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Synthesis of sponge mesoporous silicas from lecithin/dodecylamine mixed-micelles in ethanol/water media: A route towards efficient biocatalysts

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Abstract

Mixed-micelles of long-chain phosphatidylcholine and surfactants are of considerable scientific and biomedical interest. Lecithins are natural phospholipids from egg or soybean. Lecithin/dodecylamine mixed-micelles in an alcoholic/aqueous media allow to template the formation of sponge mesoporous silica (SMS) materials through a self-assembly process between mixed-micelles and tetraethoxysilane (TEOS). SMS synthesis adds a porosity control to the classical sol–gel synthesis used for enzymes encapsulation. We are reporting here the key parameters of SMS synthesis procedure (amount of amine, TEOS, ethanol, water, lecithin nature, salt addition, etc.), as well as a fine description of SMS structure by TEM. SMS features an isotropic 3-dimensional (3-D) pore structure similarly to SBA-16, but with a lower degree of mesoscopic structural order. Its porosity results from cavities and connecting channels, whose length is controlled by the synthesis conditions. Cavity diameters can reach 4.7 nm in accordance to the lecithin maximum alkyl chain length. Surface areas range from 300 to 800 m^2/g, and pore volumes from 0.30 to 0.85 mL/g. The use of lactose as an enzyme stabilizing agent does not change the pore structure of SMS. A very fragile enzyme, alcohol dehydrogenase, has been successfully encapsulated by this way, providing the first example of successful entrapment of this enzyme in an inorganic matrix. SMS encapsulation procedure is biomolecules friendly and opens a bright perspective for biomolecules processing for biocatalysis, biosensors or biofuel cell applications.

Keywords: Phospholipid; Mesoporous Silica; Sponge phase; Enzyme encapsulation; MCM-41

1. Introduction

Transcription of self-assembled surfactant mesophases to inorganic materials has been the goal for the researchers in the colloidal surface science area for many years. The first successful preparation of mesoporous micelle-templated silica (MTS) materials was achieved using alkylquaternary ammonium surfactants in 1971 by Sylvania Electric Products, Inc. [1] and their properties were disclosed in 1991 by Mobil Oil Company [2]. To date silica remains the most studied material [3]. Nowadays the research is also well advanced for many other elemental compositions in term of surfactants and inorganic species and is in addition motivated by promising results obtained in the field of chromatography, catalysis, and more recently for applications as biomaterials in biocatalysis or biosensors [4–6], and biomedical devices as drug delivery [7]. More than 150 publications are related to proteins or enzymes immobilization in MTS materials. Most of biological or pharmaceutical molecules have been immobilized by adsorption or

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by grafting in MTS. However, the adsorption of biological species is often susceptible to a progressive leaching, whereas the grafting procedure can lead to denaturation of proteins. Proteins are particularly sensitive to environmental conditions and may not be stable at temperature higher than physiological temperature (37 °C), in organic solvent, in pH conditions outside $6 < pH < 8$, in oxidation or drying conditions, and exposure to shear forces as stirring or other physical forces. Protein dehydration can affect both its conformational and its chemical stability. The stability of a protein refers to both its conformational stability, which is reflected in the protein 3-D structure, and its chemical stability, which refers to the chemical composition of the amino acids of the protein. Chemical instability can result from processes such as deamidation or oxidation of the amino acids, or cleavage at any peptide amide linkage. The use of any protonated additive should be avoided, and therefore direct interaction with silanols should be minimizing. Conformational instability can result from aggregation and subunit dissociation. It is known in the art that proteins can be stabilized in solution by addition of soluble excipients that stabilize the monomeric, correctly folded protein conformation. Disaccharides such as trehalose, sucrose or lactose, and surfactants such as phospholipids, Tween and Triton are examples of excipients useful for stabilizing proteins. Polysaccharides or synthetic polymers having a plurality of carboxyl groups are also known to stabilize proteins [8]. In silica sol–gel encapsulation, enzymes [9] or bacteria [10–12] have been successfully immobilized and are biologically active, owing to additives such as sugars, glycerol, charged polymers (poly-vinylimidazole, -ethyleneimine, -ethyleneglycol) or gelatin. Additives help to stabilize proteins against the denaturing stresses encountered upon sol–gel entrapment. Sugar produces significant increase in thermal stability and in biological activity of enzymes [9], and also helps to increase the pore size of the sol–gel silica matrix, which improves substrate delivery and thus activity. Successful studies have been performed by sol–gel methods, for lipases encapsulation [13,14], by using poly(vinyl alcohol), but the diffusivity of the substrates are limited due to the uncontrolled porosity.

Recently, a mesoporous silica material, referred to 'SMS', was synthesized using lecithin/dodecylamine mixed-micelle in the presence of lipase [15–17]. The lipase-SMS exhibited high catalytic activity, compared with traditional sol–gel procedure or post-synthesis immobilization in MTS. It was demonstrated that lactose was the more efficient excipient to preserve enzyme-SMS activity. The high enzyme activity was attributed to the ability to include simultaneously two enzyme stabilizing agents: lactose and lecithin. The high catalytic acitivity was interpreted by the use of lecithin/dodecylamine mixed-micelle permitting facile diffusion of substrates, through the generation of 3-D open mesoporosity in a controllable way. The structure of the lipase-SMS was certainly disordered, as evidenced by XRD and transmission electron micrograph (TEM) images. However, the detailed structure was not characterized. Particularly, the '3-D open mesoporosity' was suggested but unproved yet. The role of each reactants, as in particular ethanol, in directing SMS synthesis was not elucidated.

In the present work, we investigated influence of the detailed synthesis parameters on SMS structures. We have confirmed that, under favorable conditions, SMS has open mesopores that are branched in a 3-D disordered way. The branching shape of mesopores has been visualized by a Pt TEM imaging technique. Furthermore, we show that the biocompatible method of SMS formation can even allow encapsulation of very fragile enzyme needing a cofactor such as alcohol dehydrogenase.

2. Experimental section

2.1. Materials

2.1.1. Lecithins

Egg yolk lecithin (Fluka) and soybean lecithin (Aldrich) are natural and low cost phospholipids called also 3-*sn*-phosphatidylcholine (Fig. 1). They consist in fact of a mixture of phospholipids with different chain lengths and insaturations on the fatty acyl chain. The acyl chains will be noted Cn:m with n, the number of carbon atoms of the chain and m, the number of insaturation in the chain. Egg yolk lecithin is composed of 1.8% myristic acid (C14:0), 36.5% palmitic acid (C16:0), 14% stearic acid (C18:0),

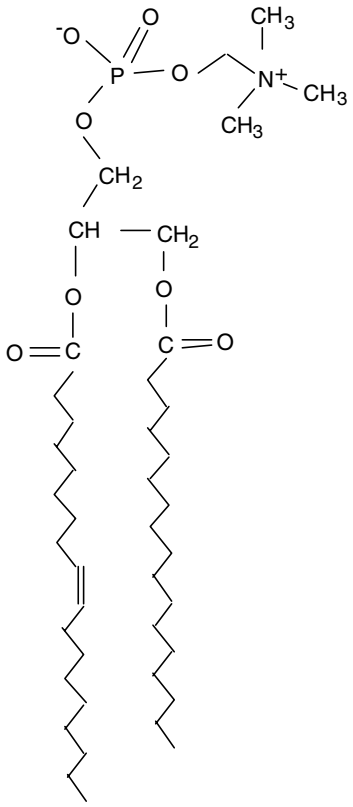

Fig. 1. Schematic model of lecithin.

