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Simple Fabrication of a Highly Sensitive and Fast Glucose Biosensor Using Enzymes Immobilized in Mesocellular Carbon Foam**

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Immobilization of biomolecules in tailor-made nanometer-scale structures can significantly improve the performance of biocatalytic processes. For efficient biocatalytic processes, it is highly desirable to develop nanostructured materials that enable high loading and long-term stability of the biocatalysts, as well as low mass-transfer resistance. In this regard, inorganic mesoporous materials[1] with well-controlled pore structures have gained much attention as appropriate, high-capacity hosts for biocatalysts.[2–5]

Template synthesis has frequently been used to synthesize novel porous carbon materials.[6] Recently, a new class of nanoporous carbons, ordered mesoporous carbon (OMC), has been synthesized using ordered mesoporous silica materials as

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inorganic templates. Considering their unique properties, such as high surface area, large pore volume, and inherent electrical conductivity, OMC materials have great potential as hosts for enzyme immobilization.[7–11] We previously synthesized mesocellular carbon foam, MSU-F-C,[11] which has a large pore size, by means of the controlled incorporation of a carbon precursor into the pores of a mesocellular silica foam[12] template.

In this communication, we report the unique characteristics of MSU-F-C and its application as a host matrix for enzyme immobilization. Transmission electron microscopy (TEM) images and pore-size-distribution analyses revealed that MSU-F-C possesses two distinct pore sizes: large cellular pores of around 31 nm are interconnected by smaller windows (≈21 nm in diameter) and are surrounded by small pores about 5.6 nm in diameter as illustrated in Figure 1A. These unique characteristics of MSU-F-C offer several favorable features as a host matrix for enzyme immobilization. The large cellular pores can provide sufficient space to accommodate large enzymes, leading to high enzyme loading. Furthermore, the existence of the small pores, which are too small for large enzymes to penetrate, is expected to alleviate the mass-transport resistance by providing so-called "substrate-transport channels", and consequently enhancing the catalytic efficiency.

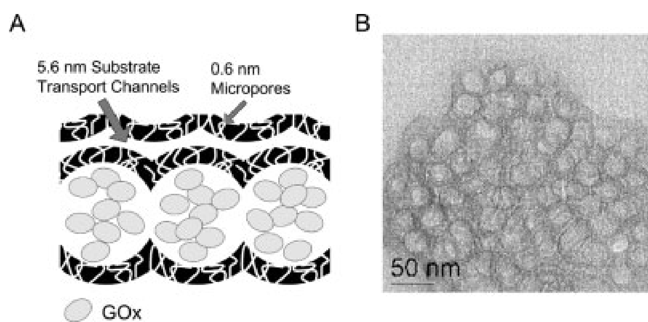

Figure 1. A) Schematic representation of MSU-F-C/glucose oxidase showing effective retention of enzymes within large mesopores and the role of small pores in the facile transport of a substrate. B) TEM image of MSU-F-C.

To demonstrate the potential of the bimodal pore structure of MSU-F-C as a unique host for enzymes, glucose oxidase (GOx), with molecular dimensions of 5.2 nm × 6.0 nm × 7.7 nm,[13] slightly larger than the small pores (diameter 5.6 nm) and smaller than the window pores (diameter 21 nm), was immobilized in MSU-F-C. The resulting enzyme-immobilized porous carbon was used to fabricate a glucose biosensor. To our knowledge, this is the first report on the use of conductive and bimodal mesoporous carbon for enzyme immobilization and, especially, their implementation in bioelectrocatalysis.

A MSU-F silica[12a] template was used to synthesize MSU-F-C, preserving the large cellular pores derived from the template. This was achieved by partial filling of the cells with carbon precursor by controlled polymerization. A TEM image of MSU-F-C (Fig. 1B) clearly shows the ordered arrangement of hollow spherical cells with diameters of around 31 nm. The existence of two different sized pores is apparent in the pore-size distribution calculated from an argon adsorption/desorption isotherm using the BJH (Barrett–Joyner–Halenda) method (Fig. 2). The size of the window was estimated from the desorption branch to be 21 nm. MSU-F-C also has micropores with diameters of around 0.6 nm, which occupy about 15 % of the total pore volume (Fig. S1 in the Supporting Information and Table 1). Distinct physical properties of MSU-F-C are listed in Table 1, together with those of several other carbon materials, such as graphite and activated carbon.

