reduced only 25% regarding to the naked piezoelectric surface, demonstrating that the biomaterial layer slightly affects the performance of the piezoelectric surface, but still allows the conversion of mechanical into electrical energy. When the CAT was entrapped in the protein film, the energy outcome (at 750 mM H₂O₂) was 70 nJ x cm⁻²; a value 3 and 1.6 times lower than the soluble enzyme acting over the naked piezo and the piezo casted with the protein film, respectively (Figure 5A). The optimal fuel concentration for the system using the soluble enzyme was 50 mM H₂O₂ whereas the system with the immobilized enzyme required 750 mM H₂O₂. In spite of that lower output and the more fuel demanded, the entrapment of the catalase enables its repeated use, maintaining more than 50% of the initial energy production after 10 energy generation cycles, replacing the fuel (H₂O₂) and rinsing the reaction chamber after every use; which is not possible with soluble catalase (Figure 5B). These results demonstrate that the catalase remains active in the protein film and can effectively convert the chemical energy into an electrical output.

Figure 5. A) Electric energy output of bioinorganic generators with soluble and immobilized enzyme. In all cases 30 μ g of soluble or immobilized catalase on SiO₂ coated

piezoelectric surface. 710 μg of CTPR protein were used when immobilized catalase. Reaction mixture consists in 0.4 mL chamber volume at 750 mM H_2O_2 in 50 mM sodium phosphate at pH 7. B) Reusability of bioinorganic generators with immobilized enzyme. 710 μg of CTPR protein were used to immobilized catalase. Reaction mixture consists in 0.4 mL chamber volume at 50 mM H_2O_2 in 50 mM sodium phosphate at pH 7.0, for SiO_2 . C) Effect of reaction volume in the produced electric energy of bioinorganic generators with soluble and immobilized enzyme. In all cases 30 μg of soluble or immobilized catalase on SiO_2 coated piezoelectric surface. 710 μg of CTPR protein were used when immobilized catalase. Reaction mixture consists in 750 mM or 50 mM H_2O_2 in 50 mM sodium phosphate at pH 7.0 when immobilized or soluble enzymes were used, respectively.

Figure 6. A) Effect of different immobilized enzyme loads in the produced electric energy of bioinorganic generators with immobilized enzyme. In all cases SiO_2 coated piezoelectric surface was employed. 710 μg of CTPR protein were used when immobilized catalase. Reaction mixture consists in 1.5 mL of 50 mM H_2O_2 in 50 mM sodium phosphate at pH 7. B) Effect of hydrogen peroxide concentration in the produced electric energy by bioinorganic generators with immobilized enzyme. In all cases SiO_2 coated piezoelectric surface was employed. 710 μg of CTPR protein were used when immobilized catalase (30 μg). Reaction mixture consists in 1.5 mL of hydrogen peroxide at the indicated concentration in 50 mM sodium phosphate at pH 7.

Although the bioinorganic generator functionalized with the entrapped CAT generates 3 times less energy than the previously reported system using the soluble catalase with 0.2 mL reaction volume, this effect is ameliorated when the reaction volume in the chamber increases (Figure 5C). Similar electric power output responses are reached when working with 1 mL (5 nJ x cm⁻²), while the immobilized enzyme using 1.5 mL reaction volume was able to produce 2 times more energy than the soluble enzyme working under the same conditions. This effect suggests that the immobilized CAT is consuming all fuel and therefore generating all the bubbles (mechanical energy) at the

interface with the piezoelectric surface, maximizing the energy harvesting. This fact explains that the more fuel (the larger reaction volume), the higher electrical output. On the contrary when the catalase is freely diffusing through the reaction volume, part of the mechanical energy is not harvested because the vast majority of the bubbles are produced far from the piezoelectric surface. This effect explains the observed plateau for the energy generation at increased reaction volumes using the soluble enzyme. Therefore, future bioinorganic generator designs must contemplate major enzyme-piezoelectric component contact across the entire chamber in order to maximize the harvesting of mechanical energy. To optimize these new generation of bio-inorganic generators, different CAT concentrations were entrapped into CTPR protein films and casted on the piezoelectric surface (Figure 6A). Bioinorganic generators with immobilized catalase generate higher electric power outputs as the immobilize catalase load increases until reaching saturation conditions at 60 μg of immobilized catalase. Finally, we studied the response of bio-inorganic generators (with 30 μg of immobilized CAT) for different H_2O_2 concentrations. Figure 6B shows that the larger H_2O_2 concentration, the larger the energy output. The maximum electrical output was found at 750 mM, meaning that performance of the system is optimal under that conditions generating 76 nJ x cm^{-2} as maximum energy.

