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From Cytochrome *c* Crystals to a Solid-State Electron-Transfer Device[†]

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ABSTRACT: In this work, we performed the physical characterization of cytochrome *c* (transport properties and temperature dependency) by dynamic light scattering methods. Additionally, the crystallization of one of the isoforms of cytochrome *c* is presented. The electrochemical fluid cell of the atomic force microscopy (EC-AFM) and the polypyrrole films used as chemical glue allowed cytochrome *c* crystals to be fixed onto the surface of the working electrode (indium tin oxide, ITO). This also permitted development of a first prototype of a solid-state electron-transfer device based on biological crystals. Finally, using the electrochemistry modulus of the AFM, cyclic voltammetry techniques were performed to characterize the electron-transfer response on the surface of crystals of cytochrome *c* attached to the electrode. This result confirmed the successful electron-transfer process between the ITO electrode and the protein crystal attached to the working electrode by a film of a conducting polymer (ppy).

1. Introduction

The electrochemistry of redox proteins is now well established for a variety of applications.[1] When the chemical conditions are available, they allow electron-transfer reactions of all simple proteins to proceed rapidly and reversibly at the electrodes.[2,3] This has been important both in the development of biosensors and in the field of bioelectrochemistry, which are based on new detection principles.[4] The cytochrome *c* is one of the best-studied proteins in biochemistry. This is probably due to its electron-transfer properties involved in the respiration of living organisms.[5] Thanks to its red color, the purification is easier, particularly for the cytochrome *c* from bovine heart which is a small protein, soluble in different buffer solutions, and it is commercially available. In spite of being a relatively easy protein to manipulate from the biochemical point of view, its electron-transfer properties had to be studied in solution[6,7] and immobilized on the electrodes.[8] However, they have never being explored in solid-state (crystals). Our group has recently described the crystallization and the three-dimensional (3D) structure of this protein at high resolution.[9] The fixation of these crystals is a first step in the study of the electron-transfer phenomena of biological crystals of cytochrome *c*. The electron-transfer phenomena were performed via electrochemical methods coupled to atomic force microscopy (AFM).

The electrochemical oxidation of heterocyclic molecules such as pyrrole or thiophene generates conducting polymer deposits on the electrode.[10] This is a very accurate technique for controlling the thickness of the deposit. The majority of these polymers, obtained by electro-polymerization, show both good conductivity and chemical stability. This is important for technological applications.[11,12] Among these heterocyclic molecules, polypyrrole (ppy) has been used as a biocompatible conducting polymer in biosensors construction, which makes it a promising material for bioengineering.[13–15] Conducting polymers have been used as chemically conducting glue. For example, polythiophene is a transparent adhesive for a lamination process,[16] and ppy is able to immobilize catalase crystals on electrodes.[17]

2. Experimental Procedures

2.1. Dynamic Light Scattering. Cytochrome *c* was purified following the protocol previously described by Mirkin et al.[9] Its purity was initially checked by gel electrophoresis. This showed a MW of about 12.6 kDa verified by mass spectrometry methods. Additionally, the data of the hydrodynamic radius, the diffusion coefficient, and the molecular weight determination by total intensity light scattering (TILS) were obtained from the software DTS (Nano) (Malvern, Ltd.). A Malvern Nano S apparatus with a NIBS laser technology was used. To perform these studies, solutions of cytochrome *c* at different concentrations were prepared, both in distilled water and in phosphate buffer pH 7.0. The thermal stability of this protein and the precipitating agent were analyzed ranging from 5 to 37 °C in steps of 1 °C.

2.2. Cytochrome *c* Crystallization. The crystallization was prepared by using the hanging-drop vapor diffusion method in NEXTAL plates (Code NCP-24-100). The crystallization conditions and the 3D structure of this protein are those published by Mirkin et al.,[9] who crystallized the oxidized form of native cytochrome *c* (usually obtained in the presence of oxygen). The reservoir (1000 μL) contained 30% (w/v) PEG1000 in 50 mM sodium phosphate pH 7.0. Droplets of 4 μL (2 μL of protein + 2 μL of precipitant) containing 25 mg/mL of repurified cytochrome *c* with 25% (w/v) PEG-1000, both in the buffer phosphate pH 7.0, were incubated at 4 °C for 32 days. The first results were poorly shaped crystals that were used as microseeds and then transferred (by means of a cat whisker) to pre-equilibrated droplets containing 22 mg/mL of protein and 25% (w/v) PEG-1000 (both prepared in 50 mM buffer phosphate pH 7.0). After one week, several well-shaped single crystals were obtained reaching maximum dimensions of 0.25 × 0.1 × 0.1 mm.

