Direct One-Step Immobilization of Glucose Oxidase in Well-Ordered Mesostructured Silica Using a Nonionic Fluorinated Surfactant

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This work describes the immobilization of glucose oxidase (GOD) in mesostructured silica. The enzyme is incorporated into the silica framework via a direct one-step immobilization method. Results obtained by SAXS and nitrogen adsorption−desorption analysis clearly show that the channel arrangement of the recovered materials depends on the GOD loading. Indeed, when the hydrothermal treatment is performed at 60 °C for 2 days, well-ordered materials are obtained if the GOD concentration is lower than 3.2 mg per mL of micellar solution, and higher loading leads to the formation of wormhole-like structures. The efficiency of the immobilization was revealed by fluorescence and FTIR spectroscopy. It appears that there is a maximum loading of GOD, about 11 wt %, that can be incorporated into the matrix. Results also show that the surfactant plays the role of a pore-forming agent. Finally, we have shown that the entrapped enzyme maintains its activity.

1. Introduction

In recent years, much interest has been attributed to the development of biosensors at the nanoscale level and promising results have first been obtained by Clark and Lyones.[1] These authors reported in 1962 an enzyme electrode for measuring glucose concentrations. Since this study, many papers dealing with enzyme-based biosensors have been published in the literature.[2−6] Because of its importance in the human metabolism, glucose is the most studied analyte.[7] Indeed, the development of a stable in vivo sensor could improve the regulation of glucose concentration and reduce complications related to diabetes. Therefore, many researches are focused on the immobilization of glucose oxidase (GOD).

The making of biosensors requires the immobilization of a biomolecule on a solid surface. The immobilization should be irreversible and stable under potentially adverse reaction conditions. At the same time, high activity, good accessibility to analytes, and rapid response times should be kept, while leaching of the biomolecule has to be avoided. Conventional methods of enzyme immobilization include physical or chemical adsorption at a solid surface,[8] covalent binding or cross-linking to a matrix,[9] entrapment within a membrane,[10] and microencapsulation into polymer microspheres and hydrogels.[11,12] However, such techniques are not generic and can be used only for a limited range of biomolecules and applications. A promising method for the design of biosensors consists of the entrapment of the biomolecules in a silica matrix prepared via the sol−gel process. Indeed, these inorganic host supports exhibit several advantages such as physical rigidity, chemical inertness, simplicity of preparation, tunable porosity, low-temperature encapsulation, optical transparency, negligible swelling, and mechanical stability. The sol−gel encapsulation of biomolecules was first reported in 1990 by Braun et al.[13] The authors showed that enzymes, trapped within a porous oxide matrix, retain their biological activity. It was also reported by Blyth et al.[14] and by Chung et al.[15] that metalloproteins, such as hemoglobin, encapsulated into porous sol−gel silica matrix kept their activity and could be used for the optical detection of small molecules. The

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chemical reactivity of these proteins is not influenced by the encapsulation and they can react reversibly with gas dissolved in water over a long period of time. However, the shrinkage of the sol−gel matrix and difficulties to control the pore size distribution of the sol−gel constitute the main disadvantage of the process. Owing to their properties such as high specific surface area and uniform pore size distribution,[16,17] mesoporous materials can overcome this drawback. Indeed, the large dimensions of these mesoporous materials offer the possibility of accommodating small enzymes within the channels. The encapsulation of the biomolecules can be made by physisorption;[18−21] in this case, weak interactions such as hydrogen bonding, van der Waals, or electrostatic forces occur between the biomolecules and the matrix. For example, Li et al.[18] have reported the immobilization of penicillin acylase on MCM-41 through the interaction between hydroxyl groups of MCM-41 and carbonyl or amino groups in the biomolecules. In a paper dealing with the adsorption of cytochrome *c* on silica and aluminosilica mesoporous materials, Balkus et al.[19,20] conclude that the physical adsorption of the enzyme showed a clear dependence on the composition of the molecular sieves; they have also shown that the immobilized enzyme is stable for several weeks. Moreover, the pore openings of mesoporous molecular sieves can be modified with organosilane, resulting in a reduction in the channel window diameter, which could effectively entrap the guest molecule and therefore prevent leaching of the enzyme.

Another method to entrap biomolecules in those materials consists of chemisorption;[22,23] this second pathway involves a covalent link between both moieties and most of the time the matrix has to be functionalized before the immobilization. The main disadvantage of this method is that the fixation through a covalent bond may cause some damage to the biomolecule and thereby quench its activity. Finally, the biomolecules can be incorporated in surfactant[24] or nonsurfactant templated[25] mesoporous materials through a direct one-step immobilization, although only a few papers in the literature deal with this kind of encapsulation. To show the benefit of mesoporous silica material, we report in this paper the direct one-step immobilization of glucose oxidase into surfactant templated mesostructured silica. The synthesis is performed under neutral pH conditions, using a nonionic fluorinated surfactant, $CF_3(CF_2)_7C_2H_4(OC_2H_4)_9OH$. In previous publications, we have shown that the use of this surfactant leads to mesoporous materials with a well-defined structure, a high specific surface area, and a tunable pore diameter.[26,27] Recently our group has shown that fluorinated cubic phases can be used as matrixes for the immobilization of cholesterol oxidase (COD) on electrodes.[28] The immobilized COD has been successfully employed as a sensing element for the electrochemical detection of cholesterol in its micellar solutions.

