RSC Advances

PAPER

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Cite this: *RSC Adv.*, 2015, **5**, 105800

Self-assembled lipase nanosphere templated one-pot biogenic synthesis of silica hollow spheres in ionic liquid [Bmim][PF_6]†

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The spontaneous self-assembly of hydrophobic enzymatic protein triacylglycerol acylhydrolase (commonly known as lipase and a member of the serine hydrolase family) in hydrophobic 1-butyl-3-methylimidazolium hexafluorophosphate [Bmim][PF_6] and in hydrophilic 1-butyl-3-methylimidazolium tetrafluoroborate [Bmim][BF_4] ionic liquids resulted in the formation of lipase enzyme nanocapsules of different morphology. The lipase enzyme capsules were found to retain varying enzyme activity in both cases with both kinds of lipase capsules acting as self-catalyzing functional templates for the hydrolysis of silica precursors into silica. The presence of silica and its interaction with biomolecules was proved by X-ray Photoemission Spectroscopy (XPS). Interestingly, hollow silica spheres were obtained in the case of [Bmim][PF_6] ionic liquid, while solid silica spheres were obtained in the case of [Bmim][BF_4] ionic liquid for the same enzyme. The structural orientation of the enzyme within the capsules, their functional templating to obtain silica particles of varying morphology and finally their combined catalytic activity depend on the initial lipase-ionic liquid interaction. The enzyme activity of all these materials was evaluated against the esterification reaction between oleic acid (fatty acid) and butanol, *i.e.* biodiesel production. The relative enzyme activity was found to be 93.30% higher in the case of lipase nanocapsules synthesized in [Bmim][PF_6] and its *in situ* templating action to make hollow silica spheres further enhanced the residual activity. Furthermore time dependent kinetics of esterification by hollow silica spheres has also been shown here. Hollow silica spheres can also be used as a reusable catalyst for up to 6 cycles. This work demonstrates that the choice of ionic liquid is critical in controlling the self-assembly of enzymes as the ionic liquid–enzyme interaction plays a major role in retaining capsule activity and enzyme function.

Received 28th October 2015
Accepted 9th December 2015

DOI: 10.1039/c5ra22543d

www.rsc.org/advances

Introduction

Enzymes can be considered biocatalysts and have various advantages in environmentally friendly processes due to their ability to perform their action at near ambient temperature, with high specificity and enabling otherwise non-attainable reactions.[1] There are various factors that restrict the routine use of enzymes, such as instability under high temperatures, extreme pH conditions, interaction with organic media and their specific solubility.[1] To overcome these problems, considerable research has been carried out to immobilize enzymes on various supports and nanomaterials. There are various enzyme immobilization techniques and these include adsorption,[2,3] cross linked enzyme aggregates, entrapment and encapsulation,[4] covalent attachment and addition of lipase during silica condensation.[5]

Bio-molecular self-assembly is omnipresent in biological systems and motivates the development of a range of complex biological architectures which give rise to different metabolic functions in biology. These self-assembled structures can be reproduced under laboratory conditions and are of interest for various applications in emerging areas of biomaterial sciences.[6] Various stimuli such as changes in ionic strength, temperature, pH and the addition of certain chemical entities have been used to trigger self-assembly process of molecular building blocks and especially protein[7] and peptide amphiphile structures[8] based on self-assembled structures are well-known examples.[9–11] These studies prompted the use of peptide-based surfactants and peptides with programmed sequences leading to induce β-sheet or coil–coil interactions,[12–14] which were shown to undergo self-assembly in aqueous solutions.[15] Instead

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† Electronic supplementary information (ESI) available: TEM images of protease template self-assembled hollow silica spheres, self-assembled lipase capsule at lower concentration, illustration for esterification reaction and tables and figure for time dependent kinetics of esterification reaction by hollow silica spheres. See DOI: 10.1039/c5ra22543d

of using peptides, protein design could be used to manipulate devices through a 'bottom up' approach whereas proteins/biomacromolecules are used as monomeric building blocks for the fabrication of structures of higher order *via* self-assembly.[16]

Most of the studies mentioned above have hitherto used aqueous solvents as the media to explore the self-assembly of organic moieties. It is well known that bio-macromolecular self-assembly processes are known to be affected by the surrounding continuous phase. In fact, there is a very high possibility that if regularly used aqueous solvent phases are replaced with solvents of unique properties (such as ionic liquids-ILs) additional advantages to control the self-assembly processes may be attained rather than using the arduous synthetic method for self-assembly process *via* programmed amino acid sequences.[17] Surrounding media can definitely provide a simple approach for the self-assembly of bio-macromolecules without much change in their functionality.

Ionic liquids (ILs-commonly known as room temperature liquid salts) recently emerged as green solvents for the synthesis of organic compounds and nano-biomaterials due to their unique physico-chemical properties.[18] Recently, applications of these ionic liquids have started to be explored in the field of bionanosciences, especially in areas such as enzyme stabilization,[19] protein crystallization,[20] pGFP gene transformation,[21] anti-cancer drug delivery,[22] biocatalysis,[23] enzyme-templated metal nanospheres[24] and bio-fuel cells.[25]

However, the stability of these structures remains a challenge. Self-assembled nanostructures need to be supported by inorganic materials that can provide the required stability without compromising their activity. The rapid development in the area of new advanced functional nano materials and organic–inorganic hybrid nano-scaffolds, plays an increasingly significant role in the self-assembly of biomacromolecular building blocks.[26,27] Silica has been a technologically important inorganic material for wide range of commercial applications and various inorganic silica structures have been reported utilising enzyme and self-assembled peptide fibres[14,28] as self-assembling template. The ability to tune hydrophilic enzyme phytase[29,30] to synthesize enzymatically functional (hollow silica) and non-functional silica spheres (solid silica) by using hydrophilic [Bmim][BF_4] and hydrophobic [Bmim][PF_6] ionic liquid, respectively has been shown.[19] It would be intriguing to look at the self-assembling properties of a relatively hydrophobic enzyme in hydrophilic and hydrophobic media. Control over nanoparticle shape can be achieved by tuning the template property (in the earlier study the authors tuned the solvent) while bio-molecular self-assembly in ionic liquid has not yet been shown elsewhere.

