Biocatalytic Protein-Based Materials for Integration into Energy Devices

Daniel Sánchez-deAlcázar,[a] Susana Velasco-Lozano,[b] Nicoll Zeballos,[b] Fernando López-Gallego,[a,b, c] and Aitziber L. Cortajarena*[a, d]

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 for the incorporation of functionalities. Herein, the fabrication of catalytic protein thin films by the entrapment of catalase into protein films composed of a scaffolding protein is demonstrated. Extensive structural and functional characterization of the films provides evidence of the structural integrity, order, stability, catalytic activity, and reusability of the biocatalytic materials. Finally, these functional biomaterials are coupled with piezoelectric disks to fabricate a second generation of bio-inorganic generators. These devices are capable of producing electricity from renewable fuels through catalase-driven gas production that mechanically stimulates the piezoelectric material.

Introduction

Extensive efforts are currently being made in the 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 applications.[1, 5–7] These strategies include bottom-up approaches for the generation of supramolecular biomaterials,[5] with particular efforts devoted to protein-based materials due to the amazing structural and functional diversity of these biomolecules.[6–9]

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, to date, including adsorption,[11] covalent linkage,[12] affinity immobilization,[13] entrapment,[14] encapsulation,[15] and enzyme crosslinking.[16] Among them, simple entrapment offers easy fabrication and handling, and endows high stability to the immobilized enzymes.[17] Regarding the support, the use of natural materials as scaffolds provides advantages of biocompatibility, chemical versatility, and biodegradability that organic or inorganic support materials do not offer. In this regard, proteins play a key role because they fulfill all of these characteristics and their use for the fabrication of protein-based materials has already been demonstrated.[8, 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. Few examples of enzymes immobilized on protein-based materials have been successfully reported to date.[19]

Several scaffolding proteins can be used for the fabrication of biomaterials. However, the engineering of proteins provides clear advantages in terms of tunability, reproducibility, scalability, and rational design of the final properties of the material.[5] Modular building blocks with simple interactions allow for better control of the final assemblies through the implementation of bottom-up approaches. Engineered repeat proteins present these inherent features, which makes them suitable candidates for use as scaffolding proteins.[20, 21] In particular, tettratricopeptide repeat (TPR) proteins have proven to be exceptional scaffolds because consensus tettratricopeptide repeat (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. CTPRs 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 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 bio-catalysts that can be readily integrated into devices.

Catalase (CAT) is an excellent candidate for entrapment into protein-based materials because it is extensively used for technological applications in which biocatalyst robustness is demanded. CAT catalyzes hydrogen peroxide disproportionation to release water and molecular oxygen as innocuous products.28 Such a catalytic reaction makes this enzyme industrially relevant for the development of biosensors,30,31 preventing food oxidation,28 removing hydrogen peroxide from residual water,30 and the intensification of oxidase-driven biotransformations.31,32 In a pioneering attempt to expand the applications of CAT, our group has recently exploited CAT in solution to produce bubbles upon H2O2 disproportionation as a source of mechanical energy, which is then harvested by coupling to piezoelectric materials to generate electricity.31 Because the described system integrates a soluble CAT that forms 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 upon exposure 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 continuous operation of the system. In this emerging filed, enzymes could play a key role because they are able to catalyze reactions, the byproduct of which may be used as a source of mechanical energy. However, the coupling of enzymatic systems and nanogenerators would require automatic, independent, and reusable systems to minimize costs and enhance 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; thus overcoming the major limitations of the current system for energy harvesting and ease of automation and integration of this bio-inorganic generator into more complex devices. Although enzyme immobilization is often considered to be one of the most effective methods to increase the robustness of the biocatalyst, preservation of the enzymatic activity in 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 dynamic 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 should be optimized according to their final use, and thus, require protocols that maintain the activity and increase the stability relative of the enzyme in solution.

To advance the fabrication of novel bio-inorganic generators based on CAT, herein we describe 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 is possible because the protein environment of the CTPR scaffolding protein forming the biomaterial stabilizes entrapped CAT, as demonstrated by thermal inactivation studies. The solid self-standing biomaterial with CAT activity was casted into a piezoelectric disk and proven to produce electricity from H2O2. Although entrapped CAT has a lower apparent catalytic efficiency than that of the enzyme in solution, the proximity of the bubbling enzyme to the piezoelectric surface improves the energy outcome in large operational volumes compared with the system involving use of the enzyme in solution.