In this work, we have investigated the effect of GOD loading on the structural and textural properties of the sensors and have evaluated the quantity of enzyme that is effectively immobilized. The activity of the incorporated GOD was also assessed.

2. Materials and Methods

2.1. Preparation. The fluorinated surfactant used, provided by DuPont, had an average chemical structure of $C_8F_{17}C_2H_4(OC_2H_4)_9OH$, labeled as $R^F_8(EO)_9$ (or Zonyl-FSN-100, its commercial name). Indeed, its hydrophilic chain moiety exhibited a Gaussian chain length distribution. To synthesize the sensors, first a micellar solution of $R^F_8(EO)_9$ at 10 wt % in water was prepared. The loading of glucose oxidase (GOD), added to the micellar solution, was varied from 0.4 to 7.0 mg per mL of micellar solution, which corresponds to a variation of the GOD concentration from 2.5×10^{-6} to 4.4×10^{-5} mol L^{-1}. Tetramethoxysilane (TMOS), used as the silica source, was added dropwise. The surfactant/silica molar ratio was adjusted to 0.5. The gel obtained was sealed in Teflon autoclaves and heated for 2 days to 60 °C. The final products were recovered after ethanol extraction with a Soxhlet apparatus for 30 h.

2.2. Characterization. *2.2.1 Structural and Textural Characterization.* X-ray measurements were carried out using a home-built apparatus, equipped with a classical tube ($\lambda = 1.54$ Å). The X-ray beam was focused by means of a curved gold/silica mirror on the detector placed at 527 mm from the sample holder. Nitrogen adsorption − desorption isotherms were obtained at −196 °C over a wide relative pressure range from 0.01 to 0.995 with a volumetric adsorption analyzer TRISTAR 3000 manufactured by Micromeritics. The samples were degassed further under vacuum for several hours at room temperature before nitrogen adsorption measurements. The specific surface area was calculated by the BET (Brunauer, Emmett, Teller) method (molecular cross-sectional area $= 0.162$ nm^2). The pore diameter and the pore size distribution were determined by the BJH (Barret, Joyner, Halenda) method.[29]

2.2.2. Spectroscopy Investigations. A Fourier transform infrared spectrometer Perkin-Elmer 2000, equipped with a KBr beam splitter and a DTGS detector, was used. The spectral resolution and the total acquisition time were, respectively, 4 cm^{-1} and 5 min. FTIR spectra in diffuse reflectance (DRIFTS) mode were collected using a Harrick DRA-2CI equipment and a Harrick HVC-DRP cell. To perform the analysis, the mesoporous powder was first diluted in a KBr matrix (10 wt %). Then, the sample was kept inside an evacuated chamber (10^{-5} Torr). Reflectances Rs of the sample and Rr of pure KBr, used as a nonabsorbing reference powder, were

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Table 1. Mesoporous Materials: Structure, Values of *d* spacing, Cell Parameter (a_0), N_2 Specific Surface Area (S_{BET}), Pore Diameter (∅), Wall Thickness, and % of Immobilized GOD

[GOD] in the started micellar solution (mg/ mL)	structure	*d*-spacing (nm)	a_0 (nm)	S_{BET} (m^2/g)	∅ (nm)[c]	wall thickness (nm)[a]	wt % GOD in the silica network[b]
0	hexagonal	5.4	6.2	1058	3.6	2.5	0
0.4	hexagonal	5.2	6.0	962	3.8	2.2	1.3
0.8	hexagonal	5.2	6.0	1046	3.8	2.2	6.0
1.6	hexagonal	5.2	6.0	879	3.8	2.2	8.9
2.4	hexagonal	5.2	6.0	953	3.8	2.2	11.5
3.2	hexagonal	5.2	6.0	869	3.8	2.2	10.7
3.4	wormhole	5.4		699	3.7		
3.6	wormhole	5.3		675	3.7		9.2
3.8	wormhole	5.3		738	3.8		11.9
4	wormhole	5.6		707	3.9		
4.5	wormhole	5.3		716	3.8		11.0
5	wormhole	5.3		712	3.9		10.8

[a] Values obtained by subtracting pore diameter from cell parameter. [b] Values determined by UV spectroscopy. [c] Values obtained from BJH method applied to the adsorption branch of the isotherm.

