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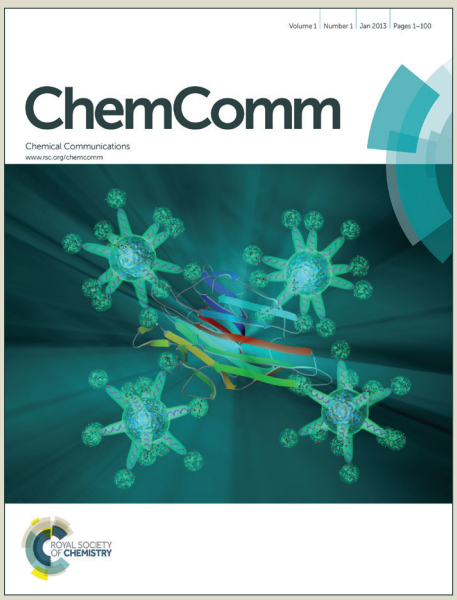

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Meso-macro compartmentalized bioreactor through silicalization of "green" double emulsions: W/O/W and W/SLN/W

Cite this: DOI: 10.1039/x0xx00000x

Received ooth January 2012,
Accepted ooth January 2012

DOI: 10.1039/x0xx00000x

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We report a straightforward approach for both structuring and entrapping enzymes into hierarchical silica materials with hexagonally ordered mesopores (12 nm) and tailored macroporosity by converting double emulsion colloidal template (tens of microns) into solid lipid nanoparticles (hundred of nanometres). The supported biocatalyst efficiently catalyzes the methanolysis of colza oil.

Enzymes are undoubtedly catalysts that meet criteria requested for an eco-compatible and sustainable chemistry. However, to develop enzyme processes for industrial applications, it is usually necessary to immobilize the enzyme into a solid support that acts as both a barrier against the surrounding environment and as a carrier. Among the solid supports reported to date, silica appears as the simplest and the most inexpensive solution. Silica, especially the one prepared by sol-gel chemistry[1], is also known to be safe, not only for the environment, but also for the human body within a certain dose[2]. Concerning the immobilization of enzymes, the main strategy explored to date consists in adsorbing the enzyme to pre-synthesized silica materials. While mesoporous silica is extensively investigated, only few examples have been reported for physically[3] or covalently immobilized enzymes[4,5] onto hierarchical silica. Results thus far suggest that the introduction of macroporosity allows for increased enzyme loading and mass throughputs relative to mesoporous silica. The protocols remain rather complex and require the pre-synthesis of the porous support. An interesting alternative to this route would be the one pot encapsulation of enzymes into a colloidal carrier, typically used as a template for the inorganic matter. In fact, this strategy was already used for enzyme-loaded mesoporous silica[6] but, to the best of our knowledge, has not yet been explored for enzyme encapsulation into meso-macroporous silica materials.

Recently, our group reported the use of solid lipid nanoparticles (SLN) as a "green" template for hierarchically structured silica, giving smaller macropores compared to emulsions (several hundred of nm *vs* tens of micrometers).[7] SLN have a limited toxicity[8] and can be produced in a cost-effective fashion, by different formulation techniques, including double emulsion[9]. This method might also enable the loading of enzymes into solid lipid nanoparticles[10]. Indeed, many enzymes have hydrophilic exterior environments and, therefore, their encapsulation into fatty emulsion droplets or the lipid core of solid particles cannot be directly achieved without either lipid-enzyme ion pairing[11] or double emulsions in which the inner aqueous droplets solubilize the enzyme[12]. We prepared herein meso-macroporous silica supported *Mucor Miehei* lipase (*MmL*) biocatalyst by a one-pot synthesis consisting of mineralizing a colloidal template pre-loaded with enzyme. Interestingly, the macroporosity could be tailored herein by templating the silica with double emulsions that have the particularity of being converted into solid lipid nanoparticles (SLN) by simple removal of the organic solvent. Also, methods were developed to control the pore size since this is a critical parameter that is known to influence diffusivity of chemicals, leaching and thus the stability of the supported catalyst.