4.3% palmitoleic acid (C16:1), 30% oleic acid (C18:1), 13.5% linoleic acid (C18:2). Soybean lecithin is composed of 11.7% palmitic acid (C16:0), 4% stearic acid (C18:0), 8.6% palmitoleic acid (C16:1), 9.8% oleic acid (C18:1), 55% linoleic acid (C18:2), 4% linolenic acid (C18:3), 5.5% C20-C22-arachido(22:4).

2.1.2. SMS synthesis

SMS syntheses were performed by using the following reactants: TEOS (Aldrich), dodecylamine (Aldrich), lecithin from egg yolk (L-α lecithin) (Fluka) or soybean lecithin (Aldrich), β-D-lactose (Aldrich). For the standard synthesis (SMS1), the molar ratios used were: 1 TEOS/0.055 lecithin/0.042 dodecylamine/0.034 lactose/28 H_2O/8.5 ethanol. A first solution of 0.15 g lactose and 7 mL H_2O was prepared and added slowly under stirring to a second solution containing 0.5 g lecithin and 0.1 g dodecylamine in 5.2 g ethanol, until a homogeneous emulsion was formed. 2.7 g TEOS was then added slowly under stirring for 15 min, and, the mixture was left for 24 h in static conditions at room temperature. The resulting powder was then centrifuged and washed five times with 10 mL of ethanol/water mixture (1/1, v/v) followed by centrifugation. The samples were then dried for 16 h at 50 °C. The calcination of the resulting materials was performed under air flow at 550 °C for 8 h.

2.1.3. Study of SMS synthesis parameters

Variations of SMS synthesis parameters were studied (Table 1) as follows:

- TEOS molar ratio of 1 (SMS1), 1/2 (SMS2), 1/4 (SMS3)
- lecithin nature: lecithin from egg (SMS1, SMS2) or from soybean (SMS9, SMS10)
- NaF addition (SMS5, SMS6, SMS11): 200 μL of 1 M NaF was added with the aqueous solution of lactose
- NaCl addition (SMS4): 0.4 g NaCl instead of NaF
- hydrothermal treatment (SMS7, SMS12): the synthesis was left in autoclave for 24 h at 50 °C
- aging time (SMS8): an aging time of 6 d in static conditions instead of 24 h.

In addition, the influence of amine amount (Table 2) and the ethanol/water ratio (Table 3) were investigated as well.

2.1.4. Blank reactions

In order to determine what is the key role of each organic component in SMS formation, syntheses have also been performed in the absence of dodecylamine, of alcohol and of lactose.

2.1.5. Direct encapsulation of enzymes into SMS

A very fragile enzyme, an alcohol dehydrogenase, (ADH) (140 kDa) from baker yeast (Sigma) has been encapsulated in SMS by direct synthesis method. ADH is specific for ethanol sensing. ADH oxydizes ethanol into acetaldehyde by the intermediacy of a cofactor, called Nicotinamide Adenine Dinucleotide (NAD^+) (Sigma), which is in turn reduced in NADH. Two different ways of encapsulation have been performed: the encapsulation of ADH alone and the co-encapsulation of ADH with its cofactor NAD^+.

ADH encapsulation alone: 5 mL of ADH solution of 0.3 mg/mL (1.5 mg ADH) in a Tris buffer solution at pH 7 were mixed with 0.15 g β-lactose, and then added to the

Table 2
Textural features of MTS synthesized with lecithin from yolk egg and the following composition: 0.5 TEOS/0.05 lecithin/x dodecylamine/0.03 lactose/8.5 EtOH/30 H_2O with different dodecylamine/lecithin ratio; and calcined at 550 °C

Samples	$C_{12}NH_2$/Si	D_{BdB} (Å)	V (mL/g)	S (m^2/g)
SMS13	0.05	42	0.43	498
SMS14	0.06	42	0.46	517
SMS2	0.08	42	0.54	615
SMS15	0.10	42	0.56	614
SMS16	0.13	42	0.69	721

Table 1
Textural features of MTS synthesized with lecithins by different routes with: x TEOS/0.05 lecithin/0.04 dodecylamine/0.03 lactose/8.5 EtOH/30 H_2O; and calcined at 550 °C

Samples	Lecithin	x TEOS	Treatment	D_{BdB} (Å)	V (mL/g)	S (m^2/g)
SMS1	Egg	1	Standard	33 (broad)	0.26	"430"
SMS2	Egg	0.5	Standard	42	0.54	615
SMS3	Egg	0.25	Standard	39 (+130 double)	0.46 (0.54)	510
SMS4	Egg	1	+NaCl	np	np	np
SMS5	Egg	1	+NaF	46	0.72	676
SMS6	Egg	0.5	+NaF	47	0.85	790
SMS7	Egg	1	50 °C	47	0.48	481
SMS8	Egg	1	6 d	38 (broad)	0.38	"567"
SMS9	Soybean	1	Standard	47	0.40	419
SMS10	Soybean	0.5	Standard	47	0.59	545
SMS11	Soybean	1	+NaF	47	0.62	603
SMS12	Soybean	1	50 °C	47	0.43	391

np: non porous; "S": equivalent surface area ±30%.

Table 3
Textural features of MTS synthesized with lecithin from egg, 1 TEOS/0.05 lecithin/0.04 dodecylamine/0.03 lactose/x EtOH/y H_2O; and calcined at 550 °C

Samples	x EtOH	y H_2O	EtOH/H_2O	D_{BdB} (Å)	V (mL/g)	S (m^2/g)
M1	6	30	0.20	np	np	211
M2	8.7	39	0.23	np	np	401
SMS1	8.7	30	0.30	36 (broad)	0.34	"538"
M3	4.4	13	0.34	np	np	38
SMS1′	8.7	13	0.68	35 (broad)	0.30	"452"

np: non porous.

alcoholic solution (5.2 g ethanol) containing 0.5 g lecithin and 0.1 g dodecylamine. 2.7 g TEOS were then added slowly under stirring for 15 min, and, the mixture was left for 24 h in static condition at room temperature. This corresponds to an initial ratio of 3 mg ADH/g silica. The resulting yellow/white powder was then centrifuged and washed five times with 10 mL of ethanol/water mixture (1/1, v/v) followed by centrifugation. The sample was then dried for 12 h at 50 °C.

ADH with its cofactor encapsulation: 0.28 mg of cofactor NAD^+ was added to the 5 mL solution of ADH. The following steps were the same as above.