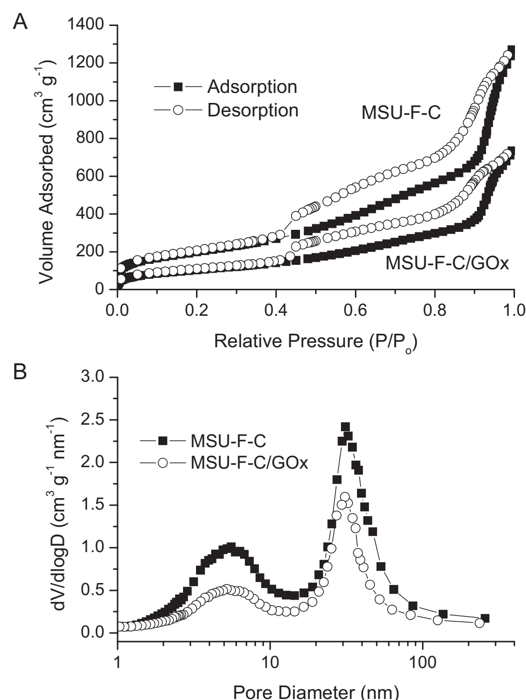

Figure 2. A) Argon adsorption/desorption isotherm of MSU-F-C and GOx-loaded MSU-F-C (MSU-F-C/GOx). B) Pore-size distribution in MSU-F-C and MSU-F-C/GOx calculated from Ar adsorption data using the BJH method; *V*: adsorbed Ar volume; *D*: pore diameter.

From the structural properties of the MSU-F-C, it is expected that the cellular mesopores (diameters around 31 nm) are able to hold large-sized enzymes such as GOx. The adsorption isotherm of GOx into MSU-F-C (Fig. 3) shows a maximum loading of 39.1 ± 0.7 % (w/w carbon, number of independent measurements, $n = 4$). Equilibrium was reached after 10 h incubation (Fig. S2 in Supporting Information). It is noteworthy that the isotherm obtained does not conform to the Langmuir type; in contrast, it displays an almost linear increase in loading with initial enzyme concentration. This suggests that the immobilization mechanism is based on the entrapment of enzymes in the mesopores of MSU-F-C, rather than on the adsorption of enzymes on the carbon surface.

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Table 1. Physical properties and GOx loadings of various carbon materials.

	BET surface area [$m^2\ g^{-1}$]	Total pore volume [$cm^3\ g^{-1}$]	Micropore volume [$cm^3\ g^{-1}$]	Mesopore volume [$cm^3\ g^{-1}$]	Pore diameter [nm]	Enzyme loading [c] [wt.-% carbon]
Graphite (Aldrich 28 266-3) [d]	16	0.06	–	–	–	0
Activated Carbon (Darco G-60) [d]	732	0.55	–	–	Broad distribution	8.2
CMK-3 [d]	1150	1.07	–	–	3.9	0.7
MSU-F-C	671	1.63	0.25	1	31.1[a]/20.7[b]/5.6	38.4
MSU-F-C/GOx	357	0.94	0.13	0.6	30.4[a]/19.0[b]/5.2	–

[a] Each material was incubated with 4 mg mL^{-1} GOx solution for 24 h; the amount of immobilized enzyme was determined after thorough washing. [b] Large cellular mesopore. [c] Connecting window. [d] The physical properties of graphite, activated carbon, and CMK-3 were measured using N_2 adsorption–desorption isotherms.

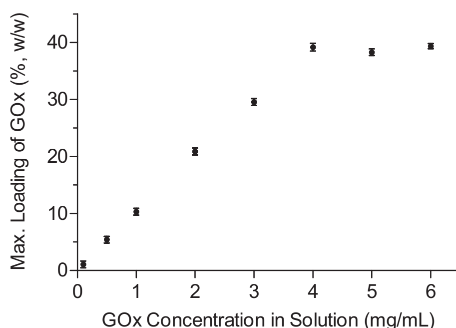

Figure 3. Adsorption isotherm of GOx in MSU-F-C. The MSU-F-C (10 mg) was incubated in GOx solution of specified concentration under shaking (at 250 rpm) at 25 °C for 48 h to get the maximal enzyme loading (error bar: ± standard deviation, $n = 4$).