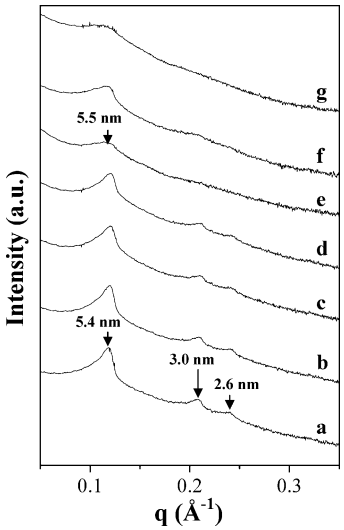

Figure 1. Evolution of SAXS patterns with the concentration of glucose oxidase in the micellar solution a: 0, b: 0.8, c: 2.4, d: 3.2, e: 3.4, f: 5.0, and g: 7.0 mg/mL.

measured under the same conditions. The mesoporous reflectance is defined as R = Rs/Rr. The spectra are shown in pseudo-absorbance (−log R) mode. Fluorescence spectra of Trp residues in the GOD enzyme were obtained with a Fluorolog-3 Jobin Yvon spectrometer. The enzyme was excited at 260 nm and the emission spectra were recorded between 280 and 800 nm. Diffuse reflectance spectra were recorded between 200 and 600 nm at 1-nm increments with an integrated sphere attached to a Cary 5G UV−Vis−NIR spectrophotometer. The relative spectral reflectance R is defined as the ratio between the flux reflected by the sample Rs and that of a PTFE reference Rr. The spectra are shown in pseudo-absorbance (−log R) mode since the use of the Kubelka−Munk function does not improve the quality of the calibration line.

3. Results and Discussion

3.1. Structural Investigation. Figure 1 depicts the variation of the SAXS pattern with the glucose oxidase loading in the starting micellar solution. It has been reported[30] that X-ray diffractograms of powdery hexagonal mesoporous materials exhibit a typical three peak pattern with a very strong feature at a low angle (100 reflection line) and two other weaker peaks at higher angles (110 and 200 reflection lines). These three reflection lines characterize a hexagonal structure. The unit cell ($a_0 = 2d_{100}/\sqrt{3}$) corresponds to the sum of the pore diameter and the thickness of the pore wall. The absence of the last two peaks suggests a disordered structure of the mesoporous molecular sieves having wormhole-like channel systems, analogous to MSU-type materials.[31] The broad peak that is observed on the SAXS pattern gives an indication of the average pore-to-pore distance in the disordered wormhole framework which presents a lack of long-range crystallographic order. As to the sample obtained without GOD, in addition to a sharp peak at 5.4 nm, two peaks at 3.0 and 2.6 nm are detected (Figure 1a). The presence of the latter peaks reveals a hexagonal organization of the channels in the material. Indeed, these peaks are attributed to the 110 and 200 reflections of the hexagonal structure. According to Bragg's rule, the unit cell dimension (a_0) can be deduced and is about 6.2 nm. With increasing the GOD concentration from 0 to 3.2 mg per mL of the starting micellar solution, the position of the peaks remains almost constant (Figure 1b−d), indicating that a_0 does not vary (Table 1). So, in this concentration range, the incorporated enzyme does not disturb the channel arrangement. However, if the GOD content is further increased, the (110) and (200) reflections disappear completely (Figure 1e−g). This suggests that the channel array is not very regular anymore and the hexagonal symmetry is lost. In this case, the introduced enzyme involves a disorganization of the channel system.

Therefore, we can conclude that to obtain biosensors with a well-ordered channel array, the GOD loading in the starting micellar solution has to be kept at less than 3.2 mg per mL ($2\ 10^{-5}$ mol L^{-1}).

3.2. Characterization by Nitrogen Adsorption−Desorption. The variations of the nitrogen adsorption−desorption isotherms with glucose oxidase concentration are depicted in Figure 2. Whatever the GOD loading, a type IV isotherm, characteristic of mesoporous materials,[32] is obtained. However, the shape of the hysteresis loop is slightly modified with increasing GOD loading. Low GOD contents rather lead to an *H1* hysteresis loop, whereas an *H2* type is observed