Results and Discussion

Protein-based biomaterial fabrication and characterization

Herein, we report on the fabrication of a bioactive material based on the self-assembly of CTPR proteins, which irreversibly entraps CAT as a functional unit, and casting onto piezoelectric materials to give rise to a new generation of energy-harvesting
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, fabrication of the protein thin film requires 3% (w/v) protein concentration of a CTPR10 variant (a CTPR protein with 10 identical repeats) to ensure appropriate packing and handling of the resulting film. The biocatalytic protein film was prepared under these conditions by homogeneously mixing CTPR10 and CAT in selected ratios, and then casting the film through two different strategies: spin-coating on a piece of quartz, or drop casting on a hydrophobic material (see the Experimental Section for further details; Figure 1A,B). 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 μm for these fabrication conditions (Figure 1C and Figure S1 in the Supporting Information). As expected, the functionalized protein film showed a peak at around 400 nm that corresponded to the maximum absorption of the heme group of CAT, which resulted in the light green color of the film (Figure 1D). The CD spectrum of the protein film entrapping CAT shows a characteristic signal of the α-helical structure of the scaffolding protein that agrees with results previously observed by our group for which the secondary structure of CTPR10 is preserved. CD data only reveal the secondary structure of the CTPR because the protein stoichiometry in the film is 1:100 CAT/CTPR; thus the contribution of CAT to the CD spectrum is negligible (Figure 1E).

It is known that CTPR proteins present intrinsic properties to form nanostructured ordered films because they macroscopically align through head-to-tail and side-to-side interactions. 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 first labeling the amine groups of CAT with the fluorescent dye rhodamine (Rh). Using confocal fluorescence microscopy, we demonstrated that entrapped CAT was homogeneously distributed across the protein film (Figure 2A) and throughout the film thickness (Figures 2B, S2, and S3). To show the tumbling freedom and potential order of CAT in the biomaterial (Table 1), fluorescence anisotropy was measured and
it was observed that the CAT–Rh conjugate in solution showed a higher anisotropy than that of free Rh, as expected from its larger mass. Interestingly, Rh-labeled CAT entrapped in the solid protein film presented a large anisotropy value, which indicated that the confined enzyme was significantly less mobile than that of its counterpart in solution. Next, the organization of CAT within the protein film was determined by measuring the fluorescence of the Rh-labeled enzyme with plane polarized light. The fluorescence intensity changed as 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; this order is imposed by the anisotropic arrangement of the CTPR scaffolding protein (Figure 2B).

The ordered self-assembled material, as described above, is soluble in aqueous media because it is formed mainly due to noncovalent interactions between CTPR proteins, which are too weak to maintain the integrity of the macrostructure. For operational purposes, the disassembly of the film and leaching of the entrapped protein need to be minimized. Therefore, the biomaterial was crosslinked with 1% glutaraldehyde (GA) for 24 h by means of vapor diffusion to result in a material that was stable in water. Upon crosslinking, the biomaterial was immersed in a buffer solution and showed no leaching of the protein into the medium; more than 95% protein was retained 7 days after immersion, which indicated effective crosslinking (Figure 3A). Moreover, the mild crosslinking reaction did not affect the structural properties of the protein, as shown in the CD spectra; thus the material is expected to preserve its activity after the reaction (Figure 3B).

Table 1. Fluorescence anisotropy measurements of CAT in solution and in solid film.

| Sample                | Anisotropy ± standard error of the mean |
|-----------------------|----------------------------------------|
| Rh control            | 0.052 ± 0.006                          |
| CAT–Rh in solution    | 0.166 ± 0.003                          |
| CAT–Rh film           | 0.819 ± 0.001                          |

Activity and stability of CAT entrapped in 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 by using peroxidase to determine the concentration of H$_2$O$_2$ that remains after the action of CAT.

