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Preparation of a Magnetically Switchable Bioelectrocatalytic System Employing Cross-linked Enzyme Aggregates in Magnetic Mesocellular Carbon Foam**

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Nanostructured magnetic materials (NMMs)[1] have attracted much attention recently because of their broad biotechnological applications including support matrices for enzyme immobilization,[2] immunoassays,[3] drug delivery,[4] and biosensors.[5] Specifically, the easy separation and controlled placement of NMMs by means of an external magnetic field

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enables their application in the development of immobilized enzyme processes[2] and the construction of magnetically controllable bio-electrocatalytic systems.[5,6] Herein, we demonstrate the use of immobilized enzymes in NMMs for magnetically switchable bio-electrocatalysis.

Magnetic mesoporous carbons, in which magnetic nanoparticles (MNPs) are incorporated into the mesoporous carbon matrix, have many desirable characteristics for preparation of immobilized enzymes for magneto-bio-electrocatalysis: large pore size and volume, the ability to be positioned with a magnet, and good electron conductivity. Herein, we synthesized magnetic mesocellular carbon foam by a simplified approach based on the in situ generation of MNPs during the synthesis of the mesocellular carbon. The resulting material, designated Mag-MCF-C, was found to possess large interconnected pores and well-dispersed MNPs. Glucose oxidase (GOx) was immobilized within the large cellular pores of Mag-MCF-C, and the resulting material was used for the construction of a magnetically switchable bio-electrocatalytic system.

The operating principle of magnetically switchable bio-electrocatalysis is illustrated in Figure 1a.[6a] When the enzymes in the Mag-MCF-C particles in a solution containing substrate (glucose) were brought into contact with the electrode by means of a magnet placed below the electrode, an anodic current was generated by electron transport from the enzyme reaction to the working electrode (switched "on"). Conversely, when positioned above the electrochemical cell, the magnet attracted the Mag-MCF-C particles away from the electrode, and the electron-transfer efficiency was significantly lowered, and the current decreased to the background level (switched "off"). By alternate positioning of an external magnet, the bio-electrocatalytic signal could be reversibly switched back and forth between the on and off states.

Various nanoporous carbon materials have been prepared using nanostructured silica materials[7] as sacrificial templates.[8] The typical synthetic procedure for Mag-MCF-C is based on a template approach and is presented schematically in Figure 2a. MCF silica[9] was used as a template, and its surface area and pore volume were estimated by the BET method to be 574 $m^2 g^{-1}$ and 1.86 $cm^3 g^{-1}$, respectively. The cellular and window (percolation-path) pore sizes determined from the adsorption and desorption branch were 24.0 and 12.3 nm, respectively (see Supporting Information). After the polymerization of divinylbenzene (DVB), the main cellular pores of the MCF silica were partially filled by a poly(DVB) coating.[10] The BET surface area of the MCF/poly(DVB) composite was reduced to 138 $m^2 g^{-1}$, which indicates that complementary micropores in the walls of the MCF silica were filled with poly(DVB).[11] Also, the decreased cell size of 19.1 nm (see Supporting Information) indicates that poly(DVB) coatings of approximately 2.5 nm thickness were formed in the main cells of the MCF silica. The next step was impregnation of $Fe(NO_3)_3 \cdot 9H_2O$ dissolved in ethanol into the pores of the MCF/poly(DVB) composite. The Fe^{3+} ions present on the poly(DVB) surface were converted into magnetic nanoparticles during the carbonization at 800 °C. Finally, the dissolution of the MCF template in 1 M NaOH at