ADH with its cofactor adsorbed in MCM-41. In order to compare SMS direct encapsulation with adsorption, 10 mg ADH and 2 mg of NAD^+ were mixed in 5 mL Tris buffer solution (0.1 M sodium dihydrogenophosphate) at pH 6.5 with 0.25 g of calcined MCM-41 featuring 12 nm pore diameter, corresponding to 40 mg ADH/g silica. The mixture was stirred for 2 h at 4 °C. MCM-41 of 12 nm pore size was synthesized accordingly to reference [18,19]. The supernatant was removed by centrifugation and the solid was washed with buffer solution and dried at room temperature.

To quantify the amount of enzyme present on the three biomaterials, the enzyme was released from the solid by proceeding to a basic hydrolysis of the biohybrid material at pH 9 followed by elution through G25 Sephadex column, and also in the supernatant after separation of enzyme and other organic molecules by G25 exclusion chromatography. The amount of enzyme was determined by the Bradford method, following the procedure used in the case of lipase-SMS encapsulation [15,16].

2.1.6. Enzymatic activity

The enzymatic activity of the encapsulated enzyme was tested towards ethanol oxidation in order to test its ethanol biosensor activity. 0.3 g of biomaterial was added to 28 ml of Tris (hydroxymethyl) aminomethane buffer (0.1 M, pH 8.8) with 1 mL of NAD^+solution (0.1 M) and 1 mL of absolute ethanol. Each minute, aliquots of 0.3 mL of supernatant was taken and put in 2.7 mL buffer solution to be analyzed in UV–Vis at 365 nm ($\varepsilon = 3400$ L ol^{-1} cm^{-1}) to determine the initial velocity of NADH formation. The initial activity of the ADH solution for SMS encapsulation was: 15.75 UI/mg ADH (IU: micromol NADH/min) and for adsorption in MCM-41: 34.68 IU/mg.

2.1.7. Pt/SMS composites

The Pt/SMS composites and replicas were prepared following the procedure reported in the literature [20,21]. The calcined SMS silica sample (0.2 g) was loaded with tetraamine platinum (II) nitrate ($Pt(NH_3)_4(NO_3)_2$, (Aldrich)) (0.16 g) by repeating alternation of the impregnation of aqueous solution (5×10^{-5} mol/L) (solution to sample ratio was 100 mL/g) and drying at 100 °C four times. The Pt/SMS samples were then heated under H_2 flow from room temperature to 300 °C for 4 h and maintained at 300 °C for 2 h using a U-tube reactor equipped with two fritted disks. The Pt/SMS samples were then put in a vacuum for 30 min at 300 °C prior to exposure to air at room temperature. The silica framework of Pt/SMS composites was dissolved completely with HF (10%). The Pt residues were filtered and washed with distilled water. An ethanol dispersion of these Pt/SMS composites and the corresponding Pt replicas was dropped onto TEM grids, and the solvent was dried at room temperature.

2.1.8. Measurements

Powder X-ray diffraction patterns were collected on a CGR Thêta-60 diffractometer with Inel drive, using monochromatic Cu Kα radiation and four 0.25 mm slits. Nitrogen adsorption-desorption isotherms at 77 K were recorded using a Micrometrics ASAP 2010 apparatus. Calcined samples were outgassed at 250 °C until a stable static vacuum of 3×10^{-3} Torr was reached. Specific surface areas were calculated by the BET method using the isotherm adsorption data in the range from $p/p_0 = 0.12$–0.25 just below capillary condensation. For pore sizes at the frontier between micropores and mesopores presenting a "knee-curve" nitrogen adsorption-desorption isotherm, the BET surface area is at its limit of validity and its calculation gives an equivalent surface rather than a real surface area which is at ±30% of its real value [22]; this so called equivalent surface will be referred as "S". Pore diameters were evaluated from the isotherm desorption branch by the Broekhoff and de Boer method [23], which has been demonstrated as one of the more accurate for MCM-41-type materials [24]. The pore volume was taken at the top of the adsorption step corresponding to the pore filling. Thermogravimetric analysis (TGA) was recorded under an oxygen flux on a TG 209C analyser (Netzsch Proteus). Elemental analysis was performed at CNRS center of Vernaison, France. TGA and EA data were used to

calculate the volume occupied by organic, and accordingly to evaluate the potential pore volume, in the as-synthesized hybrid materials. Transmission electron microscopy (TEM) images were obtained using a Philips CM 20 apparatus operating at 100 keV. Scanning electron microscopy (SEM) was performed with a HITACHI instrument.

3. Results and discussion

3.1. SMS synthesis strategy

Lecithin is a trivial name for 1,2-diacyl-*sn*-3-phosphatidylcholine which schematic formula is presented in Fig. 1. Lecithin contains residues of phosphatocholine, glycerol and two fatty acids; being a zwitterionic specie. It belongs to the phospholipids family, which form the lipid matrix of biological membranes. As a biocompatible surfactant, it is widely used in every day life, including human and animal food, medicine, cosmetics and manifold industrial applications [25]. Because of its nearly cylindrical molecular shape, lecithin cannot form micelles in aqueous media. Its tendency to curve, described in terms of its spontaneous curvature, is very low, close to zero or positive, and therefore it forms lamellar structures or vesicles or multilayers such as liposomes (Fig. 2 – left). By addition of an appropriate surfactant usually used for biological membranes solubilization, the transition of vesicles to micelles is possible due to the spontaneous curvature induced by mixed-micelles formation [26]. Therefore, nearly flat phospholipid bilayers (containing embedded proteins) transform into mixed-micelles composed of surfactant, phospholipid and membrane-bound proteins via the transient formation of a variety of kinetically and thermodynamically controlled structures, including so-called sponge phases. The most used surfactants are nonionic alkyl glucosides as dodecyl maltoside [27], octylphenylpolyoxyethylene such as Triton X-100 [28] or *n*-dodecyloctaethylene glycol ($C_{12}EO_8$) [29] and anionic bile salts [30].

With the objective of synthesizing silica MTS materials as protein hosts from these mixed-micelles, we used dodecylamine as co-surfactant. The fatty amine is anticipated to play a double role in the synthesis. Firstly, it will promote the decrease of the spontaneous curvature of the phospholipid bilayers. The spontaneous curvature C_0 of the bilayer is closely related to the geometric parameter g defined by Israelichvili [31] and is therefore related to v_0/a_0l_0 where v_0 is the chain volume, a_0 the surface of the polar head, l_0 the chain length of the organic chain. Insertion of dodecylamine between the head groups of surfactants results in a decrease of the distance between the polar heads, via strong electrostatic interaction with phosphate groups, and leads to the decrease of the spontaneous curvature (Fig. 2 – right). The phase transition between vesicles (disconnected porosity) to sponge mesophase (multiconnected porosity) is then the result of local topology transformation due to closeness of polar heads [32]. As a second main effect, the fatty amine plays the role of nucleophilic catalyst to promote the condensation of the silica, without generating elevated pH, as in the synthesis of mesoporous HMS materials [33,34]. Work at moderate pH is indeed advantageous to avoid protein denaturation.