We have used lipase enzyme in this study as lipases are considered to be of relatively hydrophobic nature.[31] Lipases have also been used as key enzymes in rapidly expanding biotechnology/bioprocess industry because of their multifaceted characteristics, which are used in various applications, such as pharmaceutical industry, bio-based surfactants in detergents, food technology, biomedical sciences,[32] and more recently in the renewable energy industry in biodiesel production.[33,34] Lipases liberate glycerol and fatty acids after acting on carboxyl ester bonds present in triacylglycerol-typically under aqueous conditions. Long-chain triacylglycerols are the natural substrates of lipases, which are much less soluble in polar solvents like water; the catalysis generally occurs at the lipid–water interface. Lipases have the exclusive ability to perform the reverse reaction, (under micro-aqueous conditions) leading to esterification and alcoholysis. There are a number of lipase immobilization methods that have been reported, namely immobilized onto a macroporous acrylic resin, covalent binding of lipase onto commercial Eupergit (R) supports and glyoxyl agarose beads.[2,35] Other examples of lipase immobilization include adsorption onto silica nanoparticles[36,37] cross-linking using glutaraldehyde[38,39] and sol–gel entrapment.[40] Many of these methods, however, suffer from tedious and expensive preparation methods, poor stability, or the use of toxic or hazardous chemicals.

In this article, we have reported the controlled self-assembly of a relatively hydrophobic enzymatic protein lipase (triacylglycerol acylhydrolase) (E.C. 3.1.1.3) from *Pseudomonas fluorescens* (a member of serine hydrolase family) in the ionic liquid 1-butyl-3-methylimidazolium hexafluorophosphate ([Bmim][PF_6]). Self-assembly of lipase enzyme in environmentally friendly green solvent [Bmim][PF_6]-'hydrophobic in nature' leads to the formation of lipase enzyme capsules further utilized as functionally active templates for the *in situ* growth of silica resulting in hollow silica spheres. These synthesized silica hollow nanospheres were found to function as nanocontainers by immobilizing lipase *in situ* and retaining its enzymatic activity for possible industrial applications. Most of the earlier studies have shown the immobilisation of lipase on the already synthesized silica or that the enzyme was added during the process of silica synthesis, however in our study we employed the principles of biomolecular (lipase) self-assembly in ionic liquid for its immobilisation in a single step (and one-pot) resulting into biogenic silica.

A plausible mechanism based on function activity of lipase for esterification of oleic acid and butanol (as a model biocatalytic reaction of commercial importance) and solid nanostructure obtained in [Bmim][BF_4], a hydrophilic IL *via* self-assembly process has been proposed and hollow silica nanocontainers further tested for enzyme reusability for the aforementioned reaction.

Results and discussion

Lipase self-assembly in ionic liquid [Bmim][PF_6]

Lipase enzyme of bacteria *Pseudomonas fluorescens* was extracted from the commercial lyophilized matrix by shaking for 2 h using deionized water as extraction media.

Lipase (20 μg mL^{-1}) was mixed with [Bmim][PF_6]-hydrophobic IL, and kept for 24 h shaking. Transmission electron micrograph has been shown in Fig. 1A, interconnected lipase capsules were obtained after controlled self-assembly of lipase enzyme in IL [Bmim][PF_6].

Sample preparation for TEM did not lead to disintegration of lipase capsules and these retained their morphology. This

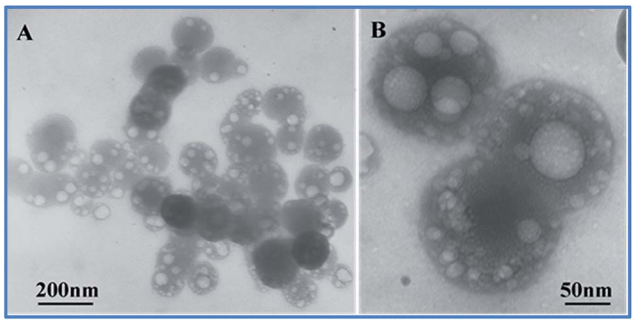


Fig. 1 Lower (A) and higher magnification (B) TEM images of self-assembled lipase (20 μg mL^{-1}) capsules synthesized in ionic liquid [Bmim][PF_6].

might be interesting in the case of biomacromolecular self-assembled soft matters. The lipase enzyme capsules are morphologically spherical, interconnected and apparently have a smooth surface with an average diameter ranging from 100–150 nm. Notably, due to the organic nature of enzyme, under the high energy electron shower of transmission electron microscope, these nanocapsules began to burst and/or burn during focusing, which is shown as a TEM micrograph of higher magnification in Fig. 1B, wherein self-assembled lipase nanocapsules start fusing with each other and showing relatively big interfused lipase hollow spheres in the centre, thus showing its interior. The self-assembly of lipase enzyme during a control experiment in water was not observed and gave a clear indication that ionic liquid [Bmim][PF_6] promoted the self-assembly process. A TEM image of a lipase capsules synthesized by using 10 μg mL^{-1} enzyme concentration has also been shown in Fig. S1.† These capsules were of similar size (~100–150 nm) and not much change in morphology was observed.