The utility of MSU-F-C for enzyme immobilization was further assessed by comparing the enzyme-loading capacity of the MSU-F-C for GOx with those of other carbon materials possessing different pore structures. Each carbon sample (10 mg) was incubated with GOx solution (4 mg mL^{-1}) for 24 h at 25 °C. Following a thorough washing, the amount of immobilized GOx was estimated by measuring the concentration of free enzyme in the solution. As shown in Table 1, MSU-F-C exhibited fairly high enzyme loading compared to graphite and activated carbon. It is known that graphite possesses no prominent pore structure, and as a result, negligible loading was observed. The activated carbon had a comparable surface area to MSU-F-C, but it has a much smaller pore volume compared to MSU-F-C. The loading of GOx in the activated carbon was about 4.7-fold lower than that in the MSU-F-C owing to the smaller pore volume.

The critical role of large pores in high enzyme loading is more clearly demonstrated by comparison with another type of mesoporous carbon, CMK-3,[8a] an hexagonally ordered mesoporous carbon, with uniform 4 nm sized pores (see Supporting Information). Even though the total pore volume of MSU-F-C is only 1.5-fold higher than that of CMK-3, MSU-F-C showed about a 50-fold higher GOx loading than CMK-3 (Table 1). This indicates that large GOx molecules cannot enter the pores of CMK-3 readily: its pores are too small (4 nm). To confirm that the GOx was incorporated mainly into the pores of MSU-F-C, rather than just adsorbed onto the exterior surface of the particles, we obtained Ar adsorption/desorption isotherms for MSU-F-C/GOx and MSU-F-C. The adsorbed amount of Ar in MSU-F-C/GOx was found to be significantly reduced (Fig. 2A) compared to that in MSU-F-C, and the total pore volume of MSU-F-C decreased from 1.63 to 0.94 cm^3 g^{-1} after the immobilization of GOx (Table 1). From this analysis, it is evident that the GOx was mainly incorporated into the pores of MSU-F-C. In addition, Figure 2B reveals that most of the GOx molecules are in the large mesopores of MSU-F-C.

To date, the mesoporous material used for the immobilization of enzymes has mainly been silica.[2] However, the sizes of immobilized enzymes in mesoporous silica were less than 100 kDa (1 Da $\approx 1.66 \times 10^{-27}$ kg), and the loading of enzymes has mostly been less than 20 % (w/w silica).[2] Immobilization of a large enzyme, such as GOx (molecular weight 160 kDa),[13] in mesoporous silica was attempted, but the enzyme loading was negligible owing to the small pore size or to a repulsion between the negative charges on the surfaces of both silica and GOx (pI = 4.6).[2g] As for mesoporous carbons, Vinu et al.[14a] used CMK-3, which was synthesized using an SBA-15 silica template, to immobilize cytochrome c (12 kDa, 26 Å × 32 Å × 33 Å) and obtained a maximum protein loading of about 23 % (w/w carbon) in CMK-3. However, the pore sizes of the CMK-3s used (3.0, 4.3, and 6.5 nm, based on the BJH method) seem not to be large enough to host large enzymes, such as GOx, as demonstrated in our comparative experiment (Table 1). We synthesized CMK-3-150 following the reported procedure,[14] but the pore size was somewhat smaller (diameter 6.0 nm, estimated using argon adsorption) than the reported value (6.5 nm). Even though the pore volume of CMK-3-150 (2.01 cm^3 g^{-1}) is higher than that of MSU-F-C (1.63 cm^3 g^{-1}), the loading (or adsorption) capacity of the synthesized CMK-3-150 for GOx was determined to be 3.8 % (w/w carbon), which was about five times higher than that of CMK-3-100[8a,14] (0.7 %) with a smaller pore size (3.9 nm). However, this loading capacity of CMK-3-150 is still much lower than that of MSU-F-C, demonstrating that the pore size of CMK-3-150 is not yet large enough to accommodate GOx in its pores. On the other hand, the high loading of GOx in MSU-F-C can be attributed to the large pore size. GOx ad-

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sorption into MSU-F-C was also quick, reaching about 26 % (w/w carbon) in 20 min (Fig. S2, Supporting Information), which seems to be due to both the large pore size and small particle size of MSU-F-C (< 500 nm diameter).[2k]