2.3. Electrochemical Investigations. Cyclic voltammetry is the most widely used technique for acquiring qualitative information about electrochemical reactions. The power of cyclic voltammetry results from its ability to rapidly provide considerable information on the thermodynamics of redox processes, from the kinetics of heterogeneous electron-transfer reactions, and from coupled chemical reactions and adsorption processes.[18] Cyclic voltammetry is often the first experiment performed in an electroanalytical study. It particularly offers a rapid location of redox potentials of the electroactive species and convenient evaluation of the effect of media upon the redox process. The electrochemical experiments were done in an electrochemical fluid cell (50 μL) of Digital Instruments MultiMode scanning probe microscopy

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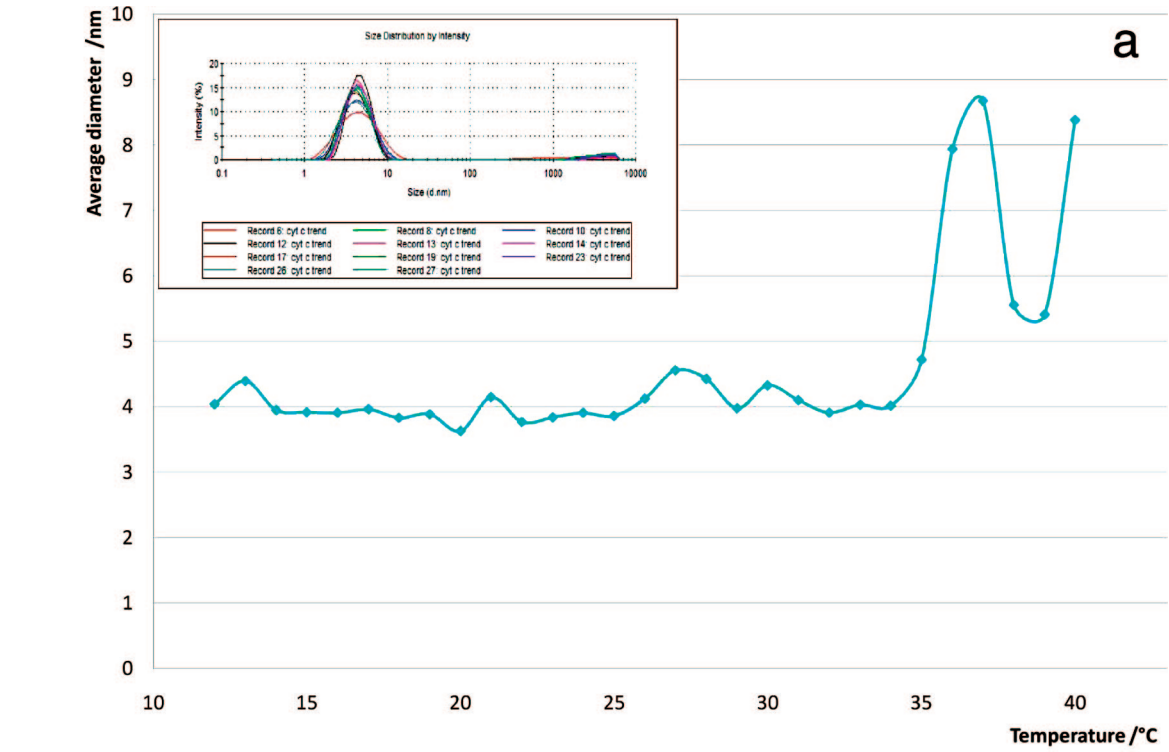