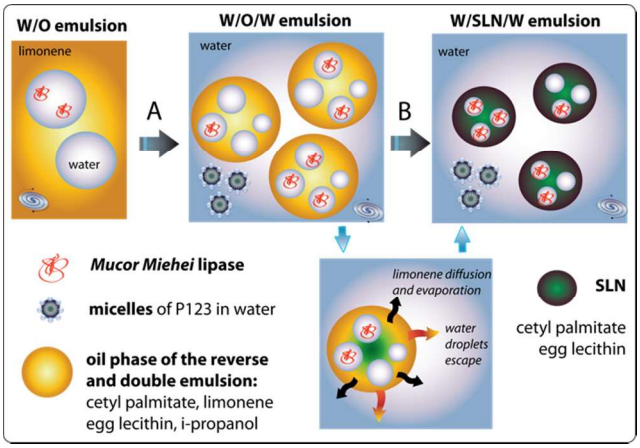

Figure 1. Illustration of the transformation of a double emulsion containing a solid lipid dissolved in an organic solvent (W/O/W) into solid lipid nanoparticles (W/SLN/W). A: addition of micelles of P123; B: evaporation of limonene

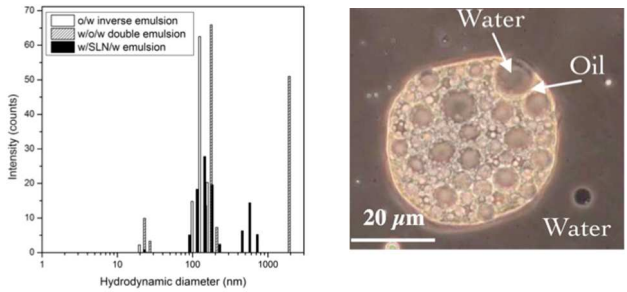

Figure 2. Hydrodynamic diameter of O/W emulsion, W/O/W and W/SLN/W emulsion as determined by DLS and POM micrograph showing the compartmentalization of the W/O/W emulsion

For this study, *Mucor Miehei* lipase was selected as a promising biocatalyst for transesterification reaction involved in biodiesel production from vegetable oils[5,13]. The activity of the *MmL* is 1.39 U/mg and it was determined by hydrolysing triolein at pH 8 and 40°C. The double emulsion (W/O/W) was prepared by adding under vortex stirring a reverse (W/O) emulsion into an aqueous micellar solution of P123. Dynamic light scattering (DLS) measurements show that the size of aqueous droplets in the W/O emulsion is of about 200-300 nm, whereas, the size of the double emulsions is centered at 2 μm (Fig. 2). The population at 29 nm could correspond to the micelles of the P123. The compartmentation of the W/O/W emulsion was also confirmed by optical microscopy (Fig. 2). W/SLN/W emulsions were prepared by first evaporating 80 wt% of the double emulsion and then, by diluting the resulting highly concentrated emulsion with an aqueous solution of micelles. As determined by DLS, SLN show a bimodal distribution centered on 130 and 550 nm (Fig. 2). By introducing the enzyme (50 mg/mL) no significant changes on the droplets size were observed. Silica materials were obtained by mineralizing the colloidal template, with or without enzyme, through a sol-gel synthesis.[†] Hence, meso-macrostructured silica materials result by a dual templating mechanism that combines self-assembly of micelles of Pluronic® P123 (EO_{20}-PO_{70}-EO_{20}) and templating of colloidal lipid spheres of either reverse emulsion (W/O, W(*MmL*)/O) or solid nanoparticles (W/SLN, W(*MmL*)/SLN). Silica materials were characterized by SAXS, N_2 sorption, Hg intrusion measurements as well as scanning and transmission electron microscopy. SAXS pattern of bare material, obtained from W/O/W double emulsion, labeled as MDE (Material from Double Emulsion) exhibits reflections at 17.2, 9.9, 8.6 and 6.4 nm (Fig. 3-1A), which can be attributed to (100), (110), (200) and (210) of the hexagonal mesopore network. In contrast, bare silica, prepared by mineralizing the dispersion of enzyme-free SLN and labeled as MSLN (Material from Solid Lipid Nanoparticle), exhibits a shift of the 1st peak towards higher q values (Fig. 3-1B), suggesting a decrease of the mesopore size. In addition, the secondary reflections are less resolved. The mesopore arrangement is therefore disturbed and the SAXS pattern is rather characteristic of hybrid mesoporous silica, containing hexagonally ordered domains embedded into wormlike silica. The presence of the mesoporosity is evidenced by N_2 sorption analyses since a type IV isotherm (Fig. 3-2A and 3-2B), characteristic of mesoporous materials, is obtained. However, during the desorption step we can see that the adsorbed volume suddenly drops. The hysteresis is thus H2 type, meaning that the distribution of pore size and shape is not well-defined. Indeed, H2 is characteristic of pores with narrow necks and wide bodies.