Synthesis of hollow silica spheres in ionic liquid [Bmim][PF_6]

Lipase capsules obtained in [Bmim][PF_6] were further used for synthesizing hollow silica nanoparticles and acted as templating nanoreactors. Hollow silica nanospheres were obtained by adding tetraethyl orthosilicate (TEOS), a silica precursor to pre-assembled lipase capsules (using 20 μg mL^{-1} lipase) in [Bmim][PF_6]. The TEM micrograph of hollow silica nanospheres synthesized *in situ* with [Bmim][PF_6]-hydrophobic IL has been illustrated in Fig. 2.

The silica particles obtained in this process were found to have a hollow interior. The diameter of these hollow silica nanoparticles ranges from 150 to 200 nm with a spherical morphology and rough surface. Thick-walled interconnected hollow silica nanospheres (wall thickness of ~40 nm) without much significant difference in overall nanosphere size were observed (Fig. 2 inset). When we checked the effect of higher lipase concentration up to 100 μg mL^{-1}, the hollow interior started to reduce in size and TEM imaging of these materials appeared to be of solid spheres or hollow spheres with a very small narrow interior having the diameter varying between 1–5 nm (image not shown for brevity).

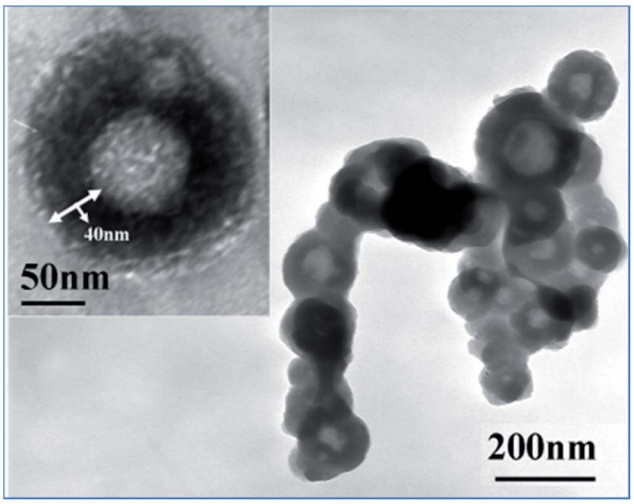


Fig. 2 TEM images of hollow silica nanoparticles synthesized in ionic liquid [Bmim][PF_6] using self-assembled lipase capsules as catalytic templates. Lipase template capsules were self-assembled using 20 μg mL^{-1} lipase enzyme and inset shows the thickness of hollow silica nanoparticles (~40 nm) at a higher magnification image of one of the particles from the same sample.

Apparently, it is remarkable that the self-assembly of a relatively hydrophobic enzyme lipase in a hydrophobic IL [Bmim][PF_6] resulted in the synthesis of enzyme nanocapsules and the aforementioned were utilized for the *in situ* synthesis of hollow silica nanospheres with variable wall thickness.

Formation of hollow silica spheres without any external hydrolyzing agent is quite notable and suggests the role of self-assembled lipase capsule as a functional template for the synthesis of hollow silica nanocapsules and simultaneously enzymatically hydrolyzing the TEOS.

XPS analysis of lipase and silica hollow spheres

XPS analysis of self-assembled lipase in IL ([Bmim][PF_6]) and lipase templated hollow silica nanostructures was further carried out to investigate the nature of interaction between the lipase-IL nanostructures and the hollow silica nanostructures. Fig. 3 shows the F 1s, N 1s, C 1s, O 1s and Si 2p core-level spectra collected from the lipase-IL nanostructures and the lipase-IL nanostructures templated silica hollow nanospheres. The presence of F 1s component (BE 686.0 eV) observed both in the case of lipase-IL and lipase-IL–SiO_2 nanostructures, clearly reveals that these nanostructures consist of IL units and an integral part of the final structures. The intensity of P 2p level spectral component was too weak to be detected. Since the source of fluorine comes only from IL, therefore the absence of P 2p signal still indicates the incorporation of IL units within the nanostructures. Another major evidence of the presence of IL units comes from the N 1s spectral data of lipase-IL and lipase-IL–SiO_2 nanostructures. Lipase-IL nanostructures clearly showed two distinct N 1s components and their binding energies observed at 399.3 eV and 401.5 eV. The higher binding energy N 1s component (401.5 eV) corresponds to the imidazolium part of IL and the lower binding energy N 1s component