Conclusions

In summary, we report a simple methodology for the immobilization of enzymes in solid protein-based biomaterials that can be casted into devices like bio-inorganic generations for the electricity production. The developed methodology has been proven with CAT as model enzyme. Herein, we describe a simple protocol that involves only mixing and drop casting of the target enzyme with a scaffolding protein followed by a mild crosslinking to yield a robust and functional biomaterial. The biomaterial shows the same intrinsic properties of protein films generated only with the scaffolding protein.[27] Upon the complete fabrication process, the entrapped CAT maintained its functionality and displayed macroscopic order within the biomaterial. In fact, this functional biomaterial was active and successfully integrated into bio-inorganic generators that convert chemical energy into electricity. The device, as previously described using CAT in solution,[11] is based on the conversion of the chemical energy of the CAT reaction into mechanical energy associated to the production of oxygen bubbles and the downstream harvesting of this mechanical energy by a piezoelectric material to produce an electrical output as open circuit voltage. Here, we demonstrate the effective functionalization of the piezoelectric surface with biocatalytic material, advancing in the fabrication of bio-inorganic generators based on a novel concept that chemical energy transforms into electricity through mechanical energy harvesting. This biomaterial allows the reusability of the device otherwise impossible using the soluble enzyme, although the energy harvesting was not as efficient as the previously reported system with CAT in solution. In addition, the CAT load entrapped into the protein film is a crucial parameter to tune the electric power output of the bioinorganic generators herein developed. Remarkably, we found that the electric power output of bio-inorganic generators with immobilized CAT was maximized by increasing the reaction volume without reaching a saturation point, unlike the system using the soluble CAT that became saturated with sub-milliliters reaction volumes. We envision the potential of this technology to advance the fabrication of more robust bio-inorganic generators where

the bioactive phase is in close contact with the piezoelectric transducer. Furthermore, the application of the described biocatalytic protein-based biomaterials can be expanded to the heterogeneous biocatalysis field to improve chemical manufacturing as well to the biosensing field to develop more sensitive devices.

Experimental Section

Materials: Reagents and substrates including the hydrogen peroxide and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS), and the enzymes catalase (CAT) from bovine liver and horseradish peroxidase (HRP) were purchased from Sigma-Aldrich (St. Louis, MO). The piezoelectric material (PZT disc) (diaphragm, 6.3 kHz, 1 K Ω , 0.01 μ F, 20 mm $\times$ 0.42 mm, cat. 7BB-20-6I0, Murata) was acquired from Farnell Element14 Components (Barcelona, Spain).

Protein expression and purification: The gene encoding CTPR10 protein was previously generated based on a consensus CTPR protein and cloned into the pPROEX-HTa vector for the expression as a His-tag fusion for affinity purification.[41,42] The protein was expressed and purified as described previously.[23] Briefly, the plasmid was transformed into BL21 (DE3) Escherichia coli and the cells were grown in LB with 0.1 mg/ml of ampicillin in agitation to an O.D. between 0.6 and 0.8. Protein expression was induced with 0.6 mM IPTG for 5 hours at 30°C, then the cells were centrifuged at 4500 rpm and resuspended in 300 mM NaCl, 50 mM Tris pH=8.0 Lysis buffer with 1 mg/ml of lysozyme, 5 mM β -mercaptoethanol, and 15 μ l DNase stock solution. The resulting lysate was sonicated during 5 minutes with 30 second intervals and 40% of amplitude, and centrifuged at 10000 rpm for 45 min. Protein purification was performed by affinity chromatography using Ni²⁺ His-TrapTM column. The eluted protein was dialyzed overnight in PBS (150 mM NaCl, 50 mM phosphate buffer pH=7.4 with 2.5 mM β -mercaptoethanol). The protein was concentrated and purified by FPLC gel filtration chromatography over a Superdex 75 HiLoad column. Fractions containing the protein were analyzed in 15% acrylamide gels to confirm the purity of the protein. Finally, protein was concentrated until the desired concentration, from 50 to 400 μ M, using the estimated molar extinction coefficient at 280 nm from the amino acid composition.