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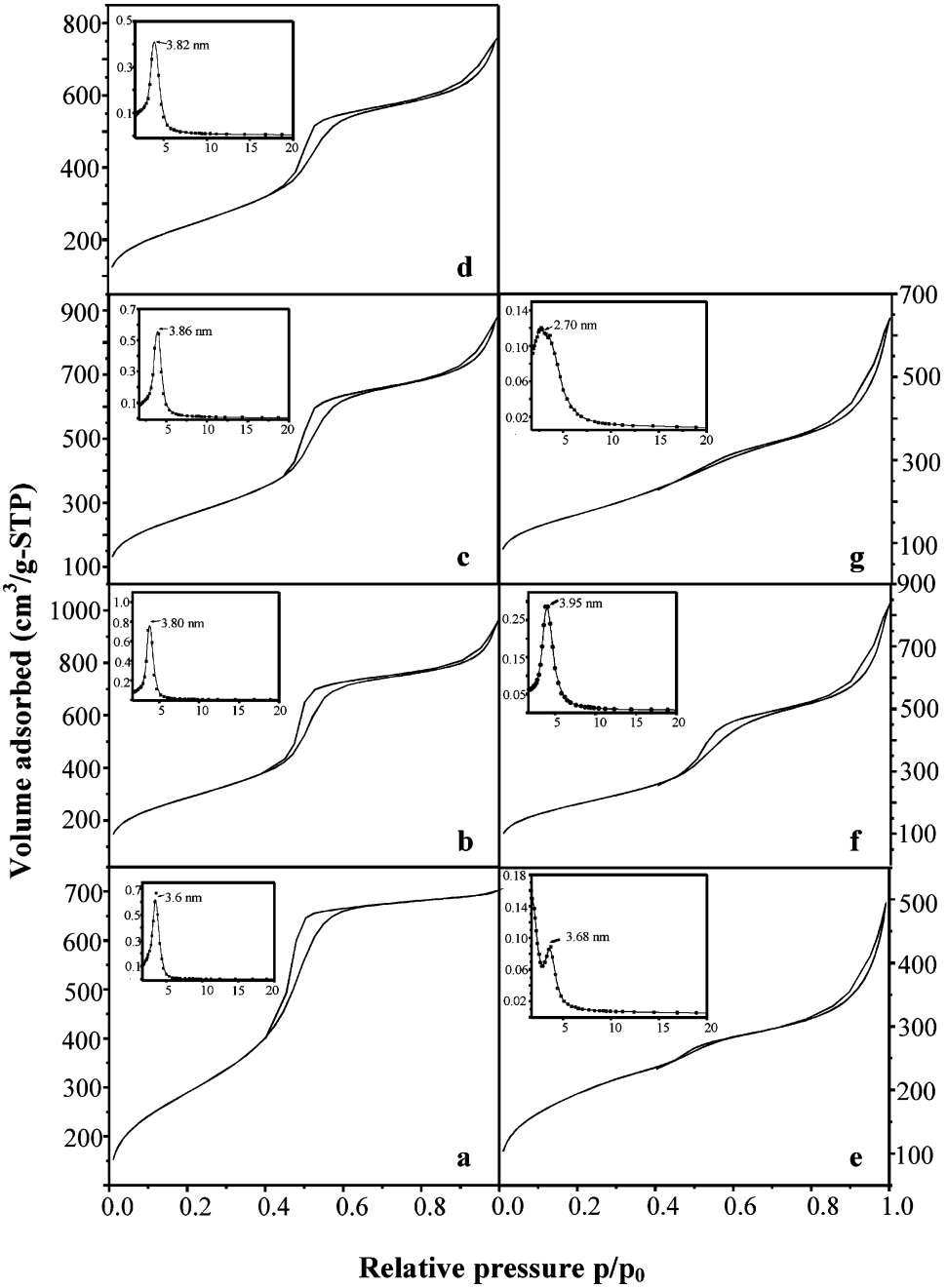

Figure 2. Evolution of nitrogen adsorption−desorption isotherms and pore size distribution (insert) with the concentration of glucose oxidase in the micellar solution a: 0, b: 0.8, c: 2.4, d: 3.2, e: 3.4, f: 5.0, and g: 7.0 mg/mL.

for higher loadings. This change can be related to the transition from a well-ordered to a disordered channel array. Indeed, *H2* is often encountered for disordered materials with a wormhole structure. These results are in good agreement with those obtained by SAXS; indeed, we have shown that for concentrations of GOD greater than 3.2 mg/mL a transition from a well-ordered channel array to a wormhole-like structure occurs.

In any case, the adsorbed volume of nitrogen increases significantly at high relative pressures instead of remaining constant because of saturation. This can be explained by a very large secondary porosity in these compounds that could be attributed to the presence of interparticle pores. The condensation of nitrogen in these large mesopores occurs very likely in addition to the adsorption inside the channels. Its origin should be related to the conditions of synthesis and in particular to the pH value. Indeed, for the analogous hydrogenated system $C_{16}(EO)_{10}$ in a paper dealing with the effect of pH of the synthesis gel on the preparation of mesoporous silica,[33] we have reported that a considerable amount of secondary porosity appears upon a pH increase. The appearance of secondary or interparticular porosity has been correlated with the SEM observations that show the formation of very small particles with increasing pH values of the synthesis gel. This phenomenon of secondary porosity appearance with increasing pH values has also been reported in the literature by different authors.[34]

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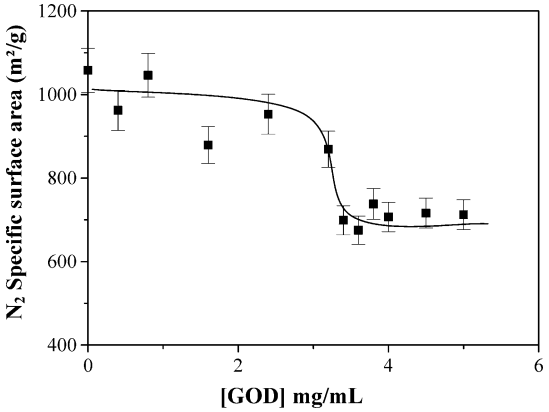

Figure 3. Variation of the N_2 specific surface area with glucose oxidase concentration.

Going from 0 to 3.2 mg of glucose oxidase per mL in the starting micellar solution, the value of the specific surface area, determined by the BET method, slowly decreases from 1000 to 850 m^2/g (Figure 3). Then, its value drops to 700 m^2/g, reflecting the progressive disorganization of the channel array.