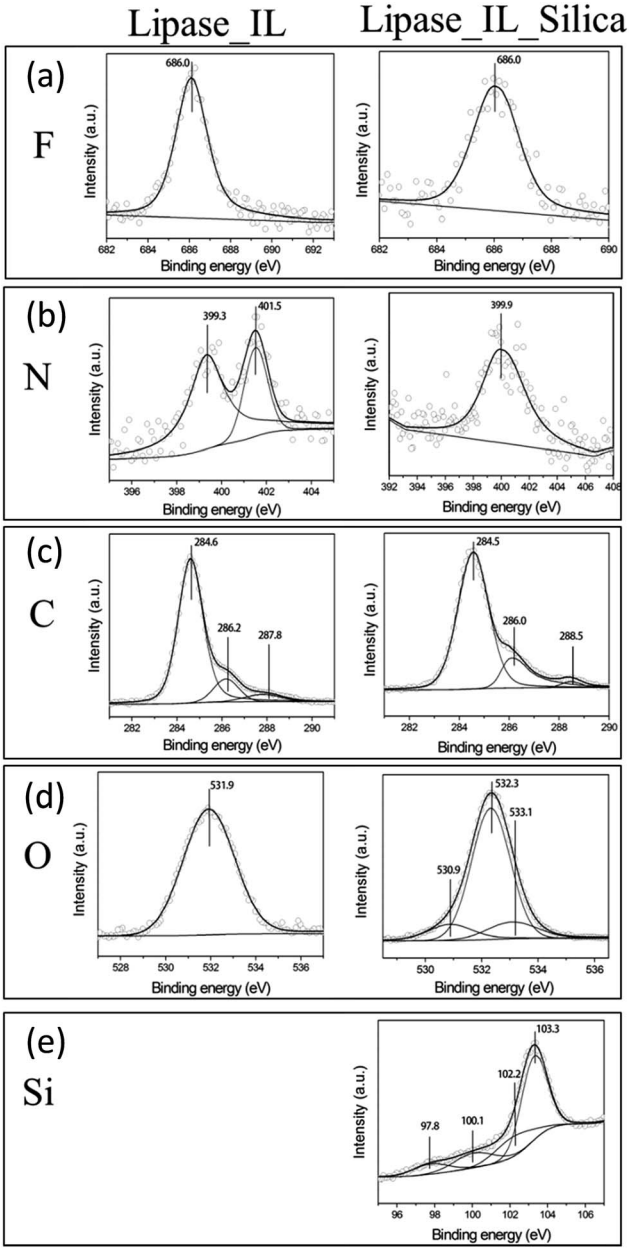


Fig. 3 XPS core-level spectra recorded from lipase and lipase–silica after interaction with ionic liquid [Bmim][PF_6]. (a) F 1s core-level spectra. (b) N 1s core-level spectra. (c) C 1s core-level spectra. (d) O 1s core-level spectra. (e) Si 2p from lipase-IL and lipase-IL–silica nanostructures. The solid lines correspond to nonlinear least-squares fits to the experimental data as shown by symbols.

originates from the amine group of amino acid residues from the lipase enzyme. However, after the silica structures formation, N 1s core level spectra was found to be different from the lipase-IL nanostructures. The two distinct N 1s components were merged into single component and the binding energy was observed at 399.6 eV. There are many reports in the literature that demonstrated that hydrolysis of TEOS to form silica can be catalysed by amine groups of amino acids and imidazolium groups of IL. Their role in TEOS hydrolysis and their electrostatic interaction with negatively charged silica nanostructures led to the major changes in N1s core-level spectra and their binding energies. This was also supported from the C 1s core level spectra, collected from lipase-IL nanostructures and lipase-IL–SiO_2 nanostructures. C 1s level that corresponds to the carboxylic acid carbon (BE 287.8 eV) and the carbon attached with the nitrogen (BE 286.2 eV) present in the lipase-IL nanostructures showed change in their binding energy after the silica nanoparticles formation around the nanostructures. This was probably due to the silica nanoparticles binding with the imidazolium group of IL moieties and amine groups of lipase enzyme, which resulted in lifting the weak electrostatic interaction between the carboxylic acid groups of the enzyme with the amine and imidazolium moieties. However, significant changes in the O 1s core-level spectra observed after lipase-IL nanostructures mediated the hydrolysis of TEOS to form silica nanostructures, as shown in the O 1s core-level spectra are shown in Fig. 3c. Since IL [BMIM][PF_6] does not contain any oxygen, the changes observed in the case of the O 1s core-level spectra were a direct consequence of any modification that occurred in the lipase-IL nanospheres and the presence of silica. In the case of lipase-IL, O 1s component exhibit its BE at 531.9 eV, which was assigned to the carboxylic oxygen atoms present in the amino acid residues of lipase enzyme. In lipase-IL–SiO_2, three chemically distinct components of O 1s were observed and the binding energy of the lipase carboxylic oxygen was shifted to 532.3 eV. The additional two O 1s components observed at BEs 530.9 and 533.1 eV, were attributed to the SiO_2 and Si–OH components. Fig. 3e shows the Si 2p core level spectra recorded from lipase-IL–silica nanostructures and the prominent peak observed at BE 103.3 eV that corresponds to silicon present in the SiO_2 nanostructures. This is a clear indication of the presence of silica structures (as a result of TEOS hydrolysis) around the lipase-IL nanostructures.

Plausible mechanism of synthesis of hollow silica spheres

Lipases are considered to be hydrophobic (due to their affinity to hydrophobic surfaces: oil microcapsules[41]) in nature and soluble in both hydrophilic and hydrophobic solvents while also having large hydrophobic amino acids domains around the active site.[31] However lipases are rather diverse from other hydrolytic enzymes because of their catalytic activity in non-aqueous systems, relatively hydrophobic nature and interfacial activation phenomenon.[31] Lipase enzyme of bacteria *Pseudomonas fluorescens* used in this study was obtained commercially and this enzyme belongs to the serine hydrolases family which is typically known to acts at the interface of lipid–water or hydrophobic–hydrophilic boundary. The catalytic active site domain comprises Ser-Asp/Glu-His and usually the serine of the active site domain is also found to be surrounded by a conserved sequence (Glyx-Ser-x-Gly). The α/β-hydrolase fold, a typical characteristic of mostly lipases, is also revealed in the three-dimensional structural maps.[42] The role of catalytically active histidine in this sequence can be proposed as it is known to hydrolyse the substrate in a two-step reaction.[31] The hydrolysis of TEOS, leading to hollow silica nanospheres can be associated with such catalytically active domains. Lipase has

more hydrophobic domains even though it is amphiphilic in nature.[31] Therefore, dispersing them in a non-polar ionic liquid like [Bmim][PF_6] tends to drive the self-assembly of many individual enzyme units by orienting their hydrophobic domains towards the [Bmim][PF_6] continuous phase (Scheme 1). Since spherical structures are known to have lower surface energy, self-assembly of lipase enzyme molecule in [Bmim][PF_6] resulted in the formation of spherical structures as revealed from the TEM images (Fig. 1).

In addition, it is also proposed that due to inter-molecular hydrogen bonding the amino acids of respective domains of enzyme may also come in proximity with each other along with the hydrophobic domains of lipase, with hydrophobic tail packing between them, finally leading to the formation of a structure that looks more like a nanocapsule of lipase.