The enzymatic activity was assayed at room temperature for the biomaterial and the enzymatic solution under the same conditions. Swelling of the film was observed upon immersion into the aqueous solution: a 0.5 mg film absorbed approximately 1 μL of water. The apparent specific activity of entrapped CAT was 0.08 U mg$^{-1}$, compared with 6.7 U mg$^{-1}$ observed for the enzyme in solution under the same experimental conditions. Moreover, the activity of films with different thicknesses was evaluated at a constant CTPR/CAT ratio. These results showed that the total CAT activity was maximized at a standard thickness of 20 μm; 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 with the values calculated for the soluble enzyme under the same conditions. Table 2 shows that the apparent $K_M$ and $k_{cat}$ for entrapped CAT towards H$_2$O$_2$ were 3.6-fold higher and 370 times lower than those for the soluble enzyme; thus confinement and crosslinking of CAT within the protein biofilm decreased the catalytic efficiency by...
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 \( K_a \) 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) enzyme entrapment in the compact protein film, which makes it less accessible to the medium;\(^{37}\) and/or 2) bubbles generated on the surface of the material by oxygen released during the reaction hamper substrate diffusion.\(^{38}\) These bubbles grow over the surface and, due to the hydrophilic nature of the protein material, stay at the film–solution interphase; thus hampering the diffusion of the substrate. In addition, this change in activity could be partially attributed to the loss of catalytic activity due to immobilization and crosslinking processes. Notably, the time-course curves show a lag time of around 400–500 s if CAT is entrapped, whereas that lag is not appreciable in the time course of the soluble enzyme (Figures S6 and S7). Mass transfer issues are also supported by loading experiments, in which the specific activity of the entrapped enzyme linearly increases with the increase in the ratio of CTPR/CAT (Figure S8). Total apparent activities of films with different ratios are similar, but the specific activity of films with higher CAT loadings is dramatically reduced. It seems that, under high CAT loading conditions, substrate molecules cannot reach all entrapped enzyme molecules due to transport limitations, rather than conformational changes related to entrapment or crosslinking. A population of inaccessible enzyme molecules may explain the low catalytic efficiency observed for entrapped CAT. Lower CAT loadings ameliorate this effect because a larger enzyme population actively participates in \( \text{H}_2\text{O}_2 \) disproportionation because of improved substrate accessibility. These diffusional restrictions have also been observed if CAT is randomly aggregated and irreversibly crosslinked in the presence of a feeder protein through crosslinked enzyme aggregate (CLEA) technology.\(^{39}\) Unlike 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 by using scaffolding proteins.

Despite exhibiting low catalytic efficiency, entrapped CAT is still active enough for further applications; thus 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 entrapped CAT in comparison with the soluble enzyme. Figure 4 shows that immobilized CAT was significantly more thermostable than its soluble counterpart because it retained approximately 100% of its initial activity after 3 h incubation at 50 °C, whereas the soluble enzyme lost 70% of its initial activity under the same inactivation conditions, which represents a stabilization factor of 3.5. According to other immobilization protocols, CAT could be stabilized by entrapment in CTPR protein thin films; thus demonstrating its potential as a heterogeneous biocatalyst that can be reused for several operational cycles.\(^{40}\)

### Energy output measurements

Recently, our group described the production of electrical energy from chemical energy by using bio-inorganic generators.\(^{35}\) Such devices are able to convert chemical energy stored in renewable molecules (as carbohydrates, amino acids, alcohols, etc.) into mechanical energy (in the form of gas bubbles), which are further harvested by a piezoelectric material based on a lead zirconate titanate (PZT) disk and transduced into open-circuit voltages. That energy output was monitored in line by using an oscilloscope. This concept has been proven with soluble CAT, which disproportionates hydrogen peroxide into oxygen and water, forming oxygen bubbles that trigger a mechanical stimulus for further harvesting by the piezoelectric material. The drawback of the reported system is its disposability, which prevents enzyme reutilization after one energy-generation cycle. To make these systems reusable, protein films entrapping CAT were cast on the piezoelectric surface coated with a nanometer-thick inorganic layer, with the aim of fabricating bio-inorganic generators that could be repeatedly used for the continuous production of electrical energy to achieve a more sustainable process.

Hence, we entrapped soluble CAT into a CTPR film that was subsequently crosslinked with GA, as above described. Primarily, we assessed the effect of coating of the piezoelectric surface with a CTPR protein layer on the energy outcome. Using the soluble enzyme as a catalyst, we observed that the energy generated was reduced by only 25% with regard to the naked piezoelectric surface; thus demonstrating that the biomaterial...

---

**Table 2.** Kinetic parameters of CAT in solution and in the solid protein thin film. \(^*\): apparent parameters.

| CAT sample | \( K_a \) [mm] | \( V_{\text{max}} \) [mm s\(^{-1}\)] | \( k_{\text{cat}} \) [s\(^{-1}\)] | \( k_{\text{cat}}/K_a \) [s\(^{-1}\)mm\(^{-1}\)] |
|------------|----------------|------------------|------------------|------------------|
| in solution | 84.02 ± 31.46 | 10.21 ± 2.74     | 2.55 \( \times \) 10\(^{-7}\) ± 0.68 \( \times \) 10\(^{-7}\) | 3.04 \( \times \) 10\(^{-7}\) ± 1.40 \( \times \) 10\(^{-7}\) |
| in film    | 301.89 ± 175.12\(^*\) | 0.24 ± 0.10\(^*\) | 6000 ± 2500\(^*\) | 19.87 ± 14.19\(^*\) |

---

**Figure 4.** Enzymatic thermal stability of CAT in a solid thin film (■) and in solution (●).  
Relative specific activity / %