The first attempts to synthesize aluminosilicate MTS materials using mixtures of lecithin/octadecylamine or lecithin/cetyltrimethylammonium bromide as templates have produced however materials with bimodal pore sizes, poor structure ordering and broad pore size distributions [35,36]. These syntheses were performed by hydrothermal treatment at 80 °C for 72 h in basic sodium hydroxyde media. The lack of pore size control and structure order control was attributed to the fact that lipid-based architec-

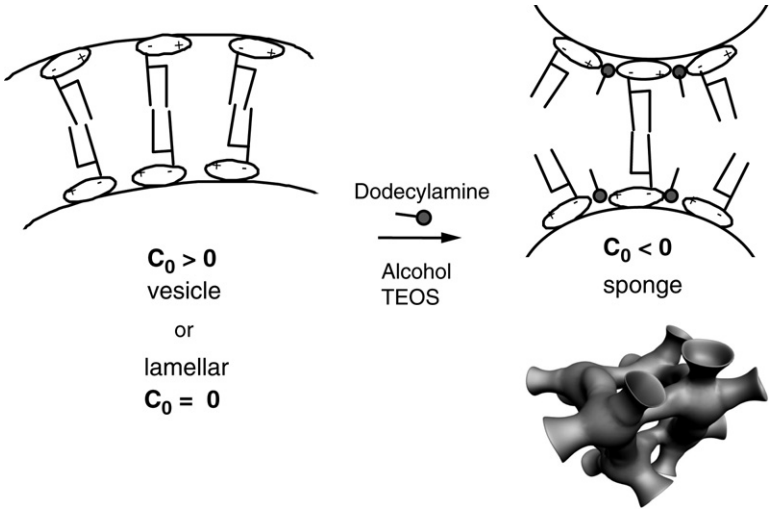

Fig. 2. Vesicle to sponge-like phase transition by addition of dodecylamines/alcohol and silica source to lecithin vesicles. C_0 being the spontaneous curvature of each phase.

tures are labile, dynamic constructions, with poor templating ability under the conditions of the hydrothermal synthesis.

Phospholipid assemblies can be however significantly stabilized in a mixed water/ethanol system by forming interdigitated layers with a significant degree of acyl chain overlap [37,38]. The phospholipids/water/ethanol systems lead to gels comprised of highly organized stacks of lamellar layers at high ethanol content, to interdigitated layers (sponge phase) at moderate ethanol content and to large multilamellar or smaller homogeneous unilamellar liposomes at low ethanol content. Liposome formation at low ethanol content is thought to proceed via formation of stalk contacts between neighboring layers, a mechanism very similar to that leading to the sponge phase. Such lipid-derived assemblies appear therefore as very promising to template the formation of materials with highly interconnected networks of pores. It is known, however, that phase diagrams in the presence of silica cannot be predicted directly from the above studies. This led us therefore to investigate in some detail the silica/lecithin/dodecylamine/ethanol/ water system, in the presence and/or in the absence of lactose and protein.

3.2. Blank experiments

The synthesis of SMS in the absence of dodecylamine, i.e. in the system silica/lecithin/ethanol/water, leads to a very small amount of solid. Only 3% of the silica engaged is recovered. The resulting solid forms a mixture of multilamellar ribbons (5–6 layers) and bilayer vesicles as evidenced by TEM (not shown). The synthesis without alcohol, (with or without amine) in pure water, also gives rise to a lamellar material. This confirms the need for the two components (synergistic influence) dodecylamine and ethanol in generating porous SMS materials.

3.3. Influence of synthesis parameters on SMS properties

Different parameters have been varied in order to investigate their influence on the properties of the organic/inorganic as-synthesized materials, the stability of the silica network upon calcination and, ultimately, to control SMS formation (Tables 1 and 2).

The reaction of what we define as our standard synthesis mixture composition: 1 SiO_2/0.055 lecithin/0.042 dodecylamine/0.034 lactose produces a material (SMS1) with molar composition: 1 SiO_2/0.044 lecithin/0.019 dodecylamine/0.012 lactose. The yield of silica is around 60%. This corresponds to an as-synthesized hybrid solid with an organic volume of 0.80 mL/g. After calcination at 550 °C, the resulting material features a pore volume of only 0.26 mL/g, a broad pore size distribution and a pore size of 3.3 nm (Fig. 3, Table 1). This pore size is small compared to that expected from the size of a lecithin/amine mixed-micelle which should be determined by the two fold of the alkyl chain length of the lecithin (4.7 nm). The 60% pore volume decrease with reference to the volume initially occupied by organic in the as-synthesized solid means that severe re-construction of the silica network happened upon calcination. The synthesis performed by engaging a lower amount of silica (SMS2), corresponding to molar ratios of 1 SiO_2/0.110 lecithin/0.084 dodecylamine/0.068 lactose led to an as-synthesized solid of the following composition: 1 SiO_2/0.093 lecithin/0.049 dodecylamine/0.022 lactose. This composition indicated an inorganic content equivalent to 1.74 mL/g. After calcination the actual pore volume was 0.54 mL/g, which corresponds to a 69% decrease. It is interesting to note that whatever the amount of TEOS engaged in the synthesis, the initial molar ratio of 1/0.8 lecithin/dodecylamine of synthesis mixture is decreased to 1/0.5 lecithin/dodecylamine in the as-synthesized material. Equivalent organic compositions were found in the SMS,

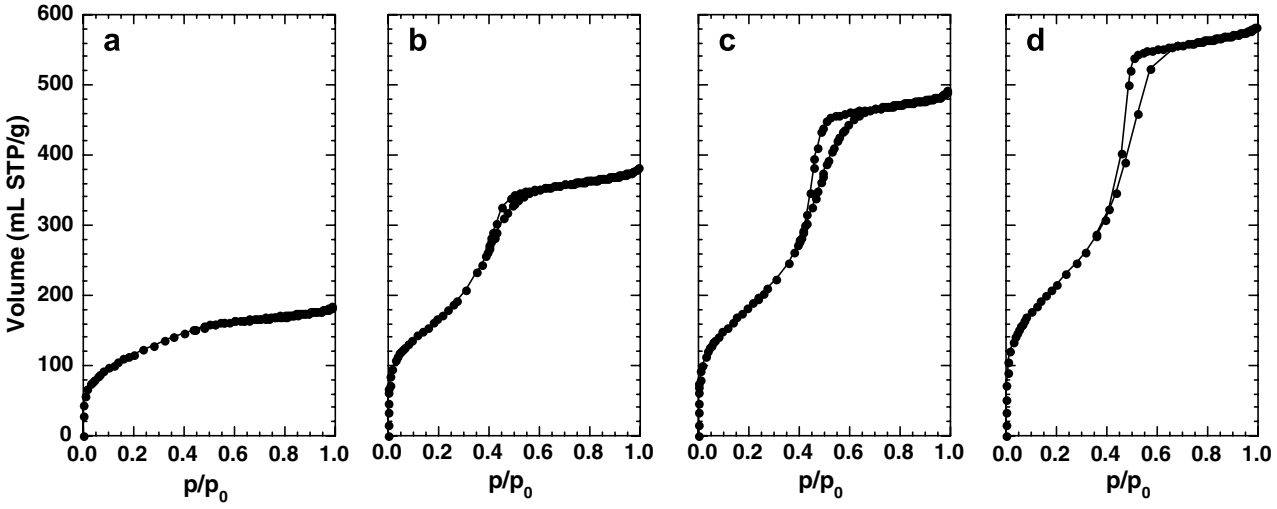

Fig. 3. Nitrogen adsorption-desorption isotherms at 77 K of (a) SMS1 – standard synthesis with 1 TEOS, (b) SMS2 – standard synthesis with 0.5 TEOS, (c) SMS5 – synthesis with NaF and 1 TEOS, and (d) SMS6 – synthesis with NaF and 0.5 TEOS.

corresponding to the molar ratios: 1 lecithin/0.5 dodecylamine/0.25 lactose. Similar large losses of pore volume (60–69%) upon calcination were observed for MCM-48 materials (pore volume loss: 64–72%) [39], whereas for MCM-41 and HMS materials corresponding losses were of only 20% and 40–50%, respectively. This observation suggests that SMS behavior is more related to that of a cubic structure than to that of a hexagonal structure. Actually, SMS materials synthesized from lecithin/dodecylamine mixed-micelles are best described (see below) as sponge-like phases which are disordered forms of the cubic phase.