Whatever the GOD loading, the pore size distribution is rather narrow and the position of its maximum remains centered at about 3.8 nm (Figure 2). This means that the pore diameter is not affected by the GOD addition. Since the values of the cell parameter and pore diameter remain constant, we can also conclude that the wall thickness does not vary and that its value is equal to 2.4 nm (Table 1). The fact that the pore diameter is not influenced by the increase of the glucose oxidase concentration indicates that GOD is probably not incorporated into the core of the micelles and that the enzyme rather interacts with the hydrophilic headgroup of $R^F_8(EO)_9$.

3.3. Evidence of GOD Incorporation and Evaluation of the Quantity of GOD Really Immobilized in the Silica Network. The FTIR spectroscopy provides a direct method to monitor the GOD incorporation into the mesoporous silica materials. All DRIFTS spectra were recorded in the ranges 2500−4000 and 900−1800 cm^{-1}.

The pure mesoporous silica exhibits bands at 3744, 3550, 2985, 2939, 2907, 1200, 1080, and 977 cm^{-1} (Figure 4a). The broad absorption around 3550 involves the OH stretching mode of H-bonded silanol groups whereas the sharp band at 3744 cm^{-1} characterizes the free silanols.[35] Bands detected at 2985, 2939, and 2907 cm^{-1} are attributed to the stretching vibrations of the CH groups of the surfactant, which was not completely removed by the ethanol extraction. However, no evidence of CF absorption is observed on the FTIR spectrum. The quantity of $R^F_8(EO)_9$, which remains after extraction, is very low.

Below 1800 cm^{-1}, the spectrum is dominated by a broad and intense band around 1150 cm^{-1} with a maximum at 1080 cm^{-1} and a shoulder at 1200 cm^{-1} characteristic of the antisymmetric stretching vibrational mode of the Si−O−Si siloxane bridges. The less intense absorption at 977 cm^{-1} is assigned to the Si−O stretching of free silanols.[35]

In addition to the bands of the silica framework reported above, we observe supplementary vibrations at 3290, 3070, 1660, 1630, and 1520 cm^{-1} in the spectra of GOD modified silica (spectra 4b−d). These absorptions characteristic of polypeptides were assigned according to results published in the literature.[36−39] All analyzed samples were outgassed under vacuum to eliminate physisorbed water. Indeed, water absorptions near 1630 cm^{-1} and around 3400 cm^{-1} would prevent clear observation of the polypeptide absorptions. The intense absorption at 3290 cm^{-1} and the weak band at 3070 cm^{-1} are, respectively, assigned to the amide A (N−H stretching) and amide B (N−H stretching in Fermi resonance with 2 x amide II) bands. The bands at 1630/1660 and 1520 cm^{-1} are assigned to the amide I and amide II bands. The amide I band originates predominantly from the C═O stretching vibrations of the peptide bond groups and the amide II band arises from N−H in-plane bending and C−N stretching modes of the polypeptide chains. As shown in Figure 4, the absorptions of amides A and B, amides I and II increase with GOD content until 3.2 mg/mL (Figure 4c), then they remain almost constant for higher loading (Figure 4d). The appearance of the later bands and their increasing absorbance with GOD concentration confirms the GOD incorporation into the silica matrix. As the amide I/amide II vibrational bands of protein are sensitive to protein structural changes, vibrational spectra give an insight into a possible conformational change of GOD immobilized with respect to the free GOD at solid state and thus an additional proof of GOD incorporation. In the spectrum of GOD at solid state, mechanically mixed to the mesopourous silica (spectrum not shown here), the amide I band exhibits a maximum at 1660 cm^{-1} with at least two shoulders near 1630 and 1690 cm^{-1} whereas immobilized GOD shows its maximum at 1630 cm^{-1}. This reveals a modification of the hydrogen-bonding interactions of the amide functions with their neighborhood when the GOD is incorporated into the silica. Nevertheless, we will show that the entrapped enzyme maintains its activity.

Incorporation of glucose oxidase was further proven by fluorescence spectroscopy. Glucose oxidase is a homodimeric enzyme. Each subunit of this protein contains one coenzyme molecule of flavine adenine dinucleotide (FAD). Each GOD monomer has two distinct domains: one that binds noncovalently but very tightly the FAD moiety, and another that binds the β-D-glucose substrate. The first area consists mainly of β-sheets and the second one of the four α-helices supporting antiparallel β-sheets. The flavinic coenzyme embedded in the protein does not generate any detectable emission,[40] unlike the tryptophan (Trp) residue which exhibits a large fluorescence.[41] The free glucose oxidase exhibits a

