Bulgarian Chemical Communications, Volume 50, Special issue H (pp. 156–160) 2018

Preparation and application of nanosized zeolite as a carrier for a lipolytic enzyme

M. D. Dimitrov[1], M. Guncheva[1]*, D. G. Kovacheva[2]

[1] *Institute of Organic Chemistry with Centre of Phytochemistry, Bulgarian Academy of Sciences, 1113 Sofia, Bulgaria*
[2] *Institute of General and Inorganic Chemistry, Bulgarian Academy of Sciences, 1113 Sofia, Bulgaria*

Received: January 16, 2018; Revised: March 02, 2018

Porous inorganic materials have been extensively applied as enzyme carriers due to their high mechanic stability and high specific surface area, resistance to microbial attack, stability in organic solvents, and stability upon heating. In recent years, enzymes immobilised on nanosized materials have attracted scientist attention with regard to new emerging applications such as biosensing and medical diagnostics. This research aims to investigate the potential of a nanosized and mostly mesoporous zeolite (nano-Zeo) as a suitable carrier for lipase from porcine pancreas (PPL). For comparative purpose, a nanosized MCM-41 silica material with spherical morphology was studied. The latter was used as a starting material to prepare the nanosized zeolite by densification of the initial amorphous MCM-41 silica nanospheres in presence of a diluted solution of tetrapropylammonium hydroxide, the nanospheres being further transformed into MFI-type of zeolite *via* steam-assisted crystallisation. Both the starting material and the nano-Zeo particles were characterised by means of X-ray diffraction, nitrogen physisorption, and scanning electron microscopy techniques. Loaded protein amount was comparable for the two carriers: 64.0±2.3 and 80.4±3.4 mg/g for MCM-41 and nano-Zeo, respectively. However, nano-Zeo showed over twofold higher specific loading with regard to the mesoporous specific surface area of the studied materials. Besides, monolayer surface distribution and a higher specific lipase activity were estimated for the nano-Zeo-PPL preparation, which is probably due to lipase molecules attached in a proper orientation.

Key words: porcine pancreas lipase (PPL), nanosized zeolite carrier, MCM-41 silica nanospheres, immobilization.

INTRODUCTION

Lipases are industrially applied enzymes in lipid modification, esterification, resolution of racemic mixtures, epoxidation, and other processes [1]. However, they find a limited application due to their high price and low stability under harsh process conditions (presence of detergents, solvents, substrate or product inhibition, high temperatures and low pressures) [2]. On the other hand, adsorption or deposition of the enzymes onto a porous support has proved to be a useful technique for improving their activity and stability [3]. Immobilisation facilitates enzyme recovery from the reaction mixture and allows multiple usages without a significant loss of activity, which makes the process cost-effective. In addition, enzymes immobilised on solid supports have many advantages over their free counterparts, namely, continuous performance and rapid termination of enzymatic reactions, controlled product formation, and easy enzyme removal from the reaction mixture. Compared with polymeric materials inorganic enzyme carriers are structurally more stable, environmentally tolerable, and resistant to organic solvents and microbial attack [4]. Besides, in recent years, enzymes immobilised on nanosized materials have attracted attention with regard to new emerging applications such as biosensing and medical diagnostics [5].

The present study aims to investigate the potential of a nanosized and mostly mesoporous zeolite (nano-Zeo) as a suitable carrier for lipase from porcine pancreas. For comparative purpose a nanosized MCM-41 silica material with spherical morphology was studied, which was used as a starting material to prepare the nanosized zeolite. This is a new idea that utilises a simple procedure to prepare zeolites with nano dimensions by transforming already pre-prepared and geometrically well-defined amorphous silica particles into crystalline entities of similar shape, diminished size, and increased density [6]. Both initial MCM-41 and nano-Zeo materials were characterised by X-ray diffraction, nitrogen physisorption, and scanning electron microscopy techniques. The activity of the immobilised preparations was estimated using 4-nirophenyl palmitate as a substrate.