By increasing the synthesis temperature to 50 °C (SMS7), it was possible to maintain a higher pore diameter (4.7 nm) and to keep a larger part of the porosity of the as-synthesized SMS. The loss of pore volume was only of 37%. An increase in temperature is expected to promote silica condensation and therefore offer a more stable structure at calcination. The addition of NaF (SMS5) plays a similar role as the temperature by enhancing the degree of silica reticulation due to its catalysis effect on silanol condensation and hence only 11% of as-synthesized volume has been lost. SMS5 exhibits a pore volume of 0.72 mL/g and a surface of 676 m^2/g (for a pore diameter of 4.6 nm). A larger pore volume (0.85 mL/g) and surface area (790 m^2/g) can be reached by decreasing the amount of silica in the synthesis mixture (SMS6). This stabilization of pore mesostructure by NaF is due to the special catalytic effect of fluoride anions and not to a salt effect since the addition of NaCl leads to a nonporous structure (SMS4). Increasing the synthesis time at room temperature for 6 d (SMS8), does not change the structuration compared to the standard synthesis performed in one day. The positive effect of dodecylamine (Table 2) in promoting silica condensation is clearly evidenced by the parallel increase of pore volumes and surface areas in calcined SMS. Both characteristics are directly proportional to the dodecylamine/silica ratio (Fig. 4). A higher degree of silica condensation limits pore restructuration during calcination. A pore volume as high as 0.70 mL/g and a surface area of 721 m^2/g have been obtained for the ratio dodecylamine/TEOS = 0.13 (SMS16). The use of lecithin from another origin, lecithin from soybean instead of egg lecithin, leads to similar results. A constant pore diameter of 4.7 nm equal to twice the lecithin alkyl chain has been found for all materials SMS9, SMS10, SMS11, SMS12. Again, the addition of NaF (SMS 11) and the use of half silica (SMS10) in the synthesis generate a better porous silica structure after calcination.

In the case of lipase encapsulation in SMS materials, syntheses performed by adding NaF or using soybean lecithin led to biomaterials with reduced catalytic activity [15]. SMS1 or SMS2 type materials appear therefore as the best candidates for further enzyme encapsulation.

3.4. Porous structure of SMS

SMS prepared from lecithin/dodecylamine mixed-micelles were characterized by XRD (Fig. 5) before and after calcination. All SMS materials reveal almost the same structural features: they exhibit a single broad diffraction peak at low angles corresponding to a d-spacing of about 7 nm (Fig. 5a). No lattice contraction is observed after calcination, but instead a slight increase in d-spacing (7.8 nm, Fig. 5b). This peak could be assigned to the distance between two pores. From only a single XRD peak, it is difficult however to draw conclusions on the pore arrangement or pore symmetry since such a signal could be associated to a 1-D wormlike, disordered hexagonal structure, as for HMS materials [34] or to a sponge-like 3-D branched, disordered cubic structure. SMS materials exhibit characteristic nitrogen sorption isotherms (Fig. 3)

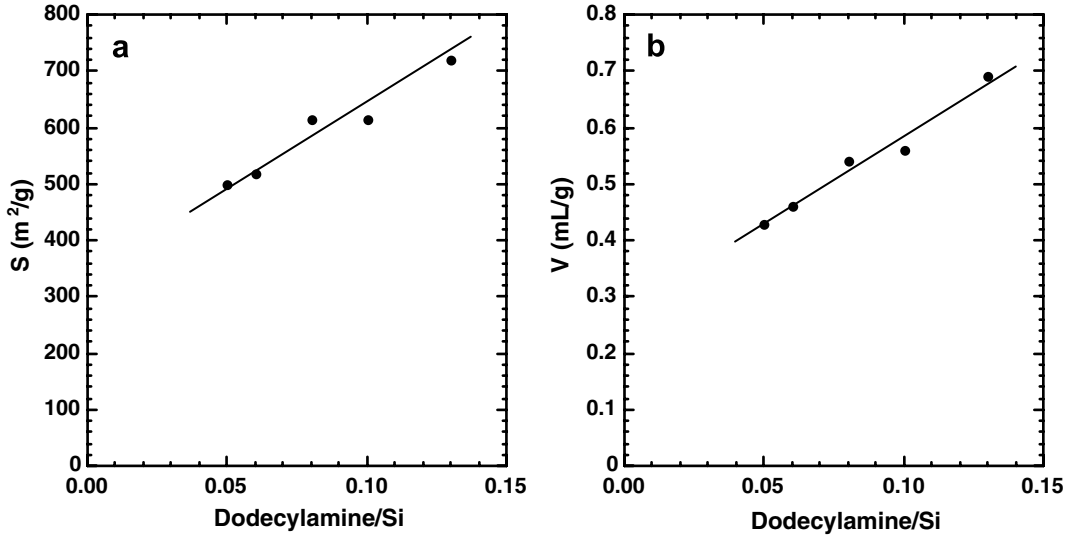

Fig. 4. Influence of dodecylamine/silica molar ratio on (a) surface areas and (b) pore volumes of SMS materials (SMS13, SMS14, SMS2, SMS15, SMS16) synthesized at room temperature with the following ratio: 0.5 TEOS/0.05 lecithin/x dodecylamine/0.03 lactose/8.5 EtOH/30 H_2O and calcined at 550 °C.