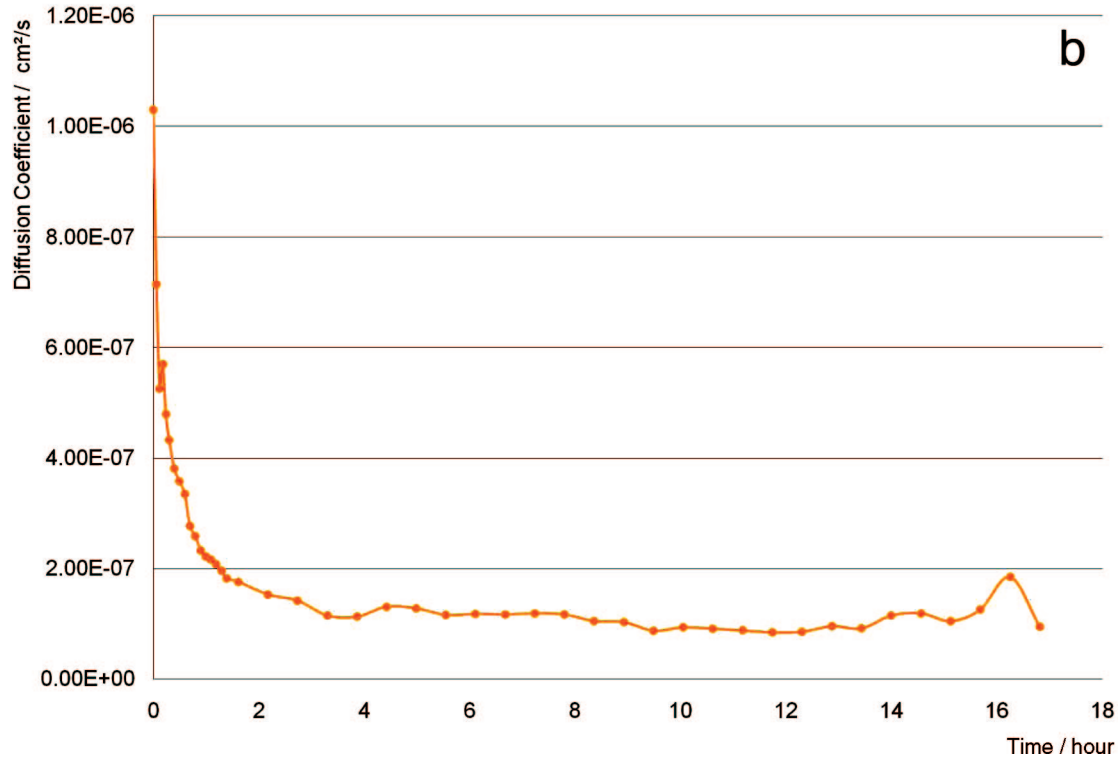

Figure 1. Hydrodynamic properties of cytochrome *c* from bovine heart: (a) average size distribution of the protein versus temperature (the inset shows the monodispersity of the protein); (b) diffusion coefficient trend when mixing protein/precipitating agent.

(SPM), (NanoScope IIIa from Veeco, formerly Digital Instruments, Santa Barbara USA) coupled with an electrochemical module (EC-AFM). The cyclic voltammetry characterization was performed with the potentiostat/galvanostat module of the AFM employing a triangular-shaped wave potential. In this configuration, a silver (Ag) wire was used as a pseudoreference electrode, a platinum (Pt) wire as a counter-electrode and an indium tin oxide (ITO) surface as a working electrode. The electrochemical potential was cycled between −750 to +750 mV at 50 mV/s in all cases. Thanks to this range of voltage, we could avoid water electrolysis. Additionally, the oxygen usually contained in aqueous solutions was removed previously to the electrochemical determination. This was done by passing through the solutions a flux of N_2 (INFRA, ultra high purity 99.999%) for an average time of 15 min.

2.4. Immobilization of Cytochrome *c* Crystals on ITO Electrodes. The fixation of the cytochrome *c* crystals was feasible by growing these crystals ex situ with the hanging-drop vapor-diffusion method, followed by the microseeding technique as mentioned in section 2.2. The cytochrome *c* crystals were introduced inside the fluid cell of

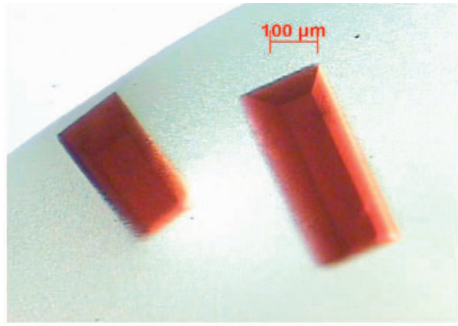

Figure 2. Crystal of cytochrome *c* from bovine heart obtained ex situ by microseeding techniques.