Heating time / min

0  30  60  90  150  180

---

ChemBioChem 2019, 20, 1977 – 1985  www.chembiochem.org 1981 © 2019 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim
Figure 5. A) Electrical energy output of bio-inorganic generators with soluble and immobilized enzyme. In all cases, 30 μg of soluble or immobilized CAT on a SiO₂-coated piezoelectric surface. 710 μg of CTPR protein were used to immobilize CAT. The reaction mixture consisted of a 0.4 mL chamber volume at 750 mM H₂O₂ in 50 mM sodium phosphate at pH 7. B) Reusability of bio-inorganic generators with immobilized enzyme. 710 μg of CTPR protein were used to immobilize CAT. The reaction mixture consisted of a 0.4 mL chamber volume at 50 mM H₂O₂ in 50 mM sodium phosphate at pH 7.0. C) Effect of reaction volume on the electrical energy produced from bio-inorganic generators with soluble (●) and immobilized (●) enzyme. In all cases, 30 μg of soluble or immobilized CAT on SiO₂-coated piezoelectric surface. 710 μg of CTPR protein were used for immobilized CAT. The reaction mixture consisted of 750 or 50 mM H₂O₂ in 50 mM sodium phosphate at pH 7.0 if immobilized or soluble enzymes were used, respectively.

Although the bio-inorganic generator functionalized with entrapped CAT generates three times less energy than that of the previously reported system with soluble CAT in a reaction volume of 0.2 mL, this effect is ameliorated if the reaction volume in the chamber is increased (Figure 5C). Similar electrical power output responses are reached upon working with 1 mL (5 nJ cm⁻²), whereas for the immobilized enzyme in a reaction volume of 1.5 mL, twice as much energy was generated than that with the soluble enzyme working under the same conditions. This effect suggests that immobilized CAT consumes all fuel, and therefore, generates all of the bubbles (mechanical energy) at the interface with the piezoelectric surface; thus maximizing energy harvesting. This fact explains why more fuel (larger reaction volume) results in a higher electrical output. On the contrary, if CAT 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 energy generation at increased reaction volumes for the soluble enzyme. Therefore, future bio-inorganic generator designs must contemplate major enzyme–piezoelectric component contact across the entire chamber to maximize harvesting of mechanical energy. To optimize this new generation of bio-inorganic generators, different CAT concentrations were entrapped into CTPR protein films and casted on the piezoelectric surface (Figure 6A). Bio-inorganic generators with immobilized CAT generate higher electric power outputs as the immobilized CAT loading increases until reaching saturation conditions at 60 μg of immobilized CAT. Finally, we studied the response of bio-inorganic generators (with 30 μg of immobilized CAT) for different H₂O₂ concentrations. Figure 6B shows that a higher H₂O₂ concentration resulted in a greater energy output.

Figure 6. A) Effect of different immobilized enzyme loadings on the production of electrical energy in bio-inorganic generators with immobilized enzyme. In all cases, a SiO₂-coated piezoelectric surface was employed. 710 μg of CTPR protein were used for immobilized CAT. The reaction mixture consists of 1.5 mL of 50 mM H₂O₂ in 50 mM sodium phosphate at pH 7. B) Effect of hydrogen peroxide concentration on the production of electrical energy by bio-inorganic generators with immobilized enzyme. In all cases, a SiO₂-coated piezoelectric surface was employed. 710 μg of CTPR protein were used for immobilized CAT (30 μg). The reaction mixture consisted of 1.5 mL of hydrogen peroxide at the indicated concentration in 50 mM sodium phosphate at pH 7.
output. The maximum electrical output was found at 750 mm H$_2$O$_2$; this means that the performance of the system is optimal under those conditions which generate a maximum energy of 76 nJ cm$^{-2}$.

**Conclusion**

We have reported a simple methodology for the immobilization of enzymes in solid protein-based biomaterials that could be cast into devices, such as bio-inorganic generators for electricity production. The developed methodology has been proven with CAT as a model enzyme. Herein, we described a simple protocol that involved only mixing and drop casting of the target enzyme with a scaffolding protein, followed by a mild crosslinking procedure, to yield a robust and functional biomaterial. The biomaterial showed the same intrinsic properties as those of protein films generated only with the scaffolding protein.[27] Upon completion of the fabrication process, entrapped CAT maintained its functionality and displayed macroscopic order within the biomaterial. This functional biomaterial was active and successfully integrated into bio-inorganic generators that converted chemical energy into electricity. The device, as previously described for CAT in solution,[28] was based on the conversion of chemical energy from the reaction catalyzed by CAT into mechanical energy associated with the production of oxygen bubbles and the downstream harvesting of this mechanical energy by a piezoelectric material to produce an electrical output as an open-circuit voltage. We have demonstrated the effective functionalization of the piezoelectric surface with a biocatalytic material to advance the fabrication of bio-inorganic generators based on a novel concept in which chemical energy was transformed into electricity through mechanical energy harvesting. This biomaterial allowed the reusability of the device, which was otherwise impossible for the soluble enzyme, although energy harvesting was not as efficient as that of previously reported system with CAT in solution. In addition, the amount of CAT entrapped in the protein film was a crucial parameter to tune the electrical power output of the bio-inorganic generators developed herein. Remarkably, we found that the electrical power output of bio-inorganic generators with immobilized CAT was maximized upon increasing the reaction volume, without reaching a saturation point, unlike the system with soluble CAT, which became saturated with sub-milliliter reaction volumes. We envision the potential of this technology to advance the fabrication of more robust bio-inorganic generators in which 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 field of heterogeneous biocatalysis to improve chemical manufacturing, as well as to the field of biosensing to develop more sensitive devices.