The relative pressure for which capillary condensation takes place is shifted toward lower values for MSLN (Fig. 3-2B). Since the p/p_o position of the inflection point is related to the pore diameter, it can be inferred that a decrease of the mean pore diameter occurs when limonene is removed from the emulsion. This decrease in pore size is further confirmed by the pore size distribution obtained by the BJH method applied to the adsorption branch of the isotherm (insert in Fig. 3-2A, 2B) and whose maximum is shifted from 12.4 to 8.4 nm when passing from MDE to MSLN silica. At high relative pressures ($p/p_o > 0.9$) the adsorbed volume of N_2 increases significantly instead of remaining constant due to saturation, indicating the presence of a secondary porosity. For both kinds of materials the specific surface area is high, 800 and 880 m^2/g for MDE and MSLN, respectively. The mesopore volumes are equal to 0.92 and 0.68 cm^3/g for MDE and MSLN, respectively.

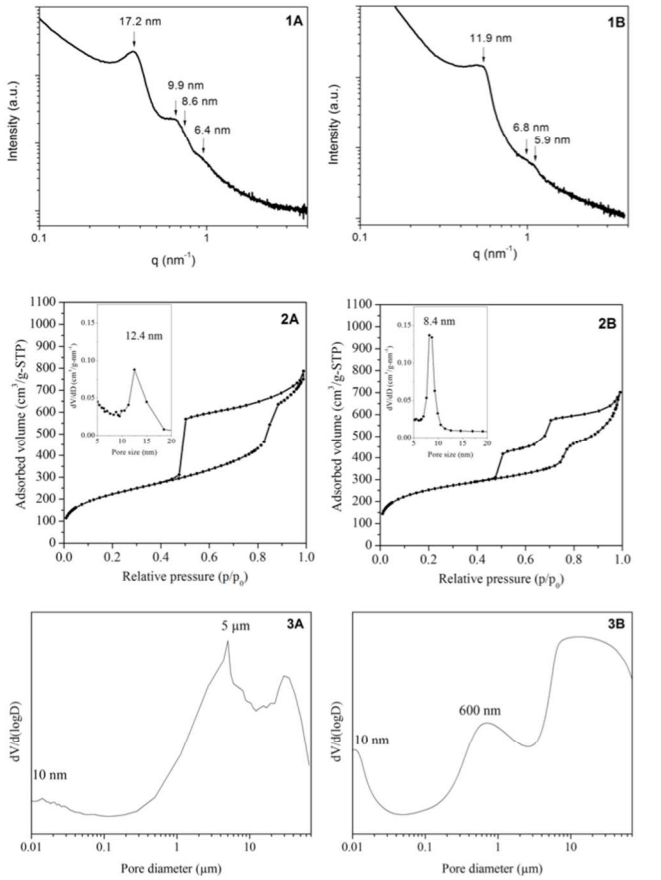

Figure3. SAXS data (1), N_2 adsorption-desorption isotherms (2) and pore size distribution calculated by the BJH method (insert 2) and pore size distribution (3) obtained by applying the Washburn equation to the Hg intrusion curves of bare silica materials ***MDE*** (A) and ***MSLN*** (B)

Looking at the MDE material, the mercury intrusion porosimetry measurements reveal the presence of macropores with two different size distributions centered at 5 and 30 μm (Fig. 3-3A). The first one can be attributed to the fingerprint of the oil droplet dispersed in the outer aqueous phase. However, it should be noted that the size of the macropore is higher than the one of the droplets of the double