To assess retention and stability of the enzyme, the MSU-F-C/GOx was incubated in buffer under stirring at 4 °C. At intervals, the sample was centrifuged, and the supernatant was replaced with a fresh buffer solution. Then, small aliquots containing MSU-F-C/GOx were sampled and assayed for GOx activity. The initial activity of GOx in MSU-F-C was maintained even after ten days (data not shown). In addition, the supernatant exhibited no detectable GOx activity during the incubation, which indicates a negligible leaching of enzymes.[15] The effective retention of GOx in MSU-F-C can be explained by the combination of several factors. First, the carbon materials, not specifically charged, provide a more favorable environment for better retention of GOx than silica. The negatively charged silanol groups in silica can strongly repel acidic proteins, such as GOx.[2g,13,16] We speculate that the bimodal structure of MSU-F-C can also contribute to the prevention of GOx leaching. The entrance of GOx molecules into the mesocellular pores is unlikely to be limited by the connecting windows (21 nm), based on the observation of quick GOx adsorption. However, once enzyme molecules are confined within the mesocellular pores (31 nm), the leaching of enzymes from mesocellular pores can be hindered because of the bottleneck resulting from the window pores.[17] Since the size of glycosylated GOx must be larger than that of the crystal structure (5.2 nm × 6.0 nm × 7.7 nm), several GOx molecules can easily plug the window pores and prevent the leaching of GOx molecules from the mesocellular pores through the window pores.

The GOx activity per mass of MSU-F-C increases almost linearly with enzyme loading, at least up to ≈ 30 % (w/w carbon) loading (from incubation in 3 $mg\,mL^{-1}$ GOx solution; see Fig. S4, Supporting Information). This suggests that filling the mesocellular pores with GOx does not significantly affect the normalized enzyme activity. However, the activity levels off above 30 % enzyme loading, suggesting that the blockage of micropores by the GOx molecules in the mesocellular pores starts to place a certain limitation on the substrate transfer from the small mesopores to the main cellular mesopores. However, the specific activity of GOx in MSU-F-C still was maintained at 30–40% of that of free GOx in solution, in spite of the high local concentration of enzyme within MSU-F-C (≈ 328 $mg\,mL^{-1}$)[18] under maximal loading conditions. This result implies that the present immobilized-enzyme system does not suffer from a serious mass-transfer limitation of substrate and products, which is likely due to the unique pore structure of the host. MSU-F-C has a variety of pore interconnections that may facilitate the transport of the substrate and products. The size of the small mesopores (diameters around 5.6 nm) is somewhat small to accept GOx, but large enough for small molecules, such as the substrate and products, providing a "transport channel", as represented in Figure 1A. Micropores with average diameters around 0.6 nm present throughout the carbon frame might also provide another route for the transport of small molecules by connecting the small mesopores and the large mesocellular pores (Fig. 1A). We can also anticipate that there is sufficient space for the diffusion of glucose molecules between large GOx molecules in large cellular pores. The relatively small size of the MSU-F-C particles (< 500 nm) might also alleviate the mass-transport resistance.

To explore the potential of MSU-F-C as a host matrix for bioelectrocatalysis, glucose biosensors were fabricated and evaluated. Glassy carbon electrodes (GCEs) were coated with the MSU-F-C/GOx in a Nafion membrane. Briefly, electrodes were first coated with an MSU-F-C/Nafion mixture, followed by adsorption of GOx into the MSU-F-C entrapped within the Nafion membrane. The Nafion membrane is known to have permselectivity and biocompatibility, which makes it useful as an electrode-modifier for biosensors.[19] Figure 4 shows the amperometric response of the enzyme electrode. The current was measured at 600 mV (vs. Ag/AgCl), where the hydrogen peroxide from the catalytic conversion of glucose by GOx was reoxidized on the electrode surface. The catalytic current increased with successive additions of glucose (Fig. S5, Supporting Information). The response was also dependent on the MSU-F-C content in the Nafion film; higher