**Experimental Section**

**Materials:** Reagents and substrates, including hydrogen peroxide, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS), and the enzymes CAT from bovine liver and horseradish peroxidase (HRP), were purchased from Sigma–Aldrich. The piezoelectric material (PZT disk; diaphragm, 6.3 kHz, 1 KΩ, 0.01 μF, 20 mm × 0.42 mm, cat. 78B-20-6l0, Murata) was acquired from Farnell Element14 Components (Barcelona, Spain).

**Protein expression and purification:** The gene encoding CTPTR10 protein was previously generated based on a CTTPR protein and cloned into the pPROEX-HTa vector for expression as a His-tag fusion protein for affinity purification.[41,42] The protein was expressed and purified as described previously.[28] Briefly, the plasmid was transformed into BL21(DE3) Escherichia coli and the cells were grown in lysogeny broth with ampicillin (0.1 mg mL$^{-1}$) under agita- tion to an O.D. between 0.6 and 0.8. Protein expression was in- duced with isopropyl-[β]-D-thiogalactopyranoside (IPTG; 0.6 mM) for 5 h at 30 °C, then the cells were centrifuged at 5000g and re- suspended in 300 mM NaCl, Tris lysis buffer (50 mM, 15 μL, pH 8.0) with 1 mg mL$^{-1}$ of lysozyme, 5 mM β-mercaptoethanol, and DNase stock solution. The resulting lysate was sonicated for 5 min with 30 s intervals and 40% amplitude, and centrifuged at 10 000g for 45 min. Protein purification was performed by means of affinity chromatography by using a Ni$^{2+}$-His-Trap column. The eluted protein was dialyzed overnight in phosphate-buffered saline (PBS; 150 mM NaCl, 50 mM phosphate buffer pH 7.4 with 2.5 mM β-mercaptoethanol). The protein was concentrated and purified by means of 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, the protein was concentrated to the desired concentration, from 50 to 400 μM, by using the estimated molar extinction coefficient at λ = 280 nm from the amino acid composition.

**Protein thin-film fabrication:** A protein solution (20 μL) of CTPTR10 (400 μM; 18.1 mg mL$^{-1}$) and 4 μM CAT (1 mg mL$^{-1}$) was deposited over a hydrophobic nonporous material by means of drop casting and left to dry for at least 4 h at room temperature to ensure formation of the protein thin film. A second approach to obtain thinner films was to use spin-coating to deposit the films. In this case, a drop (15 μL) of a solution containing 400 μM CTPTR10 and 4 μM CAT was deposited on a quartz slide of 1 × 1 mm$^2$ through a spin-coating method by using a Laurell Technologies corporation model WS-4008-6NPP/LITE instrument, at 1000 rpm for 10 min with an acceleration of 3000 rpm s$^{-1}$. A LEICA SBAPO stereomicroscope was used to image the biomaterial at the macroscopic level.

**SEM imaging:** A JEOL JSM-6490LV scanning electron microscope was used to image the surface of the protein thin film. The film was mounted on a carbon tape and imaged under vacuum conditions by applying an electron high tension (EHT) of 5.00 kV, working distance (WD) of 2.5 mm, and an aperture size of 15 μm. Sputter coating was performed on all samples by using a metallization of Au/Pd alloy.

**CD measurements:** CD was used to determine the secondary structure of the CTPTR10 units within the films by using a Jasco J-815 spectropolarimeter. Solid films were deposited on a sandwich quartz cuvette (0.1 mm path length) through spin coating. CD spectra were acquired at 1 nm increments and 10 s average time over a wavelength range of 190 to 260 nm.