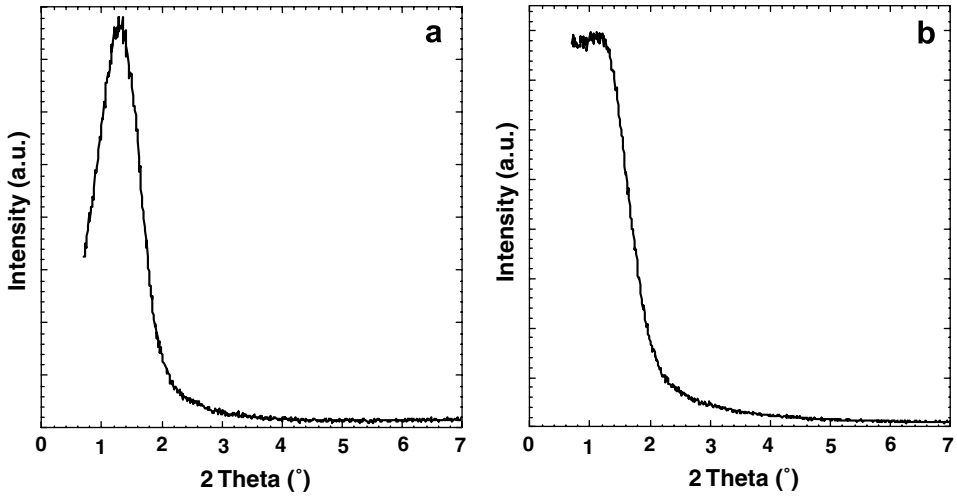

Fig. 5. XRD pattern of (a) as-synthesized, (b) calcined sponge-mesoporous silica SMS2 synthesized from mixed-micelles lecithin/dodecylamine. Same XRD pattern are found for the other SMS materials.

depending on their synthesis conditions. Larger pore size, larger pore volume and larger specific surface area were obtained for samples prepared in the presence of NaF (SMS5 and SMS6) (Table 1). The pore volume is increasing in the following order: SMS1 < SMS2 < SMS5 < SMS6. Pore sizes of SMS1 and SMS2 are close to 3–4 nm, whereas for SMS5 and SMS6, the pore diameter reaches 4.7 nm with some delay in the desorption suggesting some pore restrictions or cages formation. Pore diameter of 4.7 nm corresponds to twice the length of the octadecyl hydrophobic chain of the lecithin. Such pore diameter is in accordance with a templating effect of a mixed-micelle, which micellar diameter is directed by the length of the hydrocarbon chain (with 18 carbon atoms) of lecithin.

As the contrast of the as-synthesized and calcined solids was not high enough, the pore architecture of SMS materials has been examined by the Pt TEM imaging technique developed by Ko et al. [20]. The Pt/SMS composites have been imaged by TEM. We have chosen to examine Pt/SMS composites instead of Pt replicas in order to avoid misinterpretations on the connectivity of the porous network that could result from agglomeration of the Pt particles replicas upon dissolution of the silica (a usual phenomenon encountered in the case of spherical cavities). The TEM images of the Pt/SMS composites (Figs. 6 and 7) evidence the 3-D connectivity of the porous network of pores or channels, which is higher or lower in degree depending on the samples and therefore on the synthesis conditions. The disordered pore network of SMS materials can be featured as an "isotropically disordered pore network". The samples indicate the following decreasing degrees of isotropic order: SMS6 > SMS5 > SMS1 > SMS2 > HMS. (A HMS material with similar pore diameter, 4.1 nm, synthesized according to Ref. [40] with a molar ratio $EtOH/H_2O = 0.19$ [40], was used as comparison). The pore structure of SMS6 (NaF and 1/2 TEOS) is spherical with narrow windows, featuring a disordered SBA-16 [41]

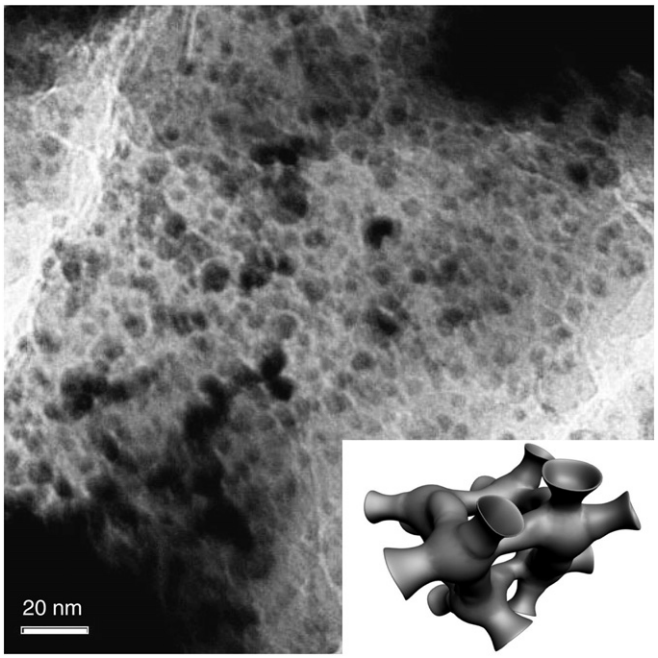

Fig. 6. TEM pictures of SMS6 materials showing the sponge structure. *Inset*. Schematic representation of the pore structure of SMS materials.

type. The shape of the Pt network inside silica pore system of SMS6 is closer to spherical (or globular shape) as revealed by the shape of the Pt agglomerates formed inside the porous silica material. SMS5 (NaF and 1 TEOS) contains very short cylindrical and branched channels, forming a completely isotropic 3-D network. SMS1 (1 TEOS) possess more elongated channels. In SMS2 (1/2 TEOS) the channels are even longer, close to the structure of HMS. The channel shape becomes more elongated as the connectivity decreases. In HMS, the pore structure is a channel type. However, the elongated channels of HMS are somewhat branched, but it is not completely isotropic as in SMS materials. HMS appears as disordered hexagonal

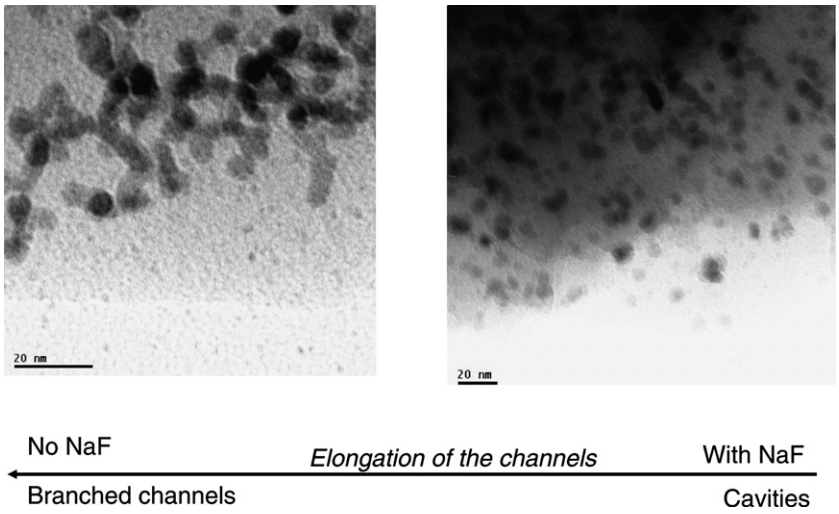

Fig. 7. TEM pictures of (left) SMS2/Pt and (right) SMS6/Pt composites, revealing the formation of cavities for synthesis with NaF and interconnected elongated channels for synthesis without NaF.

structure, whereas SMS5 and SMS6 as disordered cubic structures. SMS1 and SMS2 are intermediate structures with branched elongated channels. NaF addition in SMS synthesis reaction mixture favors cages formation by increasing silica condensation rate and therefore stabilizing previous kinetic intermediates featuring longer channels.