EXPERIMENTAL

Materials

Nano-Zeo material was synthesised by a two-step procedure. In the first step, MCM-41 silica with

* To whom all correspondence should be sent
E-mail: *maiag@orgchm.bas.bg*

spherical morphology was synthesised using TEOS as a silica source and hexadecyltrimethylammonium bromide as a structure-directing reagent [7]. Then, in the second step, the obtained MCM-41 spheres were treated with a dilute solution of tetrapropylammonium hydroxide and densified for 24 h at 383 K *via* a steam-assisted crystallisation under hydrothermal conditions [6]. Lipase from porcine pancreas (15–30 U/mg, 20% protein content) from Sigma was used to assess protein absorption capacity of the novel zeolitic material and the initial MCM-41 silica nanospheres.

Methods of characterization

Both the starting MCM-41 silica and the nano-Zeo material were characterised by X-ray diffraction, nitrogen physisorption, and scanning electron microscopy techniques. Powder X-ray diffraction patterns were collected on a Bruker D8 Advance diffractometer equipped with Cu Kα radiation and LynxEye detector. Nitrogen sorption measurements were recorded on Quantachrome NOVA 1200e and Quantachrome Autosorb iQ MP instruments at 77 K. Before physisorption measurements, the samples were outgassed overnight at 423 K under vacuum. Pore size distributions and pore diameters were calculated by non-local density functional theory (NLDFT). Scanning electron microscopy (SEM) images were obtained with FEI Quanta FEG 250 and JEOL-JSM-6390 scanning electron microscopes. Lipase from porcine pancreas was immobilised via physical adsorption. In a typical procedure, 20 mg of the carriers were gently shaken with 1 mL of PPL (1–20 mg/ml) dissolved in sodium phosphate buffer (pH 7.0) After 12 h incubation, the immobilised preparations were filtered, washed twice with 0.5 ml of sodium phosphate buffer, freeze dried, and their activity was tested in assay reaction. Protein content in the initial PPL solutions and in the supernatant after immobilisation was estimated using Lowry's method [8]. For both materials, the absorption capacity (1) and the immobilisation yield (2) were determined following the equations:

$$\text{Absorption capacity} = \frac{(Co-Cf)}{m}V, \text{ mg/g}_{\text{carrier}} \quad (1)$$

$$\text{Immobilisation yield} = \frac{(C0-Cf)}{C0}100, \% \quad (2)$$

where C_o and C_f are the initial and final concentration of the protein in the solutions in mg/ml, respectively, V is the volume of the loading enzyme solution in mL, and m is carrier weight.

The activity of the immobilised preparations was estimated using 4-nirophenyl palmitate as a substrate. One unit is the amount of protein, which catalyses the conversion of 1 μM substrate for 1 minute at 25 °C.

RESULTS AND DISCUSSION

X-ray diffraction (XRD) technique was applied to determine sample mesoporous ordering, crystallinity, and phase composition (Fig. 1). Small angle X-ray diffraction patterns of the calcined materials are presented in Fig. 1a. A MCM-41 sample shows one main reflection (100) at 2.64 2θ and two small reflections with maxima at 4.64 and 5.28 2θ, respectively, arising from quasi-regular long-range hexagonal arrangement of the obtained mesopores that are characteristic of the MCM-41 structure.