**Crosslinking of the protein thin films:** GA at a concentration of 1% was used as crosslinking agent for gentle vapor diffusion crosslinking.[43,44] The reaction was performed in wells of 1 mL for 24 h at room temperature. At the bottom of each well, a solution of GA (500 μL) was added and the protein film was fixed on the coverslip used to seal the well. After the reaction, the films were recovered.
and, to evaluate the crosslinking efficiency, dipped into an aqueous solution to monitor potential release into the solvent. The biomaterial was dipped into an aqueous solution for different times of 1 h, 24 h, and one week, and the amount of protein in the supernatant was quantified for each time by using the Bradford assay on a Varioskan Flash spectrophotometer with a scanning multimode reader (Thermo Scientific).

Distribution of CAT in thin films determined by means of fluorescence imaging and fluorescence anisotropy: CAT was fluorescently labeled with Rh 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 using a NAP-5 column (GE Healthcare) and concentrated with an Amicon ultra-0.5 mL centrifugal filter MWCO 10 K (Merck Millipore). The enzyme concentration was calculated by measuring the absorbance at λ = 280 nm (ε_{280 nm} = 246 000 M⁻¹ cm⁻¹) on a UV/Vis spectrophotometer (Thermo Fisher). Fluorescence confocal microscopy (Zeiss NLO 880) was performed to determine the homogeneity of the CAT–Rh-labeled biomaterial. The image was acquired at an excitation wavelength of 550 nm with a magnification of 20×. The z stack for the 3D reconstruction was acquired with a 40× oil objective at an excitation wavelength of 561 nm and emission wavelengths between 565 and 700 nm, 40 μm Z-slides, and an Airyscan detector at maximum intensity projection and 512×512 frames. Fluorescence anisotropy of the solid film was measured by using a PerkinElmer (LS55) fluorimeter with automated polarizers. A film of CTPR10 and CAT labeled with Rh was used and polarization was determined at excitation and emission wavelengths of 550 and 572 nm, respectively.

Enzymatic activity measurements: The CAT activity either in solution or in the protein thin film was indirectly analyzed by quantifying the amount of hydrogen peroxide produced over time. Samples of the CAT reaction were withdrawn at different times and incubated with peroxidase and ABTS (ε = 36 000 M⁻¹ cm⁻¹) for a fixed time. HRP used the CAT-produced H₂O₂ to oxidize ABTS, which increased the absorbance of the reaction mixture at λ = 420 nm. The increase in absorbance was measured by using a Varioskan Flash spectral scanning multimode reader (Thermo Scientific) and quantified to determine the H₂O₂ concentration upon the action of CAT. For the soluble enzyme, a 0.01 mg mL⁻¹ solution of the enzyme (20 μL) was mixed with a solution of substrate (980 μL; 35 mM H₂O₂ in 100 mM phosphate pH 7.4); aliquots (50 μL) were collected at different times and heated at 90 °C for 2 min to inactivate the enzyme. Upon enzyme inactivation, each sample (20 μL) was mixed with ABTS (200 μL, 0.066 mg mL⁻¹) and HRP (200 μL, 0.013 mg mL⁻¹) in phosphate buffer (100 mM, pH 7.4) at room temperature and incubated for 5 min. In the case of immobilized CAT, a sample of supernatant (20 μL) from the film incubated with the substrate solution was mixed with ABTS (200 μL, 0.066 mg mL⁻¹) and HRP (0.013 mg mL⁻¹) in phosphate buffer (100 mM, pH 7.4) at room temperature and incubated for 5 min. Hydrogen peroxide consumed in the reaction was calculated by using a calibration curve. The specific activities of both CAT in solution and entrapped CAT were determined from the time-course curves obtained through monitoring the H₂O₂ concentration over time (Figure S3). The initial reaction rates were obtained from the slope of the curve, considering zero-order kinetics, and converted into specific activity by considering the total amount of enzyme tested. The kinetic parameters of immobilized CAT and that in solution were determined by measuring the reaction rates at various H₂O₂ substrate concentrations, ranging from 0.25 to 168 mM at pH 7.4, while keeping the amount of enzyme constant. The kinetic parameters Kₘ and Vₘₘₐₓ were calculated from Michaelis–Menten fitting. To determine the thermal stability of the enzymatic activity, incubations at 50 °C for different times were performed to measure the decay in activity. The activity of each sample withdrawn at each inactivation time was measured through the HRP-coupled colorimetric assay described above, and fixing the CAT reaction times to 15 and 3 min for immobilized and soluble enzyme, respectively.