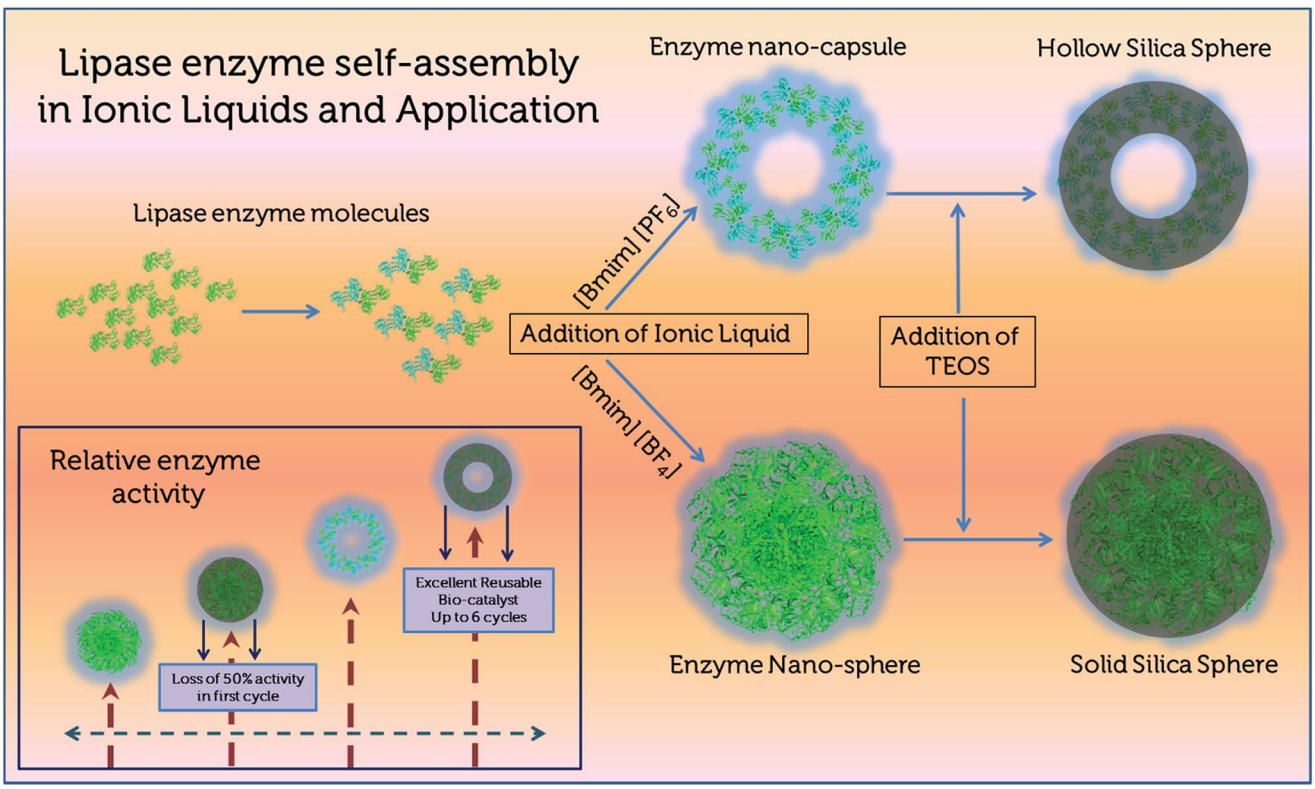
In hydrophobic IL [Bmim][PF_6], this hierarchal self-assembly of lipase should result in a hydrophilic core moving away from [Bmim][PF_6], and its hydrophobic groups aligning to [Bmim][PF_6].

Catalytically active sites and surrounding amino acid domains of lipase[31] are considered hydrophobic in nature and these domains of active site are most probably expected to be in close vicinity with [Bmim][PF_6]. When silica precursor (TEOS) is added, its hydrophobic nature tends to stay on the hydrophobic domains of the enzyme capsules, which also comprises the active site domains for hydrolysis of TEOS.[43]

For the enzyme encapsulation and formation of hollow silica nanospheres with a thin silica layer, the TEOS hydrolysis was initiated on the surface of a nanocapsule template made of self-assembled lipase units that grew from the surface to inwards. Its TEM image has been illustrated in Fig. 2 and thick walled (~40 nm) hollow silica spheres (Fig. 2 inset) were obtained primarily due to the availability of active sites on the surface of the template; also more lipase lamellar layers formed in the capsule so that TEOS hydrolysis was facilitated on the surface of the lipase nanocapsule. This also interestingly proposes that the self-assembled lipase nanocapsules obtained in [Bmim][PF_6] acted as a template for silica growth while concurrently enzymatically hydrolyzing the silica precursor.

Synthesis of silica solid spheres in hydrophilic ionic liquid [Bmim][BF_4] and plausible mechanism

As per the above-mentioned mechanism, when lipase enzyme was added to a IL [Bmim][BF_4], hydrophilic in nature using similar reaction conditions, it was anticipated that the hydrophobic lipase enzyme molecules would undergo a different kind



Scheme 1 Schematic illustration for the structure–function relationship of self-assembled lipase enzyme in hydrophobic [Bmim][PF_6] and hydrophilic ionic liquids [Bmim][BF_4]. Lipase enzyme structure from *Pseudomonas* (http://www.ebi.ac.uk/pdbe/entry/pdb/3a70) taken from a protein data bank in Europe (with due permission) is shown here to represent enzyme molecules. Self-assembly using hydrophobic IL [Bmim][PF_6] leads to lipase nanospheres with-active hydrophobic domains facing ionic liquid; while in hydrophilic [Bmim][BF_4] catalytically-active hydrophobic domains are sequestered towards the center. Addition of TEOS to the self-assembled lipase leads to the formation of a hollow silica nanosphere in [Bmim][PF_6], but results in a solid silica sphere in [Bmim][BF_4]. An interesting correlation between the synthesized nanostructures and relative enzyme activity was also observed.

of structural packing during the self-assembly process within the lipase capsules, which differs from the structural packing that was observed in the case of lipase capsules formed in hydrophobic IL.