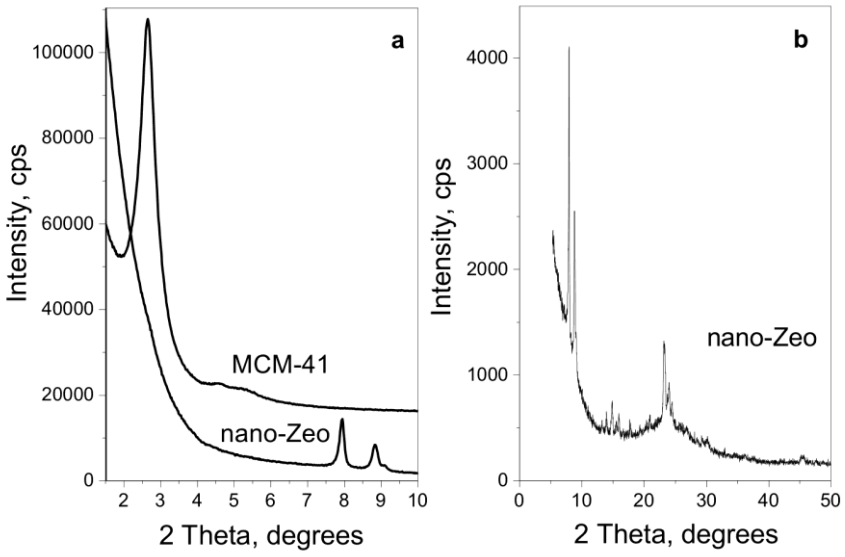


Fig. 1. XRD patterns of the studied samples.

At the same time, the pattern of the nano-Zeo sample displays no reflections of the initial MCM-41 material to indicate transformation of the latter. However, two new reflections are observed at about 7.9 and 8.8 2θ (Fig. 1a). Further information about the newly found structure was obtained from wide-angle XRD pattern of the nano-Zeo sample (Fig. 1b). The observed reflections are characteristic of a MFI zeolite structure [6]. Hence, a complete transformation of the initial amorphous MCM-41 silica material into a crystalline MFI-type zeolite was realised. To follow changes of the textural characteristics of both studied samples two additional techniques were used: nitrogen physic-sorption and scanning electron microscopy (Figs. 2 and 3). Besides, some physicochemical characteristics of the obtained samples are presented in Table 1.

Data on nitrogen physisorption measurements of the studied samples are displayed in Fig. 2 and Table 1. The initial MCM-41 sample manifests a typical isotherm that is characteristic of this type of mesoporous silica materials with a narrow adsorption/desorption step within 0.2-0.3 relative pressure (Fig. 2a). It is characterised by a high specific surface area (985 m^2/g), total pore volume of 0.47 cm^3/g, and narrow pore size distribution with main pore diameter of 2.9 nm (Fig. 2 and Table 1).

SEM analysis showed that this material is composed of well-defined spherical nanoparticles of relatively equal size, most of which are in the range of 400-600 nm (Fig. 3). However, after steam-assisted crystallisation certain changes occurred with the MCM-41 starting material. The isotherm of the nano-Zeo sample is also of type IV that is characteristic of mesoporous materials (Figure 2a). Yet, a much broader adsorption/desorption step within 0.5-0.9 relative pressure was registered for this sample accompanied by a hysteresis loop most probably owing to interparticle mesoporosity (Fig. 2a). Besides, the nano-Zeo material loses part of the very high specific surface area of the initial MCM-41 sample at the expense of the appearance of certain microporosity (270 m^2/g), however, the mesopore volume increases to 0.68 cm^3/g due to the presence of very broad pore size distribution and much larger mesopores (Fig. 2b, Table 1). At the same time, the SEM image proves preservation of the spherical morphology accompanied by some size reduction phenomena as expected during transformation from amorphous to crystalline phase (Fig. 3). However, larger spherical particles are also found with the nano-Zeo sample that we ascribe to agglomeration of some initial MCM-41 spherical particles during transformation (Fig. 3).

1,3-specific lipase from porcine pancreas was selected to evaluate the absorption capacity of the two silica supports. Industrial application of this enzyme is limited due to its lability in organic solvents and upon heating above 45°C; therefore, methods for PPL stabilisation are of current research interest. The enzyme structure is compact with dimensions *ca*. 4.6×2.6×1.1 nm^3, and earlier studies by other authors have shown that pore size is one of the major factors that should be considered upon selection or design of the excellent enzyme supports [9].