Electrical energy output measurements: SiO₂ thin films were deposited through nonreactive and reactive magnetron sputtering on PZT disk (1.25 cm² active piezoelectric surface) by using an AJA-ATC 1800 system with a base pressure of 10⁻¹ Pa. Deposition of the films was performed with three separate 2 inch elemental targets, with a purity of 99.999% for carbon (Demaco-Holland) and 99.95% for Nb (AJA International-USA) and 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 direct current (DC) power of 230 W to the Si target. CAT-embedded protein thin films were deposited on SiO₂ on the PZT surfaces and connected to an oscilloscope (Síglet model SHS806) to monitor the open-circuit voltage versus time, as described previously.[26] 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). To assess the enzyme in solution, a solution that contained CAT 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 corresponding energy of the electrical output was calculated by using Equation (1):[25]

\[
E = \int_0^t \frac{V(t)}{R} \, dt
\]

in which E is the generated electrical energy and V is the generated voltage from the start (t₁) to the end (t₂) of a cycle at a constant resistance load (R). The R value was fixed to 60 Ω (experimentally determined at the maximum produced voltage by the enzymes).

Acknowledgements

This work was partially supported by the European Research Council ERC-CoG-68071-ProNANO (ALC), the Spanish Ministry of Economy and Competitiveness (BIO2016-77367-R) (ALC); PCI2018-092894, and the Basque Government (Elkartek KK-2017/00008). We thank Drs. Irantzu Llarena and Luis Yate for support with fluorescence microscopy and sputtering experiments, respectively. This work was performed under the Maria de Maeztu Units of Excellence Program from the Spanish State Research Agency—grant no. MDM-2017-0720 (CIC biomaGUNE). We also acknowledge funding from the Fundación Hergar (FLG), Foundation ARAID, and Aragon Government (E37-17R) for funding F.L.G., and Marie-Curie Actions (NANOBIER project) for funding S.V.L.
Conflict of Interest

The authors declare no conflict of interest.

Keywords: bio-inorganic generators • enzymes • immobilization • proteins • thin films