Therefore, in the lipase nanocapsules self-assembled in IL [Bmim][BF_4], the hydrophilic regions of lipase enzyme should position it orienting outwards (towards the hydrophilic continuous phase of IL [Bmim][BF_4]), while the lipase enzyme's hydrophobic domains should be packed inside away from [Bmim][BF_4]. Since catalytically active sites of lipase are present in the hydrophobic region of lipase (which are sequestered towards the centre in a hydrophilic ionic liquid), as shown in Scheme 1, hydrolysis of silica on self-assembled lipase templates in IL [Bmim][BF_4] should happen in an inward to outward manner. Since TEOS is also hydrophobic in nature, TEOS tends to infuse within the hydrophobic domains of the lipase capsules, thus staying on the surface.

Transmission electron micrograph of lipase enzyme and silica nanostructures obtained in IL [Bmim][BF_4]-hydrophilic after lipase self-assembly and hydrolysis of TEOS is shown in Fig. 4 and 5, respectively. Solid silica nanoparticles of broad size distribution (diameter 100–250 nm) and quasi-spherical in shape were obtained. These are quite different in comparison with the above-mentioned hollow silica particles obtained in [Bmim][PF_6]. Fig. 5B inset shows the TEM image for silica solid spheres in higher magnification, showing a dense core while the intensity towards the periphery fades away. This TEM micrograph suggested that both the TEOS hydrolysis and nucleation of silica start from the hydrophobic protein domain that has been sequestered towards the centre as a dense core was observed in solid silica nanoparticles while using [Bmin][BF_4]. Interestingly, the same enzymatic protein lipase results in hollow silica nanospheres when in hydrophobic IL [Bmim][PF_6] and in solid silica particles when in hydrophilic IL [Bmim][BF_4].

We envisage that the self-assembly of enzymes may have led to the formation of superstructures involving structural changes on each enzyme unit in the superstructure as directed by the polarity of the ionic liquid. The relatively hydrophobic lipase undergoes self-assembly to form capsules in two ionic liquids, which differ only in their polarity as a result of their counter anions. However, the function of the enzyme capsules was found to be different because of the structural reorganization of each enzyme unit within the capsule. As a result, hydrolysis of TEOS was found to occur at catalytic sites in different regions of the enzyme capsules. There are no reports in the literature that show the synthesis of silica hollow and solid nanoparticles by using a hydrophobic enzyme. In summary, a commonly known hydrophobic enzyme lipase in a hydrophobic ionic liquid [Bmim][PF_6] results in a hollow self-assembled functional template that hydrolyses the silica precursor and results in functional hollow silica nanoparticles. The same hydrophobic enzyme in hydrophilic ionic liquid [Bmim][BF_4] results in solid silica nanospheres. This strongly suggests a complementary advantage over earlier approaches which require the modification of bio-macromolecules before their self-assembly can be performed.[44] As a result, the final structures of silica enzyme capsules were found to be different. Eventually, the structure and function of these capsules with and without silica particles was reflected in the natural enzymatic activity of the lipase.

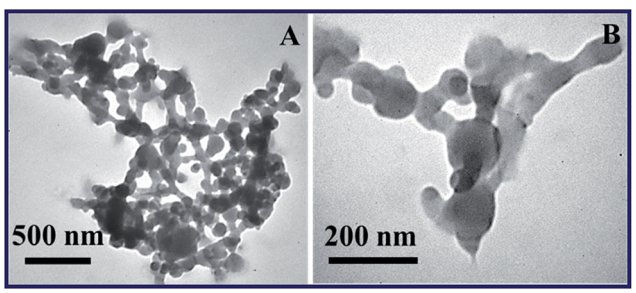


Fig. 4 Lower (A) and higher magnification (B) TEM images of self-assembled lipase (20 μg mL^{-1}) structures obtained in ionic liquid [Bmim][BF_4].

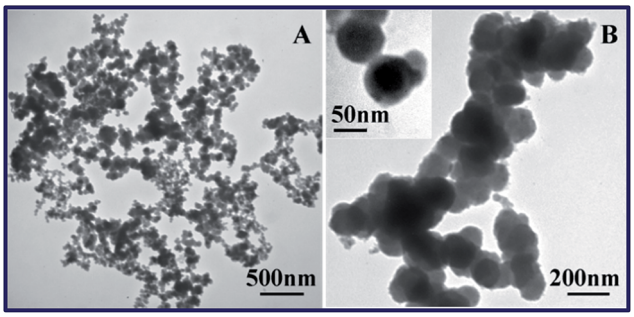


Fig. 5 Lower (A) and higher magnification (B) TEM images of solid silica nanoparticles synthesized in ionic liquid [Bmim][BF_4] using 20 μg mL^{-1} lipase enzyme.

Self-assembly of protease enzyme and synthesis of hollow silica spheres in [Bmim][BF_4]

Self-assembly of hydrophilic protease (papain) enzyme in hydrophilic ionic liquid [Bmim][BF_4] leads to the synthesis of hollow silica spheres containing enzymes (ESI Fig. S2†). This can be a versatile approach tosynthesize and immobilize enzyme into biogenic silica nanocapsules utilizing ionic liquids, in a single step.

Structure–activity relationship of lipase encapsulated hollow and solid silica spheres for esterification reaction for biodiesel production

In this work we report the results on the esterification of oleic acid with *n*-butanol (Fig. S3†) using immobilized lipase as biocatalyst (prepared by single step self-assembly process) which leads to enantiomeric esters (biodiesel)[34] with >98% of selectivity.