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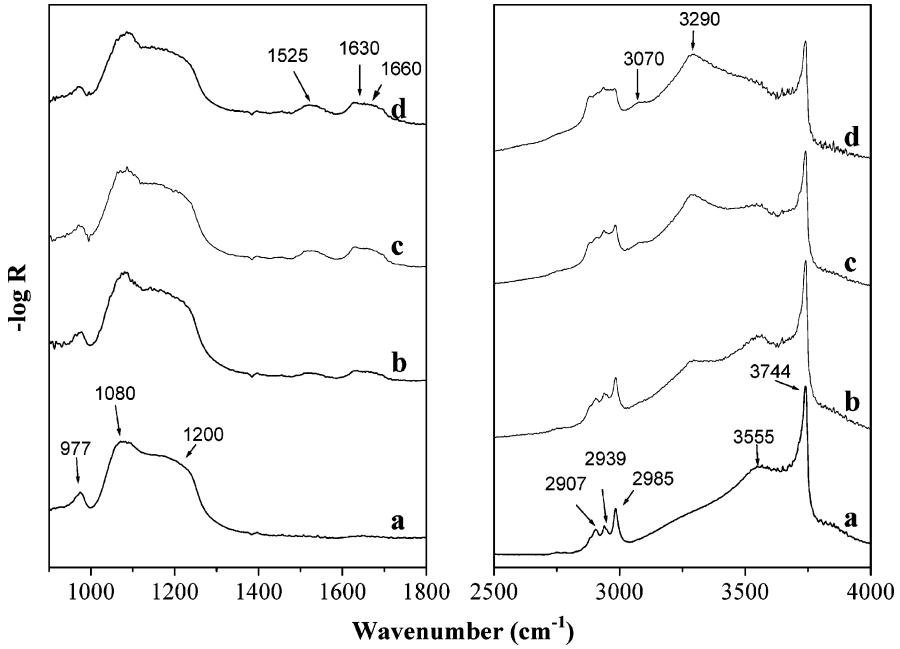

Figure 4. FTIR spectra of samples prepared with a: 0, b: 0.8, c: 3.2, and d: 5.0 mg of GOD per mL of micellar solution.

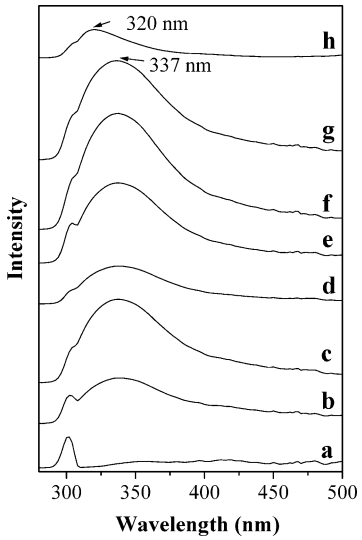

Figure 5. Fluorescence spectra of samples prepared with a: 0, b: 0.8, c: 2.4, d: 3.2, e: 3.4, f: 5.0, and g: 7.0 mg GOD per mL of micellar solution and h: free GOD.

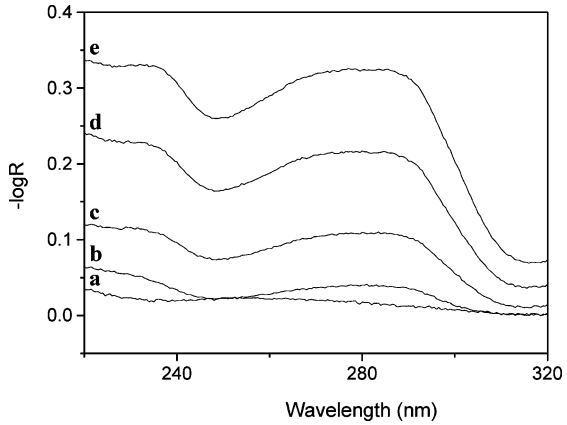

Figure 6. Diffuse reflectance UV spectra of glucose oxidase mixed with mesoporous silica a: 0, b: 1, c: 4, d: 8, and e: 14.5 wt % of GOD.

fluorescence band located at around 320 nm assigned to this tryptophan (Trp) residue (Figure 5h); no band is detected in this region on the fluorescence spectrum of the pure silica matrix (Figure 5a). Fluorescence spectra of glucose oxidase immobilized in the silica matrix exhibit the same shape as that of the free GOD (Figure 5b−g). The absence of changes in the emission maxima between the free and immobilized enzyme shows that Trp residues remain localized close to the protein surface.[37] The fluorescence spectra give evidence that the surrounding environments of the Trp residues are not affected by the immobilization. This result is in good agreement with those reported by Alpert et al.[42] For the enzyme in solution, a Förster coupling mechanism produces a partial energy transfer from seven Trp residues to the flavin, according to Alpert et al. Since the Trp emission does not change with immobilization, there is no alteration in this coupling and no change in the relative Trp-isoalloxazine orientations. Thus, we can conclude that the tertiary organization in the immobilized GOD is conserved.