3.5. Role of ethanol in SMS synthesis

The important role of ethanol in controlling the porosity of materials using fatty amines as templates has already been pointed out in the case of HMS materials [34,40]. Syntheses of SMS with different $EtOH/H_2O$ ratios have been performed in order to check if if the synthesis of SMS in the presence of lecithin/dodecylamine mixed-micelles follows the same trend as that of HMS with regards to the role of the $EtOH/H_2O$ ratio (Table 3) [40]. In HMS synthesis, pore size, pore volume, surface area and particle size are determined by the $EtOH/H_2O$ ratio in the range 0.19–0.95 [40]. At low $EtOH/H_2O$ ratio, such as $EtOH/H_2O = 0.19$, HMS materials with large pore diameter (4.1 nm), large pore volume (0.86 mL/g) and small particle size (0.2 μm) are obtained. By contrast, at high $EtOH/H_2O$ ratio, such as $EtOH/H_2O = 0.95$, HMS features small pore size (3 nm), small pore volume (0.50 mL/g) and larger particle size (1 μm). SEM pictures of SMS synthesized using lecithin/dodecylamine mixed-micelles show aggregation of microspheres of about 2 μm in diameter (Fig. 8), which size is larger than the largest particle size obtained for HMS materials. The behavior of HMS vs. alcohol was explained by the position of EtOH, close to the head of the dodecylamine, leading to a larger polar head of surfactant for higher amounts of EtOH and therefore to a decrease of the micelle size. In the case of SMS formed with lecithin/dodecylamine mixed-micelles, a somewhat different behavior is revealed (Table 3). At low $EtOH/H_2O$ ratio ($EtOH/H_2O = 0.20$), the materials are nonporous. At high

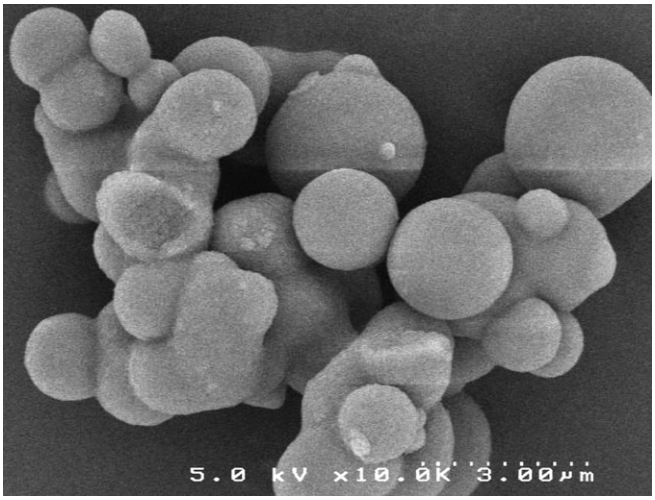

Fig. 8. SEM pictures of SMS2 materials.

$EtOH/H_2O$ ratio ($EtOH/H_2O = 0.70$), the material (SMS1′) becomes porous and similar to the standard materials (SMS1) obtained with $EtOH/H_2O = 0.30$. Furthermore, for a synthesis (M3) performed with a similar $EtOH/H_2O$ ratio ($EtOH/H_2O = 0.34$), but with a lower amount of alcohol, the solid obtained is nonporous. A minimum amount of EtOH ($EtOH/Si > 8.7$) and a maximum amount of H_2O ($13 < H_2O/Si < 30$) are required to achieve SMS synthesis. Thus, SMS synthesis using lecithin/dodecylamine mixed-micelles is not controlled by the $EtOH/H_2O$ ratio like for HMS materials. The lecithin/dodecylamine mixed-micelle, which originally forms a lamellar silica mesophase, changes its curvature to the negative side as shown in Fig. 2 thanks to the addition of ethanol. The structuring mixed-micelle will therefore generate mesoporous silica structures for only a certain composition of alcohol and water. The fact that the SMS mesostructure appears in some range of composition is characteristic of

the existence of a phase diagram for the system SiO_2/lecithin/dodecylamine/EtOH/H_2O and not in favor of a co-surfactant effect of EtOH. EtOH has a dehydration effect on phospholipids layer as in the phase diagram phospholipid/EtOH/H_2O[37] and favors the closeness of phospholipid bilayer and the interdigitation of phospholipid chains, responsible for the sponge phase formation.

3.6. Influence of lactose

The presence of lactose in the starting reaction mixture has no influence at all on the properties of the as-synthesized and calcined SMS materials. Lactose does not contribute to pore expansion of the mixed-micelle lecithin/dodecylamine and therefore should be more probably located at the micelle/silica interface and so replaces some water molecules. The main role of lactose is then to protect the enzymes (when added) without affecting the synthesis process.

3.7. Activity of SMS-based biocatalysts

Lipases [15,16] immobilized by direct synthesis into SMS-type materials demonstrated much higher activity for ester hydrolysis than any other biomaterials prepared by classical sol–gel synthesis or by adsorption into mesoporous silica such as MCM-41s with varying pore sizes and surface polarities. The hydrolytic activity of lipase-SMS catalysts was even higher than commercial immobilized lipase (Fig. 9) [16].

In the present work a much fragile enzyme, alcohol dehydrogenase (ADH) has been immobilized either alone or co-immobilized with its co-factor nicotinamide adenine dinucleotide (NAD^+) in SMS. The activity of the biocatalyst was measured for alcohol oxidation (Fig. 10) and compared to that of a similar material prepared by co-adsorption of ADH and NAD^+ in a MCM-41 material

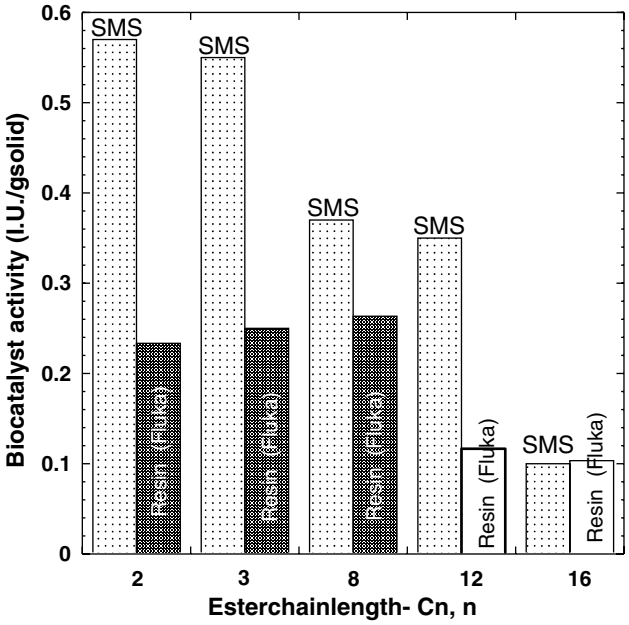

Fig. 9. Biocatalyst activity of lipase encapsulated in SMS materials compared to a commercial biocatalyst containing the same lipase (ion-exchange resin from Fluka) towards hydrolysis of esters of *p*-nitrophenol as a function of the ester chain length Cn. (IU: micromol of ester hydrolyzed per min). Figure adapted from Ref. [16].

with 12 nm pore apertures featuring 2.1 mL/g pore volume and 900 m^2/g surface area.