Gas Chromatography (GC) was used as the method to quantify esters of fatty acids produced and residual fatty acid *i.e.* oleic acid. Fig. 6 represents the comparative activities of the lipase enzyme in its different environments for the esterification of oleic acid with *n*-butanol. Lipase in butanol is much active than in water due to its better solubility in butanol. A

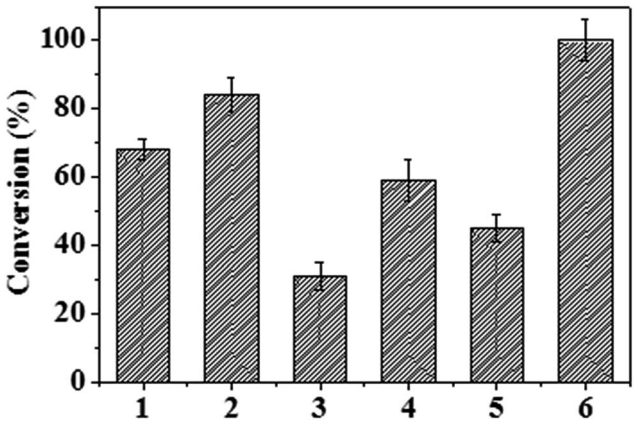


Fig. 6 Comparative lipase enzyme activity for the esterification of oleic acid with *n*-butanol (in terms of conversion of oleic acid). 1. Lipase dissolved in de-ionized water, (2) lipase dissolved in butanol, (3) self-assembled lipase in [Bmim][BF_4], (4) self-assembled lipase capsules synthesized in [Bmim][PF_6], (5) lipase encapsulated solid silica nano spheres synthesized in [Bmim][BF_4], (6) lipase encapsulated in hollow silica nanocapsules synthesized in [Bmim][PF_6].

cartoon illustrating the structure and activity relationship of native enzyme, self-assembled lipase enzyme capsules, silica nanospheres (hollow-synthesized in IL [Bmim][PF_6] and solid-synthesized in IL [Bmim][BF_4]) and residual enzymatic activity (tested for esterification reaction) for all the samples is elucidated in Scheme 1. Lipase-encapsulated solid silica nanospheres synthesized in hydrophobic IL were found relatively 55% less active than hollow silica nanospheres formed in hydrophilic IL (conversion of oleic acid to ester by hollow silica spheres was considered 100%).

The highly porous walls of hollow silica nanospheres facilitate the dispersion of lipase on support and substrate and therefore products are diffused during enzymatic reaction *via* these pores. This might lead to the possibility of *in situ* TEOS hydrolysis by the lipase capsules formed in hydrophobic [Bmim][PF_6], once enhanced enzymatic activity was expressed in hollow silica capsules (enzyme–silica nanobio-hybrid) formed in [Bmim][PF_6] in comparison to solid silica nanostructures synthesized in [Bmim][BF_4] (Fig. 6 and Table S1†). Scheme 1 represents these results in an illustrative way for better clarity of the structure–function relationship of lipase hollow and silica nanospheres' enzymatic activity.

Reusable biocatalysts and peptide loaded nano-vehicles[45] have attracted a lot of attention due to their widespread applications in therapeutics with emphasis on the immobilization of commercially and technologically significant biomolecules onto robust supports as an area for wide-ranging research and development. However, the main challenge linked with enzyme/protein immobilization strategies is to preserve the native enzyme activity onto a reusable substrate without any leakage or denaturation of the enzyme. One way to address these issues and to preserve enzyme activity is to use ILs with unique solvent properties.[46] The lipase enzyme employed in this particular study are known for their significant industrial and biomedical[31] value in the synthesis of enantiomeric therapeutic compounds and potential drug intermediates.[31] As there was *in situ* growth of silica nanospheres in ILs, in which self-assembled lipase capsules were acting as templates, there is a very high possibility of finding embedded enzyme molecules in both hollow and solid silica nanostructures during their synthesis mediated by lipase enzyme (in [Bmim][PF_6] and [Bmim][BF_4], respectively). Hence, enzyme reusability studies were performed with hollow and solid silica nanocontainers. Hollow silica nanospheres synthesized in [Bmim][PF_6] significantly showed bio-catalytic conversion of oleic acid and 1-butanol by a well-known esterification reaction and retained its 88% activity at-least up to six cycles of enzyme reusability (Fig. 7).

After the first cycle solid silica spheres retained only 45% of their initial activity (Fig. 7) and hence the enzymatic activity of these particles was not tested further. It is particularly interesting to know the retention of lipase enzyme activity onto hollow silica spheres as the activity of the native enzyme generally highly sensitive to environmental factors such as solvent system, temperature and pH, variation in any one of these factors can lead to changes to the enzyme's native structure leading to loss of enzymatic activity. Time dependent kinetic studies have also been performed (ESI Fig. S4†) with hollow silica spheres. The conversion of oleic acid was checked every 15 minutes and tested for 2 hours. The maximum conversion in 2 h was 85% (ESI Table S2†), that normalised to 100% and the subsequent conversions were plotted for the different time intervals. It has been demonstrated that the choice of ionic liquids is critical in controlling the self-assembly of enzymes due to their specific interaction with the enzymes, its major role in retaining the function of the enzyme and the activity of the capsules.