Solid-state UV spectroscopy was used to evaluate the amount of glucose oxidase that is really incorporated into the silica matrix. First, to make a calibration, various amounts of GOD (from 1 to 16 mg) were dispersed in 100 mg of pure silica mesoporous molecular sieves. Spectra were recorded (Figure 6), and the pseudo-absorbance of the band at 285 nm versus the amount of GOD was plotted (Figure 7). From Figure 8, which depicts the percentage (wt) of GOD immobilized as a function of the enzyme concentration in the starting micellar solution, it can be seen that, at first, when the content of GOD varies from 0 to 2.4 mg/mL the percentage of biomolecules that can be incorporated into the silica network increases from 0 to 11 wt %. For higher concentrations of the enzyme, it reaches a plateau and the added GOD is not incorporated anymore into the mesoporous matrix. This observation is in good agreement with FTIR analysis. Nevertheless, even if the added enzyme is not

(42) Haouz, A.; Glandières, J. M.; Alpert, B. *FEBS Lett.* **2001**, *506*, 216.

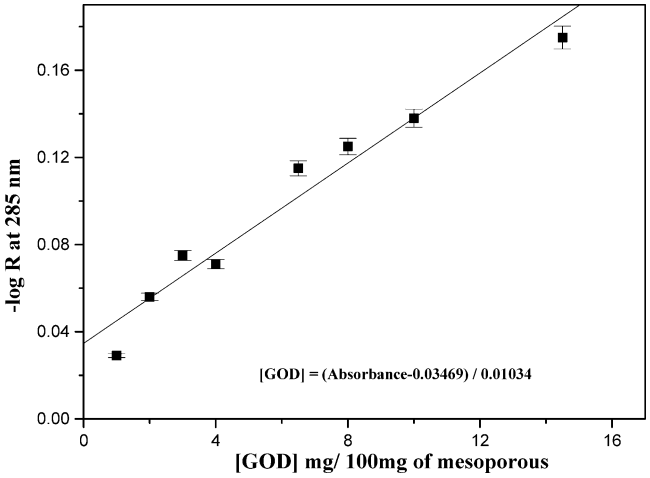

Figure 7. Calibration line used to determine the quantity of glucose oxidase actually immobilized in the silica framework.

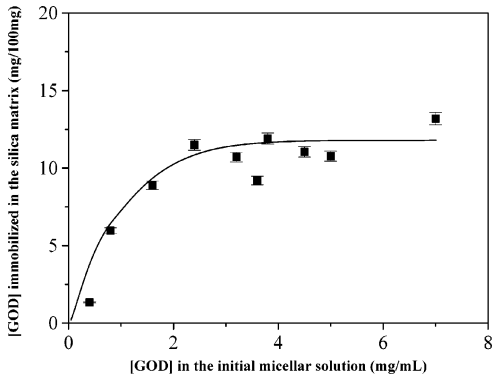

Figure 8. Quantity of immobilized glucose oxidase versus the concentration of GOD in the initial micellar solution.

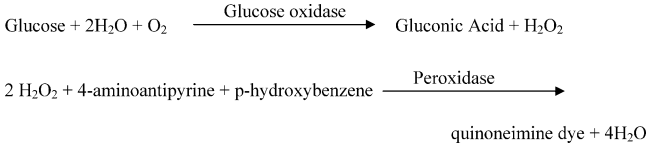

Figure 9. Scheme of the enzymatic reaction of GOD.

entrapped in the material, it disturbs the channel arrangement; SAXS experiments show that a transition from a well-defined hexagonal channel array to a wormhole-like structure appears when the content of glucose oxidase in the initial micellar solution reaches 3.4 mg/mL.

3.4. Activity of the Immobilized GOD. The aim of this part is to check if the immobilized enzyme maintains its activity. A colorimetric method was used to test GOD activity. The system involves two enzymatic reactions (Figure 9). First, glucose oxidase catalyzes, in the presence of molecular oxygen, the oxidation of β-D-glucose to gluconic acid and hydrogen peroxide. The conversion of β-D-glucose into gluconic acid involves the transfer of two protons and two electrons from the substrate to the flavin moiety. In a second step, peroxidase catalyzes the reaction of a dye precursor with hydrogen peroxide to produce a colored dye. The activity was determined from the absorbance change at 510 nm because of the formation of quinoneimine.[43] Three types of samples were prepared; the first one (parent

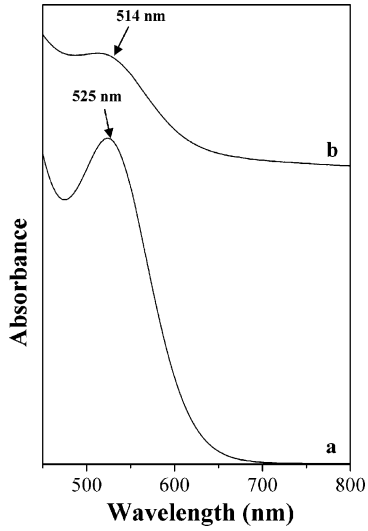

Figure 10. UV spectra showing the activity of the enzyme a: free GOD and b: immobilized GOD.

solution), used to monitor color changes in the UV cell, contained deionized water (50 mL), D-glucose (0.5 g), 4-aminoantipyrine (0.45 g), *p*-hydroxybenzene (0.47 g), and horseradish peroxidase (0.3 mL). If 9.6 mg of free GOD is mixed with 3 mL of the parent solution, the mixture obtained turns red immediately and an absorption maximum at 525 nm (Figure 10a) is observed in the UV spectra. The intensity of this band reflects the activity of free GOD.