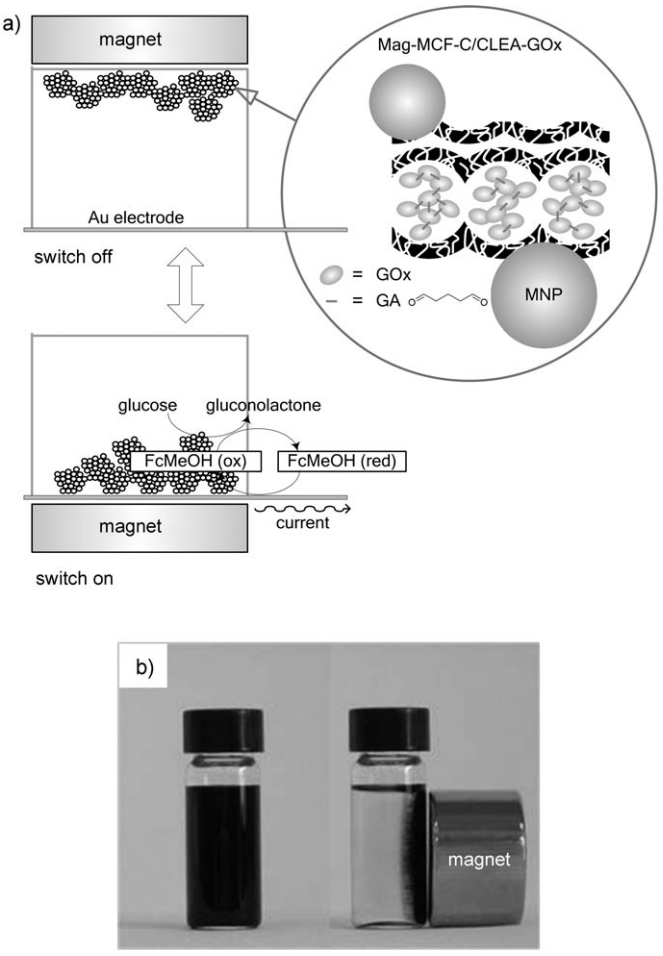

Figure 1. a) Working principle of the magnetically switchable bio-electrocatalytic oxidation of glucose by GOx immobilized in Mag-MCF-C. b) The separation of Mag-MCF-C/CELA-GOx particles by an applied magnetic field. The image shows a well-mixed suspension of Mag-MCF-C/CLEA-GOx in 0.1 M sodium phosphate buffer (ca. 10 mg mL^{-1}) before (left) and after (right) the application of a magnetic field.

100 °C yielded the magnetic mesocellular carbon foam Mag-MCF-C. The simplification of the synthetic procedure for Mag-MCF-C was attributed to the solid-state conversion of the impregnated iron salt into MNPs during the carbonization.[12] This method is in contrast with the recently reported multistep synthetic route to magnetic CMK-3 requiring separate steps for synthesis and postsynthetic deposition of the MNPs onto the carbon, and an additional carbonization step to cap the MNPs to prevent leaching.[13]

The field-emission scanning electron microscopy (SEM) image showed that the Mag-MCF-C particles had many large mesopores (see Figure 2b) open to the exterior, which was favorable for the quick incorporation of large biomolecules. The cellular mesopores were also observed in the transmission electron microscopy (TEM) image of Mag-MCF-C (Figure 2c). The cell sizes estimated from the adsorption branch of the argon isotherm were centered at 16.6 nm (Figure 3c). The window (percolation-path) sizes determined from the desorption branch were centered at 10.1 nm. The BET surface area and the single-point total pore volume were