the atomic force microscope (EC-AFM). They were then immobilized with ppy films. An equilibrated droplet of the crystallization conditions, with crystals of cytochrome *c*, were mounted on the ITO surface previous to the injection of an aqueous solution of 0.77 M pyrrole (py)/0.34 M $LiClO_4$ in the fluid cell of the EC-AFM. To ensure a close contact between the crystals and the ITO electrode, the aqueous solution was carefully (dropwise) introduced to the fluid cell to prevent the movement of the crystals away from the bottom of the electrode to the bulk of the solution. Sometimes, when crystals are moved levitating on the solution, it is necessary to allow them to come down onto the surface of the ITO before performing the experiment. When the fluid cell of the EC-AFM was closed, a potentiodynamic electropolimerization was carried out (50 mV/s) permitting the ppy film to be formed around the crystals of cytochrome *c*.

3. Results and Discussion

For many years, dynamic light scattering methods have proved their efficiency to characterize biological macromolecules. Figure 1 shows the hydrodynamic characterization versus temperature of cytochrome *c* monomers by dynamic light scattering. The monomodal size-distribution implies that the high purity of this protein is maintained in a very large range of temperatures (inset Figure 1a). A hydrodynamic radius of about 4 nm with a maximum stability that ranges between 10 and 35 °C was clearly determined (Figure 1a). Figure 1b shows the