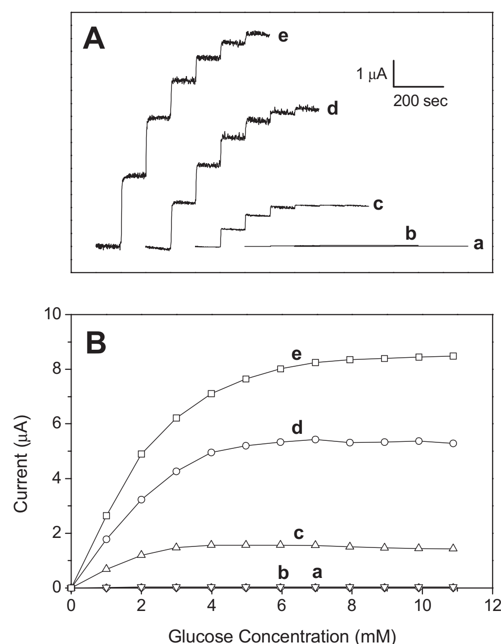

Figure 4. A) Current response of the glucose biosensor prepared with different MSU-F-C content on successive additions of 1 M glucose aliquots (10 μL each). The applied potential was 600 mV (vs. Ag/AgCl reference electrode), and the measurement was performed in 10 mL sodium phosphate buffer (0.1 M, pH 7.4) with stirring. The MSU-F-C contents are: a) 0, b) 0.5, c) 1.0, d) 2.0, and e) 3.0 $mg\,mL^{-1}$ in Nafion solution (0.5 wt.-%). B) Calibration plots from amperometric responses presented in (A). Curves (a–e) correspond to the same series as in (A).

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responses were obtained with higher MSU-F-C contents (Fig. 4A). At higher MSU-F-C contents, more enzyme is immobilized on the electrode, and consequently a higher catalytic current is expected. It is noteworthy that the electrode modified only with Nafion (without MSU-F-C) revealed a negligible response to glucose addition (Fig. 4A, curve a). This result indicates that most of the enzyme was immobilized within MSU-F-C, but neither in the Nafion film nor on the GCE directly, and that the enzyme immobilized in MSU-F-C within a preformed Nafion film retained its catalytic activity.

The dynamic range increased with increasing MSU-F-C content. The dynamic range of the enzyme electrode prepared with 3 $mg\,mL^{-1}$ MSU-F-C suspension (Fig. 4B, curve e) extended up to about 7 mM glucose, and the detection limit was about 0.07 mM based on a signal-to-noise ratio of 3. A sensitivity of 3.5×10^{-2} $A\,M^{-1}cm^{-2}$ or 0.2 $A\,M^{-1}$ (per milligram of enzyme) was registered in the linear range (up to $\approx$2 mM). These values are much higher than those of biosensors employing various polymeric matrixes that were reported to range in general from 10^{-5} to 10^{-3} $A\,M^{-1}\,cm^{-2}$. (Note that larger values ($A\,M^{-1}\,cm^{-2}$) indicate higher sensitivity).[19] The high sensitivity of the glucose biosensor seems to result from the high enzyme loading and low mass-transfer resistance derived from the unique structural characteristics of MSU-F-C. The biosensor response was fast, and the current generally reached a steady-state level less than 30 s after the glucose addition. This fast response can be explained by the effective transfer of substrate and products through MSU-F-C matrixes containing enzymes, resulting from the small particle sizes and unique pore structure of MSU-F-C as described above.

In an effort to assess the effect of the electrical conductivity of MSU-F-C on the performance of the glucose biosensor, we tested a non-conductive silica matrix, MSU-F. MSU-F was used as the template for the synthesis of MSU-F-C, and has a similar pore size to that of MSU-F-C.[12a] GOx was immobilized in both MSU-F and MSU-F-C and used to fabricate glucose biosensors. Enzyme loadings in both supports were adjusted to almost the same level for comparison.[20] As a result, no significant signal was detected from the biosensor constructed with MSU-F/GOx, even though MSU-F/GOx showed an enzyme activity comparable to MSU-F-C/GOx (data not shown). This observation demonstrates the utility of MSU-F-C with inherent electrical conductivity for biosensor applications.

In conclusion, we fabricated a highly sensitive and fast glucose biosensor by simply immobilizing GOx in mesocellular carbon foam. Owing to its unique structure, the MSU-F-C enabled a high enzyme loading without serious mass-transfer limitations, resulting in a high catalytic efficiency. As a result, the glucose biosensor fabricated with MSU-F-C/GOx showed high sensitivity and fast response. Given these results and the inherent electrical conductivity, we anticipate that MSU-F-C will make a useful matrix for enzyme immobilization for use in various biocatalytic and bioelectrocatalytic applications.

Experimental

Materials: GOx (256 $U\,mg^{-1}$; U: enzyme units) and D-(+)-glucose were purchased from Sigma. Graphite (28266-3), activated carbon (Darco G-60), sodium silicate solution (27 % SiO_2, 14 % NaOH), furfuryl alcohol (98 %), and Nafion solution (5 wt.-%) were obtained from Aldrich. All chemicals were used as received.