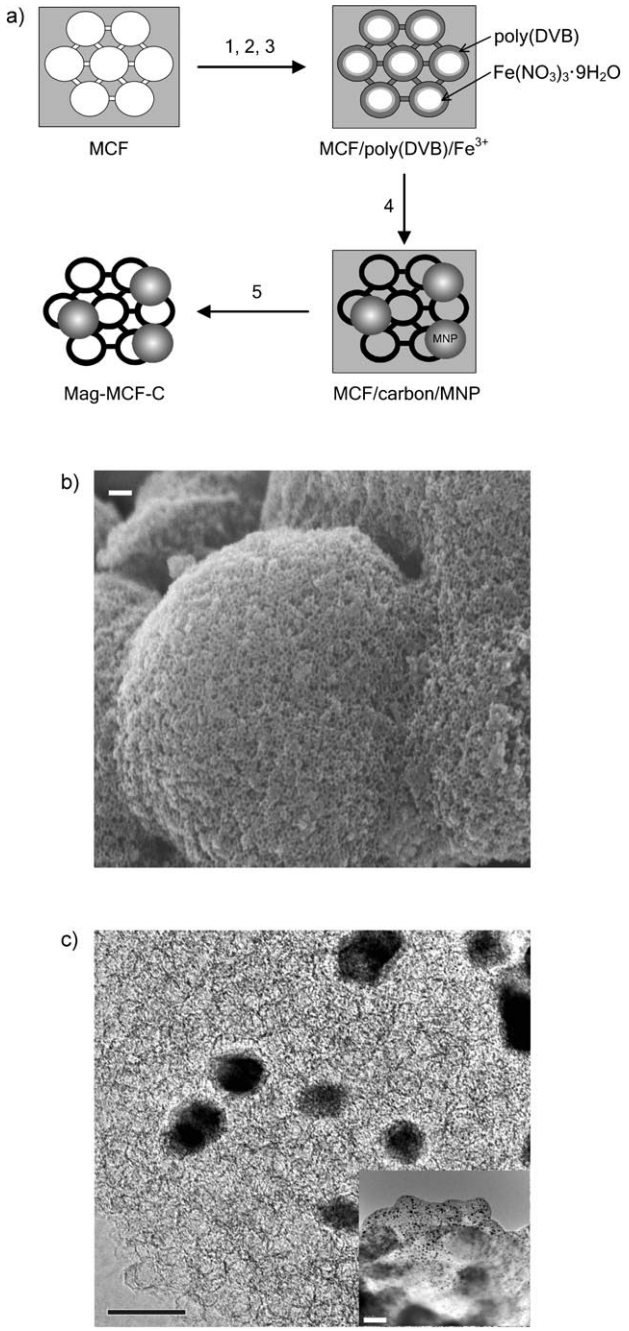

Figure 2. a) Schematic representation of the synthetic procedure for Mag-MCF-C: impregnation of DVB/AIBN (1); polymerization (2); impregnation of $Fe(NO_3)_3 \cdot 9H_2O$ (3); carbonization (4); removal of silica template (5). b) SEM image of Mag-MCF-C (scale bar: 200 nm). c) TEM image of Mag-MCF-C (scale bar: 50 nm); inset: low-magnification TEM image of Mag-MCF-C (scale bar: 500 nm). AIBN = azobisisobutyronitrile.

revealed to be 865 $m^2 g^{-1}$ and 1.43 $cm^3 g^{-1}$, respectively. The MNPs formed in situ were found to be well dispersed over the Mag-MCF-C surface (see inset of Figure 2c). The TEM (Figure 2c) and SEM images at high magnification (Supporting Information) showed clearly that the magnetic nanoparticles were formed on the external surface of the MCF-C. The iron content of the Mag-MCF-C measured by inductively coupled plasma atomic-emission spectroscopy (ICP-AES) was 26.9 wt%. The X-ray diffraction (XRD) pattern of Mag-MCF-C showed that body-centered-cubic (bcc) iron (α-Fe) nanoparticles with an average diameter of 30 nm, estimated by using the Debye–Scherer equation, were well dispersed in the mesocellular carbon foam matrix (see Supporting Information). The XRD pattern also showed small peaks attributable to magnetite, which demonstrates that the iron was partially oxidized during the NaOH etching process. Further characterization by means of TEM, convergent-beam electron diffraction (CBED), and energy-dispersive X-ray spectroscopy (EDX) showed that the nanoparticles had an Fe/Fe_3O_4 core/shell structure. The formation of large, 30-nm sized particles, which are larger than the cell diameter of MCF-C, seems to be due to sintering of the initially synthesized small nanoparticles on the surface of the mesocellular carbon foam during the carbonization at high temperature.

The magnetization curves (see Supporting Information) exhibited hysteresis over the complete temperature range of the measurements from 325 K down to 2 K, which demonstrates the ferromagnetic character of Mag-MCF-C at room temperature. The zero-field cooled and field-cooled magnetization data measured in an applied field of 100 Oe also revealed that Mag-MCF-C is ferromagnetic at room temperature (Supporting Information). This ferromagnetic property, derived from the large size of the magnetic nanoparticles, turned out to be very important to the facile and fast migration of the nanoparticles to a desired position by means of an external magnetic field (see below). The saturation magnetization value was 8.5 emu g^{-1} at 300 K, which was sufficient for magnetic separation. Furthermore, the remnant magnetization was low (< 1 emu g^{-1}), which was important for the easy redispersion of the Mag-MCF-C particles when the magnet was removed.