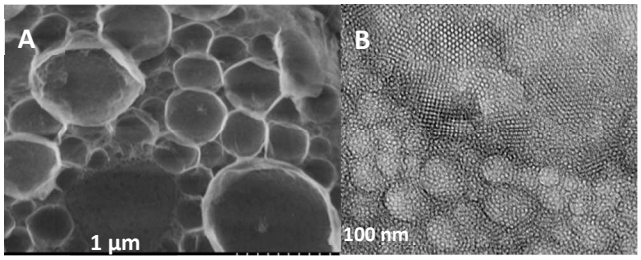

Figure 4. SEM (A) and TEM (B) micrographs of bare silica ***MDE***

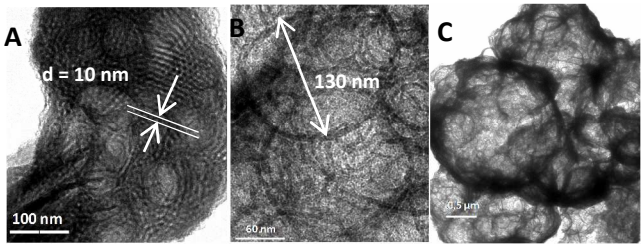

Figure 5. TEM micrographs of bare silica material, ***MSLN*** (A and B) and of enzyme loaded silica material, ***MmL-MSLN*** (C)

emulsions. This phenomenon can be attributed to a coalescence of the droplets that can take place during MDE synthesis. The second peak (30 µm) might arise from inter-particle porosity induced by aggregation of particles. Finally, the peak at 13 nm corresponds to the mesopore size (12.4 nm from the N_2 sorption measurements). The pore volumes determined by Hg intrusion porosimetry are equal to 6.10 and 5.26 cm^3/g for MDE and MSLN, respectively.

The presence of the macropores is further confirmed by SEM and TEM (Figure 4). Mercury intrusion porosimetry experiments also indicate a shrinkage of the macropores upon limonene removal. Indeed, the macropore size distribution of the MSLN sample shows peaks at 10 nm, 600 nm and 15 µm. The latter one arises from the interparticle porosity and the first one is due to the mesopores. The pore size distribution centred at 600 nm corresponds to the imprint of SLN. Similar observations can be made from TEM analysis (Fig. 5). The above results indicate that the transition from MDE to MSLN is accompanied by a perturbation of the mesopore network and a decrease of both meso- and macropore size. These results clearly indicate the hierarchical structure of the silica matrix. The structuring is maintained while adding the lipase into the colloidal template. Indeed, SAXS patterns of *MmL*-loaded materials (data not shown) were similar to the bare silica, indicating that the enzyme is loaded into macrocavities rather than into mesopores.

Finally, the resulting materials, *MmL*-MDE and *MmL*-MSLN, were used as biocatalyst in the methanolysis of colza oil. The reaction was performed in hexane[14], at 25°C and the molar ratio methanol: colza oil of 3. In both cases, the biocatalyst contains 0.054 mg *MmL*/mg silica. The entrapped lipase retains its catalytic activity and the complete conversion is reached within 50 h (Figure 6 left). For comparison, the reaction was also performed with *MmL* that was physisorbed onto pre-synthesized mesoporous SBA-15 (Santa Barbara Amorphous) (0.43 mg *MmL*/mg), having similar mesopore size but exempt of macroporosity. The complete conversion is reached within only 6 h. The difference in behavior between SBA-15

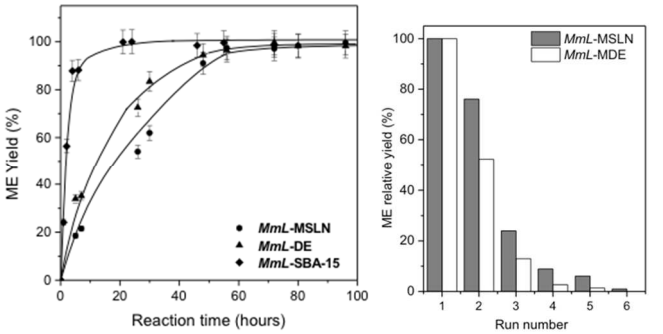

Figure 6. Variation of the methyl esters (ME) yield as a function of time (left) and variation of methyl esters (EM) yield over the recycling runs obtained with ***MmL*-MDE** and ***MmL*-MSLN** supported biocatalysts, respectively (right).