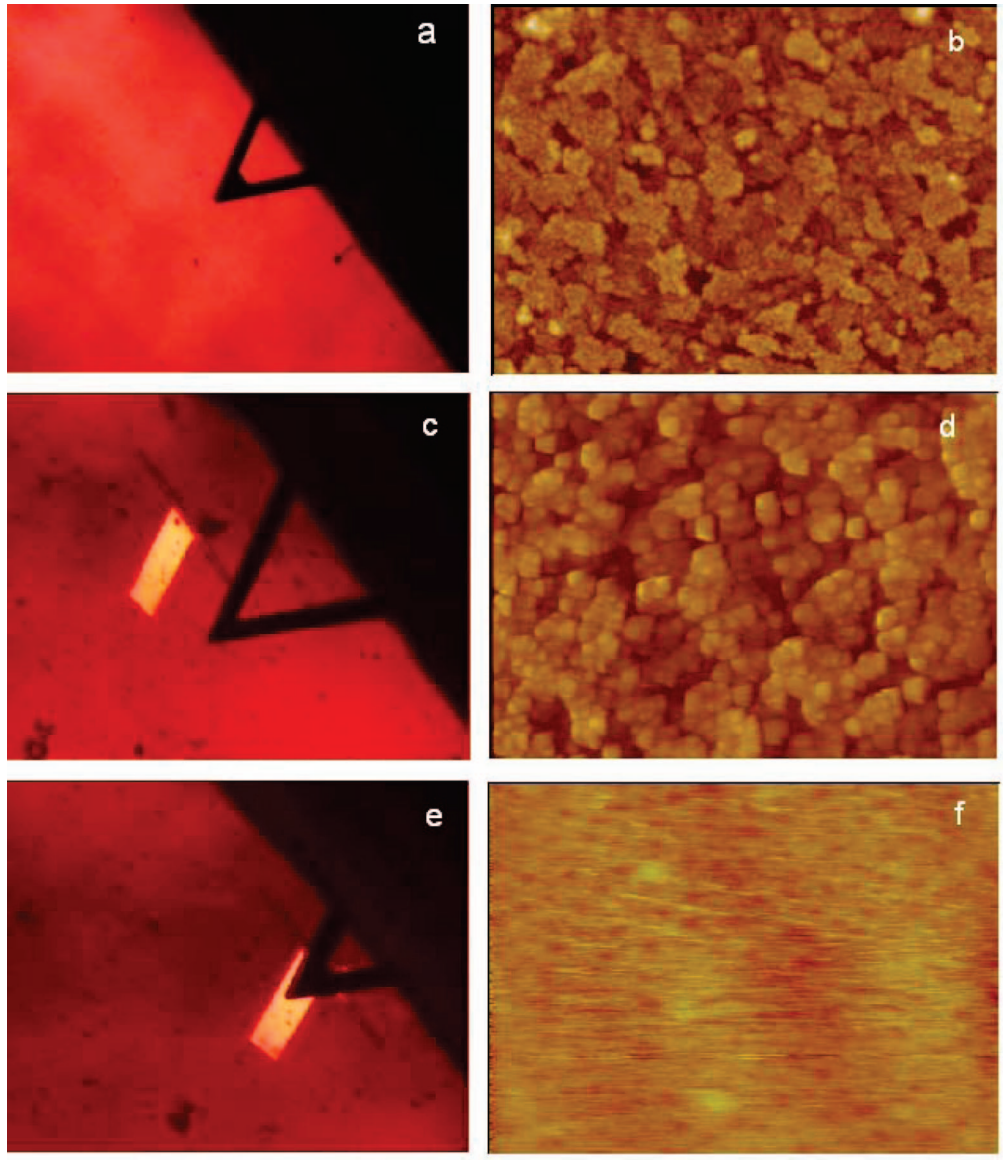

Figure 3. AFM micrographs before and after analyzing the crystal surface: (a) cantilever on the surface of the ITO electrode; (b) 2.5 × 2.5 μm image of the surface of the ITO electrode; (c) this image shows the polypyrrole area where the scanning on the film was performed; (d) 2.5 × 2.5 μm image of the surface the polypyrrole film; (e) after polymerizing the pyrrole the crystal was fixed by this polypyrrole film; (f) 2.5 × 2.5 μm image of the crystal surface.

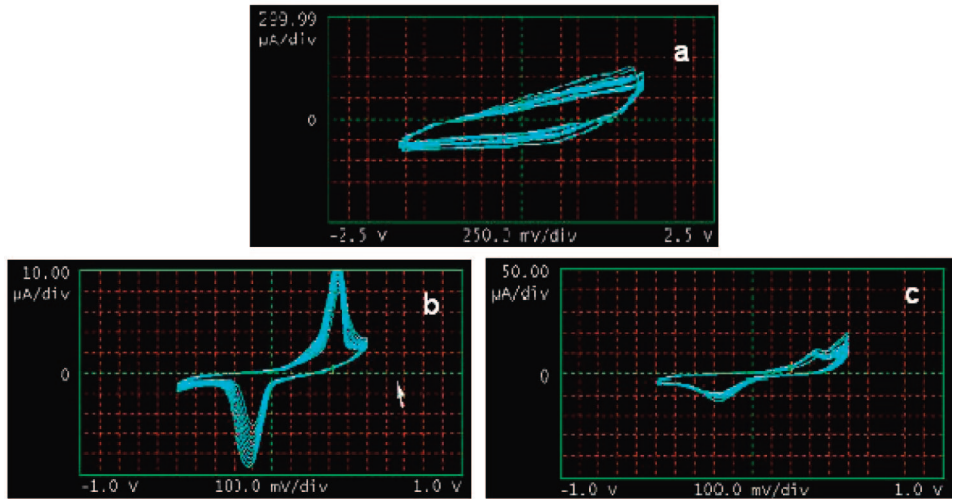

Figure 4. Two different electrochemical responses of cytochrome *c* by using cyclic voltammetry techniques: (a) ppy voltammperogram (control); (b) signal of layers of few nanometers of cytochrome *c*; (c) voltammogram of cytochrome *c* crystals fixed by ppy films. The reader must take into account that the scales are different. The software of the AFM equipment technically adapts all electrochemical signals.

evolution of the diffusion coefficient when the protein is mixed with the precipitating agent (PEG-1000). This coefficient diminishes due to the growth of the size of protein aggregates. On the basis of this hydrodynamic behavior, we decided to set all crystallization experiments at 18 °C, where this protein is much more stable. These studies are useful for both the AFM and the electroanalytical experiments of cytochrome *c*.