To demonstrate the utility of Mag-MCF-C in the construction of a magnetically switchable bio-electrocatalytic system, glucose oxidase (GOx), which is a well-studied redox enzyme, was immobilized within the Mag-MCF-C particles. A schematic representation of Mag-MCF-C with immobilized and cross-linked GOx in the large mesocellular pores is presented in Figure 3a. From the molecular dimensions of GOx ($6.0 \times 5.2 \times 7.7$ nm^3),[14] it was expected that the enzyme molecules would easily enter the large cellular pores (16.6 nm) of Mag-MCF-C through the connecting windows (10.1 nm). In fact, the amount of argon adsorbed in Mag-MCF-C was found to be significantly decreased after loading with GOx (Figure 3b), which indicates that most of the GOx molecules were incorporated into the pores of Mag-MCF-C, especially into its large cellular pores (Figure 3c). The adsorption isotherm of GOx-immobilized Mag-MCF-C (see Supporting Information) shows a maximum loading of about 53% (w/w carbon). Equilibrium was reached after a 5 h incubation period (see Supporting Information). Notably, the isotherm obtained did not conform to the Langmuir-type with an almost-linear increase in loading with initial increase in enzyme concentration. The results suggest that the immobilization mechanism involves the entrapment of enzymes in the mesocellular pores of the Mag-MCF-C particles rather