and MDE or MSLN can be due to a higher enzyme loading, almost a factor 8. In addition it should be noted that the lipase is not immobilized by the same method. In the case of SBA-15 the enzyme is physisorbed in the mesopore while it is entrapped for MDE and MSLN. So SBA-15 presents a better accessibility of the MmL. The main advantage of the method reported here is that a lower quantity of enzyme is needed and that the efficiency of the immobilization is 100%. When the *Mucor miehei* lipase is physisorbed into SBA-15 a higher quantity of enzyme is required and the efficiency of the physisorption is only 25 %.

Figure 6 shows that, even if its activity decreases rapidly, the colloidal-entrapped biocatalysts shows a better efficiency than the corresponding physisorbed enzyme, with up to 5 consecutive runs in the case of *MmL*-MSLN and to 4 consecutive runs in the case of *MmL*-MDE, *vs* only 2 consecutive runs for *MmL*-SBA-15, as reported elsewhere[13].

Conclusions

The templating of silica with double emulsions containing an enzyme in the aqueous core of the hydrophobic droplets provides a viable pathway for the synthesis of supported biocatalysts. By using an oily mixture of a green solvent (*i.e.* limonene) and a solid lipid (*i.e.* cetylpalmitate), one can easily tune the size of the macrocavities from 5 µm in the case of the emulsion to 600 nm in the case of the solid lipid nanoparticles, by simply reducing the size of the colloidal template by evaporation of the organic solvent. The resulting supported biocatalyst was able to catalyze the methanolysis of colza oil, reaction of interest for biodiesel production. This strategy is also opening the way to achieve other materials that could be loaded with large size (tens to hundred of nanometers) bioactifs ingredients.

Acknowledgement

Authors would like to thank M. Emo for SAXS measurements, J. Ghanbaja for TEM analysis and F. Michaux for Master Size measurements facilities. The work was financed by the ANR project n°ANR-09-JCJC-0024-01.

Notes and references

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†*General procedure for W/O/W emulsion:* 600mg of buffer solution of TRIS (pH 6) containing 33mg of lipase was added to a mixture of limonene/ cetylpalmitate (1000mg/105mg) under vortex. Then the mixture of i-propanol/egg lecithin (237mg/57mg) was added. The composition (wt%) of reverse emulsion is limonene/TRIS containing lipase/egg lecithin/NHP/isopropanol: 50.03/30.01/11.86/ 5.25/2.85. To prepare the double emulsion, O/W emulsion previously prepared was added into ~~an~~ 12g of aqueous solution of 3.45 wt% of Pluronic P123 under continuous stirring. Hence the composition of the W/O/W double emulsion (wt%) is limonene/ TRIS containing lipase/egg lecithin/NHP/i-PrOH/P123/water: 4.29/7.14/0.75/1.69/0.41/2.96/82.76.

General procedure for W/SLN/W emulsion: approximately 80wt% of solvent of the W/O/W emulsion was evaporated and then water was added to adjust P123 concentration as 3.45 wt%.

General procedure for material preparation: 350mg of TMOS was added, at neutral pH to 8mL of double emulsion (vP123/vTMOS = 0.018). The mixture was enclosed in an autoclave at 40°C for 24 h to allow the hydrolysis/ polycondensation of silica. After washing the material was then washed with ethanol at room temperature ~~for~~ during 12h, the final granular powder is recovered.

General procedure of the transesterification reaction: To 10mL of hexane were added 100 µL of colza oil (1 eq., M=881.6 g/mol), 13 µL of methanol (3 eq.) and 100 mg of supported biocatalyst (0.054 mg MmL/mg). The reaction mixture was kept in closed glass vials at 25 °C under stirring at 300 rpm. Reaction progress was followed by GC-MS.[13]

‡ Mercury intrusion porosimetry measurements (MIP) were performed on on Pascal 140/240 Instrument (Thermo Scientific).

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