Protein thin films fabrication: A 20 μ l protein solution of 400 μ M CTPR10 (18.1 mg/ml) and 4 μ M CAT (1 mg/ml) were deposited over a hydrophobic non-porose material by drop casting and left to dry for at least 4 hours at room temperature to ensure the formation of the protein thin film. A second approach to obtain thinner films was using spin-coating to deposit the films. In this case, a drop of 15 μ l of 400 μ M CTPR10 and 4 μ M CAT were deposited over a quartz slide of size 1x1 mm² by spin-coating method using a Laurell Technologies corporation Model WS-400B-6NPP/LITE, at 1000 rpm for 10 min with an acceleration of 3000 rpm/s. Stereomicroscope LEICA S8APO was used to image of the biomaterial at macroscopic level.

Scanning electron microscopy (SEM): SEM JEOL JSM-6490LV was used to image the surface of the protein thin film. The film was mounted over a carbon tape and imaged under vacuum conditions, applying an electron high tension (EHT) of 5.00 Kv WD of 2.5

mm and an aperture size of 15 μm . Sputter coating technique was performed in all samples using a metallization of Au/Pd alloy.

Circular dichroism: Circular dichroism (CD) was used to determine the secondary structure of the CTPR10 units within the films. CD was performed using a Jasco J-815 spectropolarimeter. The solid films were deposited on a sandwich quartz cuvette (0.1 mm path length) by spin coating. The CD spectra were acquired at 1 nm increments and 10 seconds average time over a wavelength range of 190 to 260 nm.

Crosslinking of the protein thin films: Glutaraldehyde (GA) at 1% was used as crosslinking agent for a gentle vapor diffusion crosslinking.[43,44] The reaction was carried out in wells of 1 ml for 24h at room temperature. At bottom of well 500 μl glutaraldehyde solution were added while the protein film was fixed on the cover slip used to seal the well. After the reaction, the films were recovered and, in order to evaluate the cross-linking efficiency, dipped into water solution to monitor the potential release into the solvent. The biomaterial was dipped into a water solution for different times, 1h, 24 h, and one week and the amount of protein in the supernatant was quantified at each time by Bradford assay using the spectrophotometer Varioskan Flash spectral scanning multimode reader (Thermo Scientific).

Distribution of CAT in thin films by fluorescence imaging and fluorescence anisotropy: CAT was fluorescently labeled using rhodamine B isothiocyanate (Sigma-Aldrich). The labeling reaction was performed for 24 h at 37°C under constant shaking. The enzyme was purified from the free dye by a NAP-5 column (GE Healthcare) and concentrated with Amicon® ultra-0.5 ml centrifugal filter MWCO 10K (Merck Millipore). The enzyme concentration was calculated measuring the absorbance at 280 nm ($\epsilon_{280\text{nm}} = 246000 \text{ M}^{-1} \text{ cm}^{-1}$) using a UV-vis spectrophotometer (ThermoFisher). Fluorescence confocal microscope (Zeiss NLO 880) was performed to determine the homogeneity of the biomaterial CAT-rhodamine labeled. The image was acquired using an excitation wavelength of 550 nm and with a magnification of 20x. The z-stack for the 3D reconstruction was acquired with 40x oil objective using an excitation of 561nm and emission between 565-700 nm, 40 μm Z-slides and an Airyscan detector at maximum intensity projection and 512x512 frames. Fluorescence anisotropy of the solid film was measured using a fluorimeter Perkin Elmer (LS55) with automated polarizers. A film of CTPR10 and CAT labeled with rhodamine was used and the polarization was determined using excitation and emission wavelengths of 550 and 572 nm, respectively.