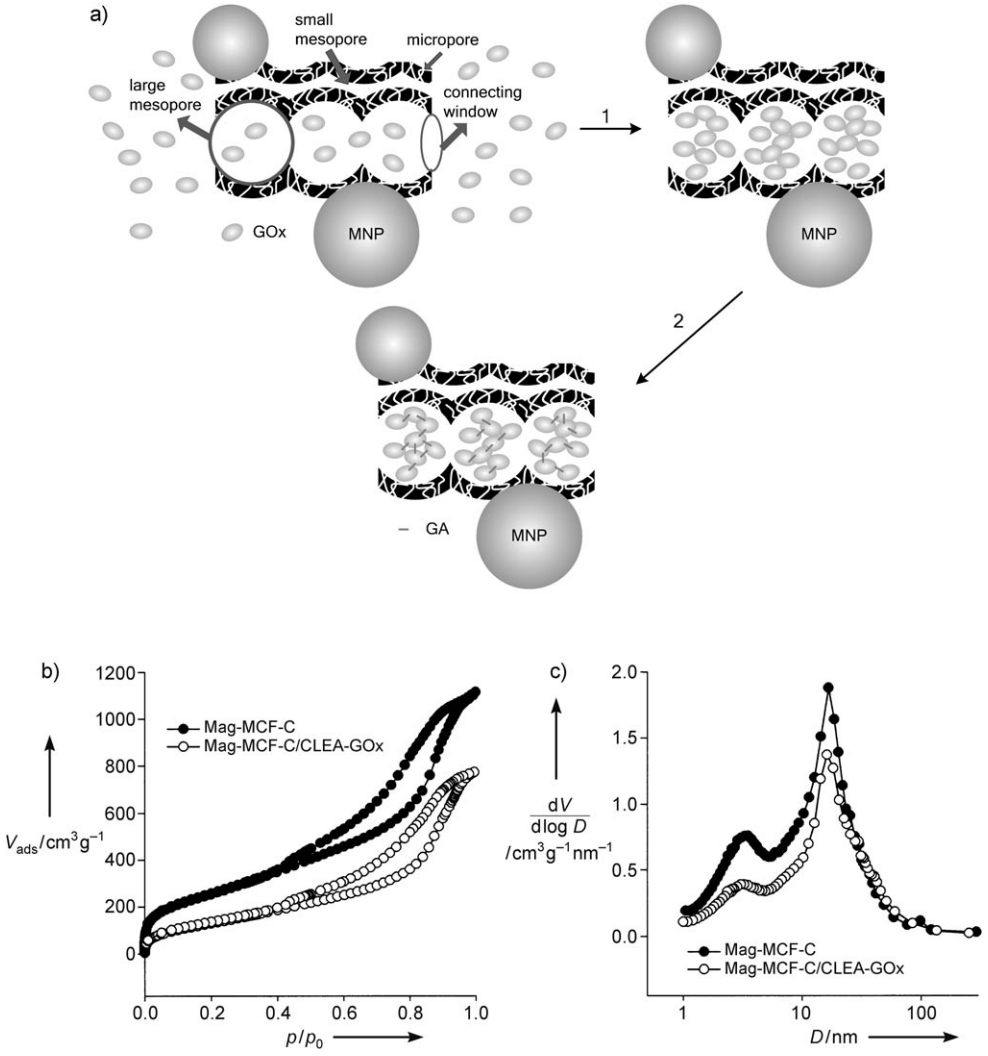

Figure 3. a) Schematic representation of Mag-MCF-C with immobilized and cross-linked GOx in large mesocellular pores. Also depicted are the incorporated MNPs and the small pores: adsorption of GOx in the mesocellular pores of Mag-MCF-C (1); cross-linking of GOx using glutaraldehyde to make enzyme aggregates (2). b) Argon-adsorption-desorption isotherms and c) pore-size distributions (PSDs) of Mag-MCF-C and Mag-MCF-C/CLEA-GOx. The PSDs were calculated from Ar adsorption data by using the Barrett–Joyner–Halenda (BJH) method. The amount of adsorbed argon was significantly reduced after loading of GOx.

than the adsorption of enzymes on the carbon surface. It was observed that the migration of the GOx-immobilized Mag-MCF-C (Mag-MCF-C/GOx) under the same strength of external magnetic field slowed down somewhat as the GOx loading increased. Consequently, Mag-MCF-C/GOx with a GOx loading of approximately 30% was used in this study to take into account the migration of Mag-MCF-C/GOx by a magnet.

The enzymes in the pores were stabilized through cross-linking with glutaraldehyde (GA).[15] As Mag-MCF-C consisted of 17-nm mesocellular pores connected with 10-nm window pores, we anticipated that the smaller size of the window pores would prevent the leaching of the cross-linked enzyme aggregates (CLEAs) and result in high enzyme loading and good enzyme retention (see Figure 3a).[15] As a result of the cross-linking, Mag-MCF-C/CLEA-GOx maintained more than 90% of its initial activity after 12 washings as well as after continuous incubation with vigorous shaking for 22 days, whereas only approximately 50% activity was retained for GOx immobilized in Mag-MCF-C without cross-linking after shaking for 22 days (see Supporting Information for experimental details). This observation suggests that the cross-linking of the enzyme was effective in improving its operational stability by preventing leaching of the enzyme from the Mag-MCF-C composite. On the basis of these results, cross-linked Mag-MCF-C/CLEA-GOx was used for the following experiments.

The specific activity of CLEA-GOx within Mag-MCF-C (45.8 ± 7.5 U per mg GOx) was estimated to be 29% of that of free GOx in buffer (155.5 ± 12.5 U per mg GOx). This 29% recovery of activity was quite impressive when the high concentration of GOx in the pores (384 mg mL^{-1}, see Supporting Information for the detailed calculation) and possible enzyme inactivation during cross-linking by GA is considered.

It was expected that the unique pore structure of the host Mag-MCF-C could relieve the mass-transport resistance: MCF-C had a variety of pore interconnections that facilitated the transport of substrate and products as shown schematically in Figure 3a. The small mesopores (diameters around 3.0 nm), which were generated by dissolution of the silica template, were too small to accommodate GOx but large enough to accommodate small molecules such as the substrate and products and thus provide "substrate transport channels". Micropores with an average diameter of around 0.7 nm (see Supporting Information) present throughout the carbon framework[16] might also provide a route for the transport of small molecules by connecting the small and the large mesopores.

Suspended Mag-MCF-C/CLEA-GOx particles were quickly attracted in the direction from which the external magnetic field was applied (see Figure 1b), which confirmed the utility of Mag-MCF-C in magnetically controllable sensing systems such as the one presented in Figure 1a. The heavily loaded, active, and stable Mag-MCF-C/CLEA-GOx composite was used for magnetically switchable bio-electrocatalysis (see Figure 1a). Figure 4a shows cyclic voltammograms obtained when the magnet was placed in the "up"