Synthesis and Characterization of MSU-F-C: For the synthesis of MSU-F-C, MSU-F silica with nanometer-sized primary particles was synthesized by means of a modified version of the original synthetic procedure, employing hydrothermal post-treatment at 100 °C [12a]. After 4 h calcination at 550 °C, alumination (Si/Al = 20) was performed using the impregnation method to generate acidic catalytic sites for the polymerization of furfuryl alcohol inside the mesopores. Typically, furfuryl alcohol (1 mL) was impregnated with AlMSU-F through an incipient-wetness method. AlMSU-F/furfuryl alcohol was polymerized at 85 °C under vacuum for more than 12 h. The resulting composite was heat-treated under N_2 at 850 °C. HF or NaOH etching generated mesocellular carbon foam. To improve the dispersion of MSU-F-C in aqueous solution, it was treated with a mixture of HNO_3 and H_2SO_4 for 30 min. Ar adsorption and desorption isotherms were measured at 77 K using a Micromeritics ASAP 2010 Gas Adsorption Analyzer after the mesoporous materials were degassed at 423 K at 10 μtorr (1 torr ~ 133 Pa) for 5 h. The micropore (below 2 nm) and mesopore (between 2 and 50 nm) size distributions were calculated from the analysis of the adsorption branch of Ar isotherms using the Horvath–Kawazoe and the BJH formalisms, respectively. TEM images were obtained on a JEOL JEM-2010 electron microscope. Scanning electron microscopy images were obtained on a JSM-840A microscope.

Immobilization of GOx in Carbon Materials: Prior to the immobilization, carbon materials (graphite, activated carbon, CMK-3s, and MSU-F-C) were washed with sodium acetate buffer 0.2 M; (pH 5.1) three times. Washed carbon (10 mg) was incubated in GOx (4 $mg\,mL^{-1}$ in acetate buffer) with shaking (at 250 rpm) at 25 °C. Finally, the MSU-F-C/GOx was washed twice with acetate buffer, resuspended in fresh buffer, and stored at 4 °C.

Enzyme Assay: A GOx assay kit from Molecular Probes was used for enzyme assays according to the manufacturer's instructions. Briefly, samples containing GOx were diluted in sodium phosphate buffer (50 mM; pH 7.4). GOx standards and samples (50 μL each) were loaded into the wells of a 96-well microplate. Then, freshly made assay solution (50 μL; 100 μM Amplex Red, 0.2 $U\,mL^{-1}$ horseradish peroxidase, and 100 mM glucose) was added to each microplate well containing sample to initiate a chromogenic reaction. After incubation for 30 min at room temperature in the dark, the reaction product (resorufin) was quantified by measuring the absorbance at 570 nm in a microplate reader (Bio-Rad). The enzyme activity was determined from the standard curve. The activity of GOx immobilized in MSU-F-C was also determined following the same procedure after the removal of the aqueous phase by a brief spin in a centrifuge tube filter (0.22 μm pore, Corning) and resuspension/dilution in fresh buffer. In this dilution range, suspended particles had a negligible effect on the absorbance measurement. The protein concentration was determined using the Bradford method (Bio-Rad).

Enzyme Electrode Preparation: GCEs (3 mm diameter, Bioanalytical Systems) were used as working electrodes. Prior to surface modification, the GCE was polished with 0.05 μm alumina, sonicated for 5 min, and then washed with double-distilled water and methanol. MSU-F-C was dispersed in Nafion (0.5 wt.-%, in 0.1 M sodium phosphate buffer, pH 7.4) to give a homogeneous mixture. The mixture (10 μL) was then deposited upon the polished electrode surface and dried for 1 h under ambient conditions. The resulting modified electrode was incubated in GOx solution (10 $mg\,mL^{-1}$) overnight at 4 °C for the immobilization of enzymes. The enzyme electrode was thoroughly washed with cold buffer before use.

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Electrochemical Measurements: A CV-50W voltammetric analyzer (Bioanalytical Systems) was used for cyclic voltammetry and amperometry. All electrochemical measurements were performed in sodium phosphate buffer (10 mL, 0.1 M, pH 7.4) at room temperature. Ag/AgCl and a platinum wire (Bioanalytical Systems) were used as the reference and auxiliary electrodes, respectively. For amperometry, 600 mV was applied as an operating potential. After initial current decrease and stabilization, small aliquots of glucose stock solution (1 M, in 0.1 M sodium phosphate buffer, pH 7.4) were added at appropriate time intervals (usually 100 s) to stabilize the signal between additions. The glucose stock solutions were allowed to mutarotate overnight before use.

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