The results are presented in Table 4 and Fig. 11. The amount of enzyme immobilized in SMS is low (1 mg enzyme/g of SMS, which corresponds to the incorporation of 62% of the amount of enzyme engaged in the synthesis) compared to that incorporated by adsorption into the large-pore MCM-41-type materials (10 mg/g of biocatalyst, which corresponds to 28% of incorporation). This different amount of incorporation might come the different interaction of the enzyme with the supports. In MCM-41, enzyme might interact by strong electrostatic interactions

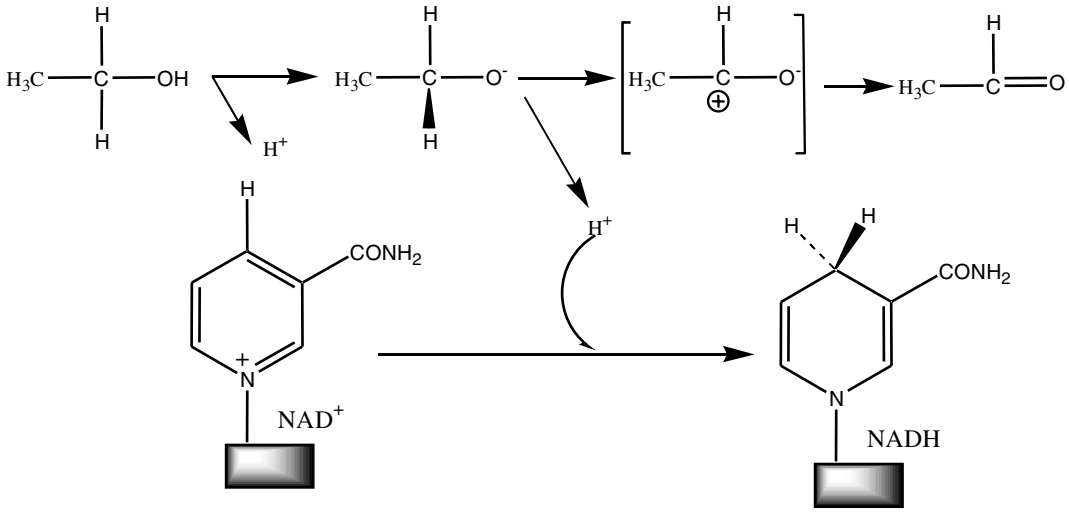

Fig. 10. Schematic representation of the oxidation of ethanol into acetaldehyde by ADH with the reduction of the NAD^+ co-factor.

Table 4
Amount of ADH in the different biomaterials, catalytic activity of the biocatalysts per g of solid, per mg of enzyme and compared to the activity of the native ADH

Biocatalysts	Amount of enzyme ($mg/g_{biocatalyst}$)	Activity ($IU/g_{biocatalyst}$)	Specific activity (IU/mg enzyme)	Relative specific activity (%)
ADH-SMS	1.08	0.44	0.41	2.6
ADH/NAD^+-SMS	0.99	1.01	1.02	6.5
ADH/NAD^+-MCM41	10.3	1.99	0.19	0.6

IU: micromole NADH/min.

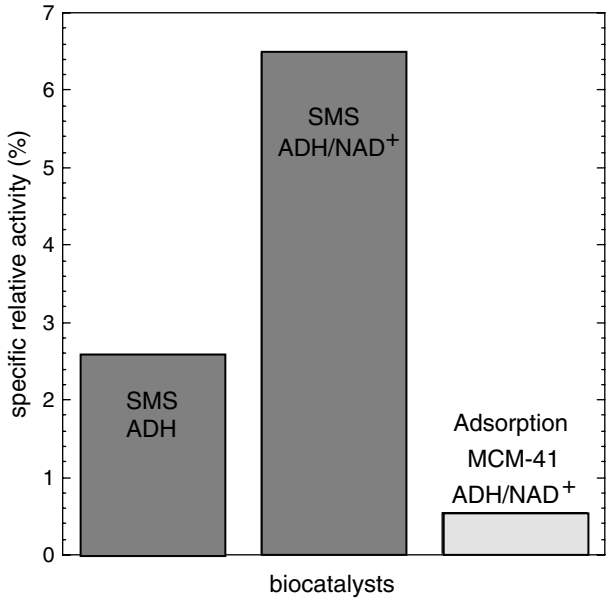

Fig. 11. Relative specific activity of ADH encapsulated in SMS with and without its cofactor NAD^+. As comparison, relative activity of ADH immobilized with its cofactor NAD^+ by adsorption in a large-pore MCM-41-type.

between NH_3^+ functions of the ADH with the SiO^- of the support, whereas in SMS, enzymes are mostly confined in micelles of lecithin such as in a membrane cell without any strong interactions, which will allow maintaining a better conformation of the enzyme and therefore a better specific activity. As a consequence of the different enzyme loading, the overall activity expressed on per gram of catalyst is the higher for the MCM-41-based material. If we consider however the specific activity of the enzyme itself, it appears clearly that the direct encapsulation route into SMS produces much efficient systems, and that co-immobilization of the enzyme and its NAD^+ co-factor is a very promising alternative. The NAD^+ participates to the formation of the quaternary structure of ADH, responsible of building of the catalytic active site, creating in the same time a hydrophobic environment necessary to its activity. The use of the ADH/NAD^+ system instead of ADH alone favors to maintain its structure and its activity during the encapsulation. With respect to the relative specific activity (activity of the encapsulated enzyme versus activity of the native enzyme, last column of Table 4), the direct SMS route leads to an enzymatic system one order of magnitude more active than the adsorption procedure. This result seems very promising with regards to the development of effective biocatalysts involving very fragile enzymatic species.

4. Conclusion

Biological and biocompatible surfactants such as egg yolk or soybean lecithins (phosphatidylcholine) have been used to template mesoporous silica materials with controlled pore size. Lecithin alone leads to lamellar materials evoking multilamellar systems and vesicles formation. The adjunction of a long chain alkyl amine and alcohol allows to form mixed-micelles of lecithin/alkylamines which self-assemble in the presence of silica into a SMS mesostructure. The phase transition from multilamellar mesostructure to sponge-like mesostructure is driven by the presence of dodecylamine which packs the heads groups of the lecithin and then change the spontaneous curvature of the lamellar phase, but also by the use of alcohol as a co-solvent which dehydrates the phospholipid bilayers and promotes interdigitation of the phospholipid chains. The pore size of the material is determined by the alkyl chain length of the phosphatidylcholine. The pore structure is isotropic and close to a disordered cubic phase. The length of the channels between cavities can be controlled by the synthesis conditions. The addition of lactose in the synthesis mixture does not affect the pore structure but helps to maintain the catalytic activity of enzymes immobilized by this new SMS synthesis route. The results in enzymatic activity show that this biocompatible method of SMS formation enhances the enzyme specific activity of immobilized enzymes and allows encapsulating even very fragile enzymes. This new encapsulation process respecting biomolecules opens large perspectives for biomolecules processing for applications such as biocatalysis, biosensors or biofuel cells.

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