Table 1. Textural characteristics of the obtained materials

Sample	S_{BET},[a] m^2/g	V_{total},[c] cc/g	S_{micro},[b] m^2/g	V_{micro},[d] cc/g	dDFT,[e] nm	D_{SEM},[f] nm
nano-Zeo	728	0.80	270	0.12	2.3; 4.5; 10.5	200–800
MCM-41	985	0.47	-	-	2.9	400–600

a - BET specific surface area; b - Micropore surface area by t-method; c - Total pore volume; d - Micropore volume by t-method; e - Main pore diameter evaluated using NLDFT method, f - Particle sizes evaluated by SEM analysis.

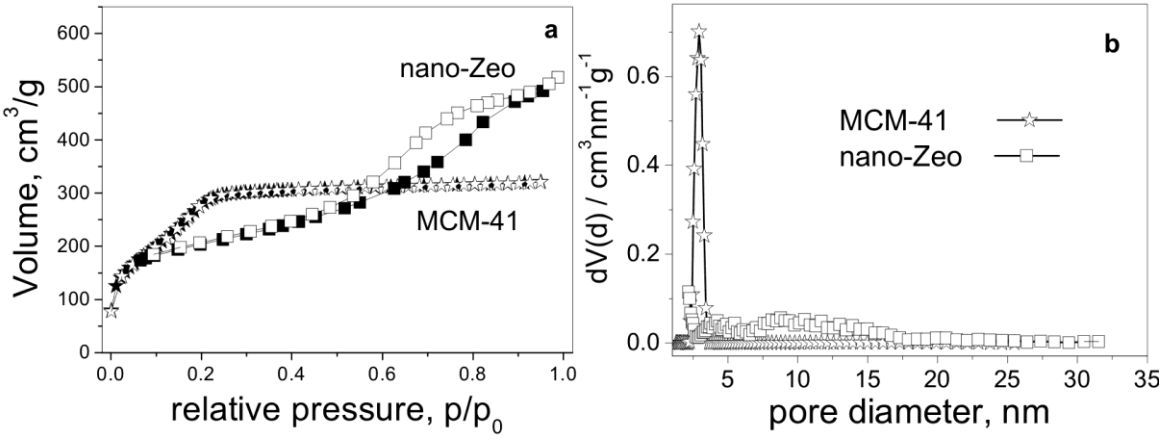
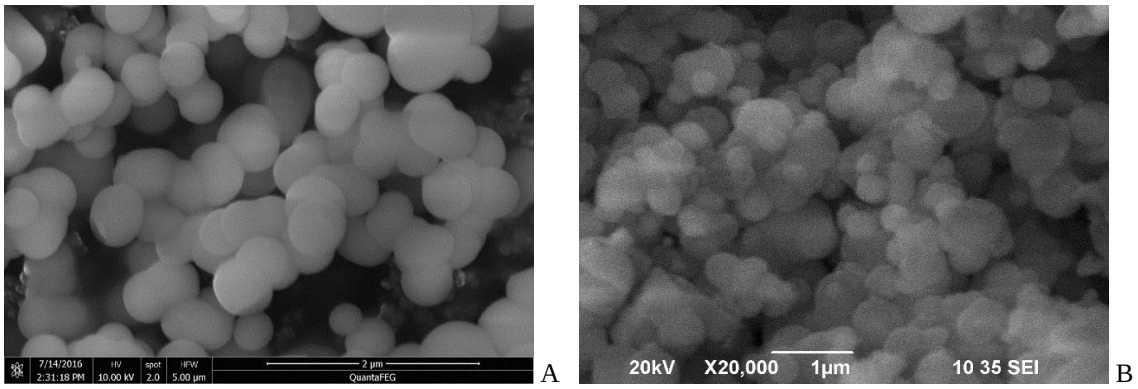


Fig. 2. Nitrogen adsorption/desorption isotherms (a) and pore size distribution (b) of the studied samples.