Enzymatic activity measurements: The catalase activity either in solution or in the thin protein film was indirectly analyzed by quantifying the amount of hydrogen peroxide produced along the time. Samples of the catalase reaction were withdrawn at different times and incubated with peroxidase and ABTS ($\epsilon_{420} = 36000 \text{ M}^{-1} \text{ x cm}^{-1}$) for a fixed time. Herein, the peroxidase (HRP) uses the catalase-produced H_2O_2 to oxidize ABTS increasing the absorbance of the reaction mixture at 420 nm. The increase in absorbance was measured using a Varioskan Flash spectral scanning multimode reader (Thermo Scientific) and quantified to determine the H_2O_2 concentration upon the catalase action. For the soluble enzyme, 20 μl of the enzyme at 0.01 mg/ml was mixed

980 μ l of substrate solution (35 mM H₂O₂ in 100 mM phosphate pH 7.4), aliquots of 50 μ l was collected at different times and heated at 90°C for 2 min in order to inactivate the enzyme. Upon enzyme inactivation, 20 μ l of each sample were mixed with 200 μ l of 0.066 mg/ml ABTS and 0.013 mg/ml HRP in 100 mM phosphate pH 7.4 at room temperature and incubated for 5 min. In the case of the immobilized catalase, a sample of supernatant (20 μ l) from the film incubated with substrate solution was mixed with 200 μ l of 0.066 mg/ml ABTS and 0.013 mg/ml HRP in 100 mM phosphate pH 7.4 at room temperature and incubated for 5 min. The hydrogen peroxide consumed in the reaction was calculated using calibration curve. The specific activities of both in solution and entrapped CAT were determined from the time-course curves obtained through monitoring the H₂O₂ concentration along the time (Figure S3). The initial reaction rates were obtained from the slope of the curve, considering a zero-order kinetic, and converted to specific activity considering the total amount of enzyme tested. The kinetic parameters of the immobilized and in solution catalase were determined by measuring the reaction rates at various H₂O₂ substrate concentrations ranging from 0.25 to 168 mM at pH of 7.4, while keeping the amount of enzyme constant. The kinetic parameters K_m and V_{max} were calculated from Michaelis-Menten fit. To determine the thermal stability of the enzymatic activity, incubations at 50°C at different times were carried out to measure the decay of activity. The activity of each sample withdrawn at each inactivation time was measured through the HRP-coupled colorimetric assay described above and fixing the catalase reaction time in 15 and 3 minutes for the immobilized and soluble enzyme, respectively.

Electric Energy Output Measurements: SiO₂ thin films were deposited with nonreactive and reactive magnetron sputtering on PZT disc (1.25 cm² active piezoelectric surface) using an AJA-ATC 1800 system with a base pressure of 10⁻⁷ Pa. The deposition of the films was done with three separate 2 inch elemental targets, with a purity of 99.999% for carbon (Demaco-Holland), 99.95% for Nb (AJA International-USA) and for SiO₂, in a confocal configuration at a pressure of 0.25 Pa of pure Ar. The substrate bias voltage and substrate holder heating facility were turned off during depositions; the distance between target and substrates was about 15 cm. Prior to deposition, the substrates were sputter-cleaned with a negative bias of 180 V (25 W) in a 4 Pa Ar atmosphere for 10 min. SiO₂ films were deposited in an Ar/O₂ atmosphere (10 seem Ar + 20 seem O₂) at total pressure of 0.4 Pa and applying a d.c. power of 230 W to the Si target. CAT-protein thin films were deposited over the SiO₂ on the PZT surfaces and connected to an oscilloscope (Siglent model SHS806) to monitor the open-circuit voltage versus time as described before.[35] Different chamber volumes were obtained by varying the liquid fuel volume inside the open chamber (0.2, 0.4, 0.6, 1, 1.5 and 2 mL). When in solution enzyme was assessed, an enzyme solution that contained catalase in 50 mM sodium phosphate buffer at pH 7 was placed inside the chamber. The reaction was initiated by the addition of the fuel, H₂O₂ at the indicated concentrations. Before triggering the reaction, the system was equilibrated until the voltage signal reached 0 V. The energy of the electrical outputs according was calculated with eq. 1.[45]

$$E = \int_{t_1}^{t_2} \frac{V^2(t)}{R} dt$$

Where E , is the generated electrical energy and V is the generated voltage from the start (t_1) to the end (t_2) of a cycle at a constant resistance load (R). The R value was fixed to 60 M Ω (experimentally determined at the maximum produced voltage by the enzymes).

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