[1] Z. Liu, Z. Zhang, R. O. Ritchie, Adv. Mater. 2018, 30, 1705220.
[2] C. Ezquerro, E. Fresta, E. Serrano, E. Lalinde, J. Garcia-Martinez, J. R. Berenguer, R. D. Costa, Mater Horiz 2019, 6, 130 – 136.
[3] A. J. C. Kuehne, M. C. Gather, Chem. Rev. 2016, 116, 12823 – 12864.
[4] I. Meglinski, Biophotonics for Medical Applications, Woodhead, Cambridge, 2015.
[5] N. C. Abascal, L. Regan, Open Biol. 2018, 8, 180113.
[6] Y. Sun, Z. Guo, Nanoscale Horiz. 2019, 4, 52 – 76.
[7] C. Zhang, D. A. Mcadams, J. C. Grunlan, Adv. Mater. 2016, 28, 6292 – 6321.
[8] M. J. Webber, E. A. Appel, E. W. Meijer, R. Langer, Nat. Mater. 2016, 15, 13 – 26.
[9] W. Huang, S. Ling, C. Li, F. G. Omenetto, D. L. Kaplan, Chem. Soc. Rev. 2018, 47, 6486 – 6504.
[10] J. Zdarta, A. Meyer, T. Jesionowski, M. Pinelo, Catalysts 2018, 8, 92.
[11] H.-T. Dang, Z.-K. Xu, Z.-M. Liu, J. Wu, P. Ye, Enzyme Microb. Technol. 2004, 35, 437 – 443.
[12] M. B. Méndez, C. W. Rivero, F. López-Gallego, J. M. Guisán, J. A. Treilles, J. Biotechnol. 2018, 270, 39 – 43.
[13] Q. Zhu, W. Zhuang, H. Niu, L. Ge, B. Villacorta Hernandez, J. Wu, K. Wang, D. Liu, Y. Chen, C. Zhu, H. Ying, Colloids Surfaces A 2018, 164, 155 – 164.
[14] R. L. de Oliveira, O. S. da Silva, A. Converti, T. S. Porto, Int. J. Biol. Macromol. 2018, 115, 1088 – 1093.
[15] S. Escolar, C. Bernal, J. M. Bolivar, B. Nidetzky, F. López-Gallego, M. Mesa, Mol. Catal. 2018, 449, 106 – 113.
[16] M. Mohy Eldin, C. G. P. Schroên, A. E. Janssen, D. Mart, J. Tramper, J. Mol. Catal. B 2000, 10, 445 – 451.
[17] A. G. Grigorov, Biochem. Eng. J. 2017, 117, 1 – 20.
[18] A. Hirtl, E. Schmich, J. A. Garrido, J. Hernando, S. C. R. Catharino, S. Walter, P. Feulner, A. Kromka, D. Steinmüller, M. Stutzmann, Nat. Mater. 2004, 3, 736 – 742.
[19] Q. Zhu, W. Zhuang, H. Niu, L. Ge, B. Villacorta Hernandez, J. Wu, K. Wang, D. Liu, Y. Chen, C. Zhu, H. Ying, Colloids Surfaces A 2018, 164, 155 – 164.
[20] Y. L. Boersma, A. Pluckthun, Curr. Opin. Biotechnol. 2011, 22, 849 – 857.
[21] T. Z. Grove, L. Regan, A. L. Cortajarena, J. Royal Soc. Interface 2013, 10, 20130051 – 20130051.
[22] T. Kajander, A. L. Cortajarena, S. Mochrie, L. Regan, Acta Crystallogr. Sect. D Biol. Crystallogr. 2007, 63, 800 – 811.
[23] A. L. Cortajarena, S. G. J. Mochrie, L. Regan, Protein Sci. 2011, 20, 1042 – 1047.
[24] S. H. Mejias, A. Aires, P. Couleaud, A. L. Cortajarena, Adv. Exp. Med. Biol. 2016, 940, 61 – 81.
[25] D. Romero, P. Couleaud, S. H. Mejias, A. Aires, A. L. Cortajarena, Biochem. Soc. Trans. 2015, 43, 825 – 831.
[26] S. H. Mejias, J. López-Andarias, T. Sakurai, S. Yoneda, K. P. Erazo, S. Seki, C. Atienza, N. Martin, A. L. Cortajarena, Chem. Sci. 2016, 7, 4842 – 4847.
[27] J. López-Andarias, S. H. Mejias, T. Sakurai, W. Matsuda, S. Seki, F. Feixas, S. Osuna, C. Atienza, N. Martin, A. L. Cortajarena, Adv. Funct. Mater. 2018, 28, 1704031.
[28] M. Alfonso-Prieto, X. Biarnés, P. Vidossich, C. Rovira, J. Am. Chem. Soc. 2009, 131, 11751 – 11761.
[29] K. K. Gaikwad, S. Singh, Y. S. Lee, Environ. Chem. Lett. 2018, 16, 523 – 538.
[30] J. Kaushal, S. Mehandia, G. Singh, A. Rina, S. K. Arya, Biocatal. Agricul. Biotechnol. 2018, 16, 192 – 199.
[31] J. M. Bolivar, S. Schelch, M. Pfeiffer, B. Nidetzky, J. Mol. Catal. B 2016, 134, 302 – 309.
[32] M. R. Chapman, S. C. Cosgrove, N. J. Turner, N. Kapur, A. J. Blacker, Angew. Chem. Int. Ed. 2018, 57, 10535 – 10539; Angew. Chem. 2018, 130, 10695 – 10699.
[33] R. C. Rodrigues, C. Ortiz, A. Berenguer-Murcia, R. Torres, R. Fernández-Lafuente, Chem. Soc. Rev. 2013, 42, 6290 – 6307.
[34] A. Starzyk, M. Cieplak, J. Chem. Phys. 2011, 135, 235103.
[35] S. Velasco-Lozano, M. Knez, F. López-Gallego, ACS Appl. Energy Mater. 2018, 1, 2032 – 2040.
[36] A. L. Cortajarena, J. Wang, L. Regan, FEBS J. 2010, 277, 1058 – 1066.
[37] C. Riccardi, S. McCormick, R. Kasi, C. Kumar, Angew. Chem. Int. Ed. 2018, 57, 10158 – 10162; Angew. Chem. 2018, 130, 10315 – 10319.
[38] G. Ozyilmaz, S. S. Tukel, O. Alptekin, Indian J. Biochem. Biophys. 2007, 44, 6.
[39] A. C. O. Mafra, W. Kopp, M. B. Beltrame, R. de Lima Camargo Giordano, M. F. de Arruda Ribeiro, P. W. Tardioli, J. Mol. Catal. B 2016, 132, 107 – 116.
[40] J. Kaushal, Seema, G. Singh, S. K. Arya, Biotechnol. Rep. 2018, 18, e00258.
[41] T. Kajander, A. L. Cortajarena, E. R. G. Main, S. G. J. Mochrie, L. Regan, J. Am. Chem. Soc. 2005, 127, 10188 – 10190.
[42] T. Kajander, A. L. Cortajarena, L. Regan, Methods Mol. Biol. 2006, 340, 151 – 170.
[43] A. G. Destaye, C.-K. Lin, C.-K. Lee, ACS Appl. Mater. Interfaces 2013, 5, 4745 – 4752.
[44] C. J. Lucky, J. Appl. Crystallogr. 1999, 32, 106 – 112.
[45] J. Briscoe, N. Jalali, P. Woolliams, M. Stewart, P. M. Weaver, M. Cain, S. Dunn, Energy Environ. Sci. 2013, 6, 3035.