A B

Fig. 3. SEM images of MCM-41 (left) and nano-Zeo (right) samples.

Table 2. Amount of the immobilised porcine pancreas lipase on the two supports and biocatalyst activity

Sample	Absorption capacity, mg prot./g carrier	Specific capacity[a], mg prot./m^2 carrier	Specific activity, U/mg prot.	Immobilization yield, %
MCM-41	64.0 ± 2.3	0.065	170	32
nano-Zeo	80.4 ± 3.4	0.176	238	40

[a] To estimate the specific capacity the absorption capacity of the carrier was divided by its specific surface area ($m^2/g_{carrier}$).

The absorption capacity of the two carriers is given in Table 2. Novel nano-Zeo proves to be a 1.25-times more efficient support than the MCM-41 precursor, most probably due to higher porosity and larger pore sizes. The two carriers were less efficient as compared to literature data on PPL adsorption on SBA-15 for which enzyme loading was between 100 and 920 mg enzyme per gram carrier regarding carrier textural characteristics and reaction conditions [10,11]. However, a monolayer deposition of the PPL on nano-Zeo and MCM-41 was observed, which resulted in a preserved (or even enhanced) lipolytic activity of the nano-Zeo-PPL and MCM-41-PPL in comparison with the free enzyme.

MCM-41-PPL and nano-Zeo-PPL were examined in the reaction of 4-nitrophenyl palmitate hydrolysis. Tested in several consecutive cycles, a fast drop of activity was observed even after the first cycle with the two immobilised preparations, which is due to enzyme leakage or desorption in the aqueous medium (Fig. 4). Functionalisation of the supports and subsequent covalent bonding of the PPL or application of these biocatalysts in esterification reactions in non-solvent or organic media could be a possible solution to enhance enzyme performance.

CONCLUSIONS

Nanosized zeolite material with MFI structure and spherical morphology was successfully synthesised from MCM-41 mesoporous spheres *via* a steam-assisted crystallisation under hydrothermal conditions at 383 K. The novel zeolite material exhibited high specific surface area and high total pore volume due mainly to very high mesoporosity characterized by broad mesopore size distribution and high amount of mesopores. Over twofold higher adsorption capacity for lipolytic enzyme from porcine pancreas was found with the novel nano-sized zeolite in comparison with the initial MCM-41 material regarding the specific mesoporous surface area of both carriers. A higher adsorption capacity and monolayer surface distribution and a higher specific lipase activity was also estimated for the nano-Zeo-PPL preparation, which is probably due to lipase molecules attached in suitable orientation.

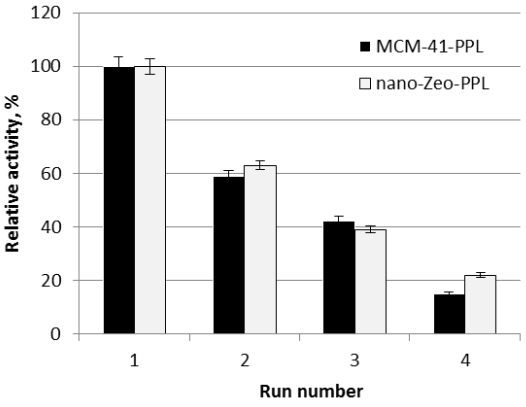


Fig. 4. Relative activity of MCM-41-PPL and nano-Zeo-PPL in the assay reaction of hydrolysis of 4-nitrophenylpalmitate.

REFERENCES

1. F. Hasan, A. A. Shah, A. Hameed, *Enz. Microb. Tech.*, **39**, 235 (2006).
2. A. Houde, A. Kademi, D. Leblanc, *Appl. Biochem. Biotech.*, **118**, 155 (2004).
3. T. Jesionowski, J. Zdarta, B. Krajewska, *Adsorption*, **20**, 801 (2014).