To apply electroanalytical techniques, crystals of cytochrome *c* were obtained following the crystallization procedure of Mirkin et al.[9] Figure 2 shows the beautiful red single crystals of cytochrome *c* used for these electroanalytical investigations. The crystals were characterized by synchrotron radiation (Brookhaven National Laboratories-NSLS, USA) and belonged to the $P2_12_12_1$ space group with the following unit cell parameters $a = 36.84$ Å, $b = 54.48$ Å and $c = 55.33$ Å. They also have four monomers into the asymmetric unit and 46% solvent content, as already published elsewhere.

A recent development on biosensors field requires a high local protein molecule concentration to prepare better devices of a higher sensitivity. One way to reach this objective is to create a solid-state electrochemical biosensor using protein single crystals. The availability of highly purified cytochrome *c* crystals allowed us to explore new applications of cytochrome *c* to create a solid-state biosensor based on the electron-transfer properties of this biomolecule. However, due to a lack of methods to fix protein single crystals on the surface of the working electrode for analytical studies, the use of conducting polymers such as ppy as chemical glue was explored. This polymer resulted in conducting chemical glue[17] useful to fix our crystals for structural and electrochemical investigations. It must be taken into account that protein crystals are a fragile material, which break easily under mechanical pressure, but they cannot be easily immobilized as other inorganic crystals.[19,20]

Figure 3a,b shows the surface of the ITO electrode where the crystals of the protein will be deposited. An homogeneous ITO deposit composed by small flowerlike microspheres was observed. After polymerizing py, the crystal was perfectly fixed on the ITO electrode. The surface morphology of ppy (Figure 3c,d) determined by AFM differs clearly from the protein crystal (Figure 3e,f). This last image could only be obtained when the cytochrome *c* crystal was steadily fixed on the ITO electrode by this ppy film. After fixing the cytochrome *c*, the deflection of the tip was also monitored applying a small force (0.3 N/m) to the crystal surface. Since no movement was detected when pushing the crystal with the tip of the cantilever during the scanning process, a good immobilization of the single crystal of cytochrome *c* to the ITO surface was ensured. The ppy surface is a set of homogeneous aggregates, whereas the surface of the protein crystal is smooth and planar. This confirms that ppy did not cover the surface of the crystal and that it was exposed toward the solution. This experimental setup permitted us to measure the electrochemical response of the cytochrome *c* crystal attached to the electrode using the in situ AFM-electrochemical module.

Figure 4 depicts the electrochemical response obtained by means of cyclic voltammetry of (a) the ppy layer (control); (b) cytochrome *c* layers adsorbed on the electrode;[9] and (c) cytochrome *c* crystal fixed with ppy. A similar shape of the voltammperogram was observed for both forms (monolayer and single crystal) of immobilization of cytochrome *c*. This experiment confirms the electric transference in solid-state of electrons from the bottom to the surface of the single crystal.

Additionally, the scanning tunneling microscopy (STM) mode from the SPM/AFM was also used to confirm this electron-transference along the crystal structure producing a signal response. However, the gel-like structure of the protein crystals did not permit us to obtain any image with this technique (STM); this is why the EC-AFM in the cyclic voltammetry mode was the most appropriate technique for our purposes.

Finally, it is worth mentioning that the crystal-electrode can be kept inside an electrochemical cell covered with light oil to avoid evaporation. The lifetime of these solid-state electron transfer biosensor has not been yet evaluated. For the exchange of electrons via the cytochrome *c* with membrane proteins such as cytochrome *c* oxidase or reductase, this biosensor can be theoretically reused (not tested). However, both the temperature and the equilibrium conditions must be kept constant to avoid dissolution of the crystals. Future experiments will be focused on protecting these crystals with a paste of graphite or Nafion (to avoid damage of the solid-state structure). This is a very common technique in bioelectrochemistry, where immobilized enzymes have been studied.

4. Conclusions

This novel immobilization technique for cytochrome *c* crystals will open a new possibility to investigate electric properties in biological materials (biocrystals). This contribution has shown the plausible measurement of an electron-transfer signal through a solid-state material (crystals) using a redox biomolecule. Additionally, the perspectives of this work will be focused on the mounting of several crystals by these ppy films, to use them as seeds for crystal growth and X-ray crystallographic research. These polycrystalline electrodes will permit the construction and the investigation of biosensors once the electric response for a variety of applications has been enhanced. Future research based on these results must be devoted to coupling different types of biological crystals to produce the first semiconductor based on proteins for the new bioelectronics in the near future.

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