The activity of the encapsulated enzyme was also demonstrated by the change of color. Indeed, when 3 mg of mesoporous silica containing GOD was added to 3 mL of the parent solution, after about 2 minutes the resulting solution turned red, as observed for the free GOD. Moreover, the activity of GOD was further confirmed by the detection of an adsorption band at 514 nm, which can be attributed to quinoneimine (Figure 10b).

The evaluation of GOD activity and comparison with the activity of free GOD and GOD immobilized in a conventional sol−gel silica matrix, that is, prepared without surfactant, will be the subject of more detailed studies.

3.5. Discussion. Mesoporous materials have been prepared with a concentration of $R^F_8(EO)_9$ of 10 wt % in water. According to the phase diagram established for $C_8F_{17}C_2H_4$-$(OC_2H_4)_9OH$, this concentration corresponds to a micellar phase (L_1).[26] It is well-known that mesoporous materials are formed through a CTM (cooperative templating mechanism) type mechanism.[44−46] This mechanism is described as follows: in the initial step, the interactions between silica and isolated spherical or cylindrical micelles lead to the formation of an organic−inorganic mesophase. Then, the condensation of the inorganic precursor at the external surface of the micelles occurs. The ordered mesophase is obtained after intermicellar condensation. Finally, the hydrothermal treatment at higher temperature completes the assembly and the polymerization of the silica source. SAXS results show that

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(44) Zhao, D.; Huo, Q.; Feng, J.; Chmelka, B. F.; Stucky, G. D. *J. Am. Chem. Soc.* **1998**, *120*, 6024.
(45) Firouzi, A.; Kumar, D.; Bull, L. M.; Besier, T.; Sieger, P.; Huo, Q.; Walker, S. A.; Zasadzinski, J. A.; Glinka, C.; Stucky, G. D. *Science* **1995**, *267*, 1138.
(46) Lee, Y. S.; Sujardi, D.; Rathman, J. F. *Langmuir* **1996**, *12*, 6202.

from 0 to 3.2 mg of GOD per mL in the initial micellar solution, biosensors with a well-defined hexagonal channel arrangement are obtained. So, in this concentration range, interactions between the enzyme and the oxyethylene groups of the surfactant do not disturb the CTM mechanism.

For glucose oxidase contents greater than 3.2 mg per mL, only wormhole-like mesostructures are formed. Thus, in this case the incorporation of GOD into the micellar solution does not favor the hydrogen-bonding interactions between the oxygen atoms of the oxyethylene group of the surfactant and hydrogen atoms of TMOS; as a consequence, this leads to the formation of a disordered hybrid mesophase instead of a hexagonal one.

We have shown by SAXS and nitrogen adsorption−desorption analysis that neither the *d* spacing nor the pore diameter are influenced by the loading of enzyme added to the starting micellar solution (Table 1), used for the preparation of mesoporous silica. Indeed, whatever the concentration of GOD, the values of *d* spacing and pore diameter remain equal to 5.3 and 3.8 nm, respectively (Table 1). Therefore, glucose oxidase, which is a hydrophilic molecule, is not solubilized in the core of the micelles but it rather interacts with the oxyethylene group of $R^F_8(EO)_9$. Therefore, the following question arises: what is the role played by the surfactant? To answer this question, we have prepared a series of samples without surfactant. No peak is detected on the SAXS pattern; consequently, biosensors prepared in this way have a randomly oriented pore structure. It appears that even if the surfactant is not used to solubilize the enzyme, it plays a key role in the organization of the channel array. Moreover, compounds prepared without $R^F_8(EO)_9$ exhibit a broader pore size distribution and a lower specific surface area than those synthesized in the presence of surfactant (Table 1). From these data, it is obvious that $R^F_8(EO)_9$ acts as a pore-forming agent and it induces a narrower pore size distribution. The surfactant also controls the pore diameter. Thus, when molecular sieves containing GOD are used for the oxidation of D-glucose, internal diffusion of the substrate and product molecules will be greatly facilitated if the biosensors are prepared via the surfactant-template method.

4. Conclusions

The present study reveals that glucose oxidase can be incorporated into silica mesoporous materials via a direct one-step immobilization method. To obtain a hexagonal pore structure, the GOD concentration should not exceed 3.2 mg per mL in the initial micellar solution. By comparison of the properties of biosensors prepared under the same conditions, both with and without surfactant, we have evidenced that the surfactant controls not only the organization of the channels but also the pore diameter.

Results obtained from FTIR and fluorescence spectroscopies indicate that the enzyme is readily incorporated into the mesostructure. We have also evidenced that there is a maximum loading of GOD, around 11 wt %, that can be entrapped in the silica network. If the concentration of the biomolecule is superior to 3.2 mg per mL of micellar solution, glucose oxidase is not incorporated anymore into the mesoporous silica, but the added GOD disturbs the CTM mechanism and wormhole-like mesostructures are recovered.

We have also shown that the immobilized biomolecule maintains its activity.

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