4. S. Y. Lee, J. Lee, J. H. Chang, J. H. Lee, *BMB Rep.*, **44**, 77 (2011).
5. M. Leitgeb, Ž. Knez, K. Vasić, Micro- and Nanocarriers for Immobilization of Enzymes, in: Nanotechnology and Nanomaterials "Micro and Nanotechnologies for Biotechnology", S. G. Stanciu (ed.), CC BY 3.0 license, 2016, Ch. 2.
6. A. G. F. Machoke, B. Apeleo Zubiri, R. Leonhardt, V. R. R. Marthala, M. Schmiele, T. Unruh, M. Hartmann, E. Spiecker, W. Schwieger, *Chem. Eur. J.*, **23**, 10983 (2017).
7. M. Guncheva, M. Dimitrov, P. Ossowicz, E. Janus, *J. Porous Mater.* (2017), https://doi.org/10.1007/s10934-017-0505-z
8. O. H. Lowry, N. J. Rosebrough, A. L. Farr, R. J. Randall, *J. Biol. Chem.*, **193**, 275 (1951).
9. H. Ma, J. He, D. G. Evans, X. Duan, *J. Mol. Cat. B: Enzym.*, **30**, 209 (2004).
10. Y. Li, G. Zhou, W. Qiao, Y. Wang, *Mat. Sci. Eng. B*, **162,** 120 (2009).
11. G. D. Yadav, S. R. Jadhav, *Micro. Meso. Mat.*, **86**, 215 (2005).

ПОЛУЧАВАНЕ И ПРИЛОЖЕНИЕ НА НАНОРАЗМЕРЕН ЗЕОЛИТ КАТО НОСИТЕЛ ЗА ЛИПАЗА

М. Д. Димитров[1], М. Гунчева[1]*, Д. Г. Ковачева[2]

[1] *Институт по органична химия с Център по фитохимия, БАН, 1113 София, България*
[2] *Институт по обща и неорганична химия, БАН, 1113 София, България*

Постъпила на 7 февруари 2018 г.; Преработена на 20 март 2018 г.

(Резюме)

Порестите неорганични материали се прилагат широко като носители на ензими, поради тяхната висока механична стабилност и висока специфична повърхност, устойчивост на микробиална атака, стабилност в органичните разтворители и стабилност при нагряване. През последните години ензимите, имобилизирани върху наноразмерни материали, са атрактивни обекти за изследване поради разширяването на областта им на приложение, например като биосензори или в медицинската диагностика.

Настоящото изследване е фокусирано върху проучване на потенциала на наноразмерен и преобладаващо мезопорест зеолит (nano-Zeo) като подходящ носител за липаза от свински панкреас (PPL). За сравнение проведохме експерименти и с наноразмерен MCM-41 силикат със сферична морфология на частиците, който е изходен материал за получаване на наноразмерния зеолит посредством процес на "уплътняване" на изходните аморфни MCM-41 наночастици в присъствието на разреден разтвор на тетрапропиламониев хидроксид и последващото им превръщане в зеолит тип MFI чрез кристализация в присъствие на водна пара. Материалите са охарактеризирани с помощта на рентгенова дифракция, физична адсорбция на азот и сканираща електронна микроскопия. Количеството на натоварения протеин е съизмеримо за двата носителя и е съответно 64,0 ± 2,3 mg/g за MCM-41 и 80,4 ± 3,4 mg/g за nano-Zeo. Струва да се отбележи, че нанозеолитът показва повече от два пъти по-голямо специфично натоварване по отношение на мезопорестата повърхност на изследваните материали. Освен това, за nano-Zeo-PPL бяха отчетени монослойно повърхностно разпределение и по-висока специфична хидролитична активност, което вероятно се дължи на закрепването на липазните молекули в по-подходяща ориентация за този носител.