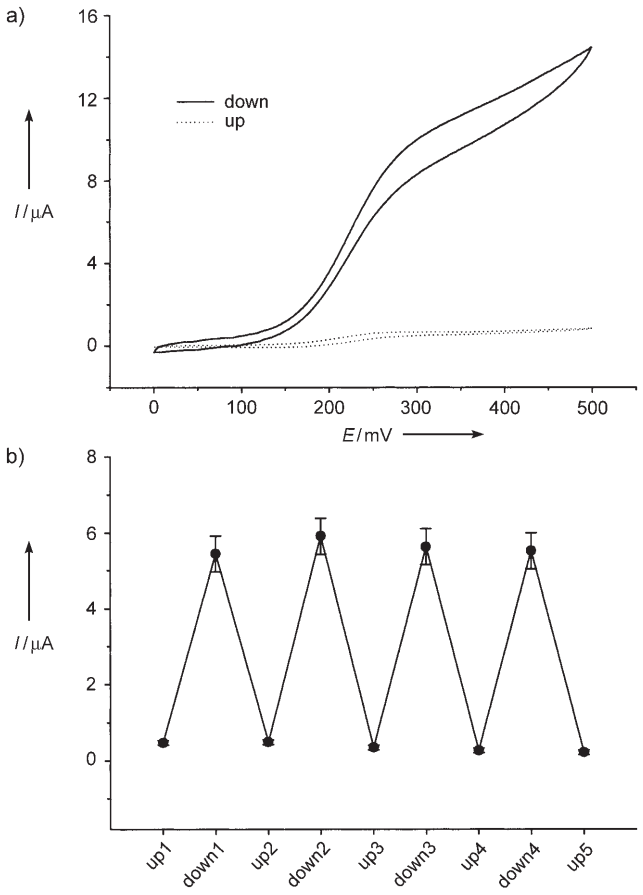

Figure 4. a) Cyclic voltammograms measured with the magnet in the "down" (Mag-MCF-C/CLEA-GOx near the electrode surface) and "up" (Mag-MCF-C/CLEA-GOx away from the electrode surface) positions. The voltammetry was performed in an electrochemical cell (4 mL) containing deoxygenated 0.1 M sodium phosphate buffer (pH 7.4) with 0.1 mM ferrocenemethanol and 5 mg of Mag-MCF-C/CLEA-GOx in the presence of 20 mM glucose. All the measurements were performed at room temperature with a scan rate of 5 $mV s^{-1}$. b) Current switching during the shuttling of the magnet between the "up" and "down" positions. The experimental conditions were the same as those described in (a).

(above the electrochemical cell) and "down" (below the electrode) positions, respectively, in the presence of 20 mM glucose. As expected, a significant increase in anodic current was observed when the Mag-MCF-C/CLEA-GOx particles were brought into contact with the electrode by a magnetic field. No such current was observed after the Mag-MCF-C/CLEA-GOx particles were pulled up by the magnet, even in the presence of glucose.

To check the reusability of Mag-MCF-C/CLEA-GOx and the reversibility of the current switching, bio-electrocatalytic oxidation of glucose was alternately repeated in the on and off states by cyclic placement of the magnet below and above the electrochemical cell. As shown in Figure 4b, anodic currents could be reversibly switched between the on and off states, and a constant signal was maintained in the on state. The magnetic capturing was also very quick; the positioning of most of the Mag-MCF-C/CLEA-GOx particles could be generally completed within 30 seconds. No catalytic current was observed even after the magnet was placed in the "up" position (off state) for several hours, which confirms that GOx was effectively retained within the Mag-MCF-C particles and leaching of the immobilized GOx was negligible, as observed earlier. The exceptional stability of the Mag-MCF-C/CLEA-GOx particles and the ability to easily control their placement by an external magnetic field allowed the construction of a highly effective magnetically switchable bio-electrocatalytic system showing a constant signal upon iterative switching.

In conclusion, we have demonstrated that the magnetic mesocellular carbon foam Mag-MCF-C can be synthesized through a simple synthetic method and used for the fabrication of a magnetically switchable bio-electrocatalytic system. The cross-linked GOx in the mesocellular pores of the Mag-MCF-C particles exhibited a high enzyme loading capacity and exceptional stability. Such advantageous characteristics of Mag-MCF-C/CLEA-GOx together with controlled positioning by an applied magnetic field were successfully utilized in the development of this system. From these results, it is anticipated that our approach will find various applications, such as bioconversion, reversible amperometric immunosensors, regeneration of enzyme electrodes, switchable biofuel cells, and site-specific immobilization of sensing elements in microsized sensing devices.

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