Experimental

Materials

Commercial Amano Lipase AK of *Pseudomonas fluorescens* and tetraethyl orthosilicate (TEOS) were procured from Sigma-

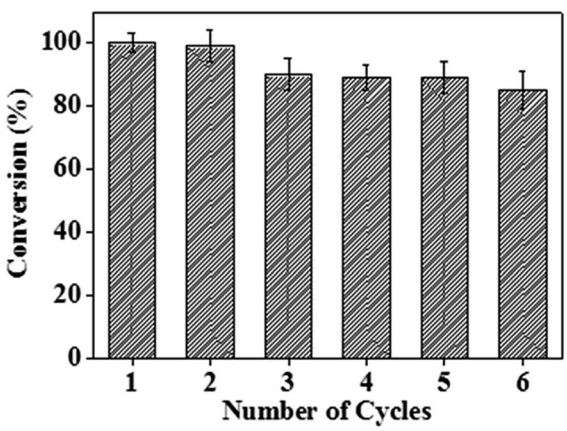


Fig. 7 Reusability study of lipase enzyme encapsulated within hollow silica nanospheres during their synthesis in ionic liquid [Bmim][PF_6] for esterification of oleic acid with 1-butanol.

Aldrich. Ionic liquids (ILs) 1-butyl-3-methylimidazolium tetrafluoroborate ([Bmim][BF_4]) and 1-butyl-3-methylimidazolium hexafluorophosphate ([Bmim][PF_6]) were purchased from Ionic Liquid Technologies (IoLiTec). All chemicals were used as received, unless specified.

Lipase extraction

Lipase was extracted from the dried commercial powder. 10 mg of lyophilized lipase on inert matrices was suspended in 1 mL of deionized water and kept shaking at room temperature for 4 h. The dissolved lipase was recovered by centrifugation at 14 000 rpm for 10 minutes in supernatant. The extracted protein concentration was checked by Bradford method[47] and diluted accordingly with deionized water to make final a concentration of 10 mg mL^{-1}.

Material characterisation

TEM studies were made on a JEOL JEM-2100F instrument equipped with a slow-scan CCD camera and an accelerating voltage of the electron beam (200 kV). The preparation of TEM samples involved sonication in isopropanol for 2–5 min, followed by deposition of a drop on a copper grid supporting a perforated carbon film and allowed to dry. The nature of the material synthesized (lipase capsules and hollow silica nanospheres) in ionic liquid [Bmim][PF_6] characterized by XPS and measurements was carried out on a Thermo K Alpha XPS instrument at a pressure greater than 1×10^{-9} Torr (1 Torr = 1.333×10^2 Pa). The general scan and N 1s, P 2p, O 1s, and C 1s core-level spectra of hollow silica spheres, as well as core-level spectra from the respective samples were recorded with monochromated aluminum Kα radiation (photon energy = 1486.6 eV) at a pass energy of 20 eV and an electron take off angle (angle between the electron emission direction and surface plane) of 90°. The core-level binding energies (BEs) were aligned with the adventitious carbon binding energy of 285 eV.

Ionic liquid-mediated synthesis

Lipase nanocapsules. A typical synthesis reaction of lipase nanocapsules in ILs was performed in 0.5 mL volume containing 490 μL of the respective IL [Bmim][PF_6] and 10 μL of purified lipase enzyme (1 mg mL^{-1} dissolved in water), thus achieving a final enzyme concentration of 20 μg mL^{-1} in the reaction. The IL-lipase mixture was incubated at 37 ± 0.1 °C for 24 h with gentle reciprocal shaking, after which samples were centrifuged at 14, 000 rpm, followed by washing with deionized water and acetonitrile to remove the viscous IL. Lipase nanocapsules thus obtained were further analysed by TEM and XPS.

Hollow and solid silica nanospheres. To obtain silica nanospheres, 20 mM tetraethyl orthosilicate (TEOS) stock solution was prepared in the respective ILs viscous [Bmim][PF_6] and [Bmim][BF_4]. 500 μL of TEOS stock solution (20 mM) in ILs was added to 500 μL of the aforementioned reaction mixture containing IL and lipase capsules. The final concentration of lipase became 10 μg mL^{-1} and TEM image of lipase capsules (10 μg mL^{-1}) has been shown in Fig. S1 under ESI.† These capsules looked similar to the lipase capsules shown in Fig. 1 (obtained with 20 μg mL^{-1} concentration). The 1 mL reaction contents were incubated at 37 ± 0.1 °C for 24 h under stirring conditions, during which all the reactions involving lipase became turbid, indicating TEOS hydrolysis. After 24 h of reaction, samples were centrifuged at 8000 rpm followed by washing with deionized water and acetonitrile to remove the viscous ionic liquid. Silica nanostructures thus obtained were further analyzed by TEM and used for enzyme reusability studies. In a control experiment, 10 μL of deionized water were added to 500 μL of TEOS stock solution and 490 μL IL, and the reaction was pursued along with the main experiment. However, no turbidity was observed in the control reaction containing water, thereby negating the possibility of water-mediated hydrolysis of TEOS in IL [Bmim][PF_6] and [Bmim][BF_4] and suggesting the role of lipase towards TEOS hydrolysis in ILs. Washing the reaction products with deionized water at 37 °C (instead of washing with acetonitrile) did not show any significant difference in the silica nanoparticles formed, however samples not washed with acetonitrile were difficult to image in TEM due to the residual presence of ILs in those samples. The TEM images shown in this article correspond to nanoparticles that were washed with acetonitrile.

Esterification of oleic acid with 1-butanol and reusability of bio-catalyst. The preparation contained only one kind of lipase and no other enzymes which had esterifying activity. To determine the enzymatic stability of lipase in hollow silica nanospheres after encapsulation, hollow silica nanoparticles synthesized using 80 μg mL^{-1} lipase in IL [Bmim][PF_6] were dispersed in *n*-butanol.

The reaction mixture (3.6 mM oleic acid with 36 mmol *n*-butanol) was then incubated at 50 °C for 2 h at 300 rpm, followed by centrifugation to obtain a clear supernatant and nanoparticles pellet. The conversion of oleic acid in to ester due to enzyme activity was determined in supernatant by gas chromatography as mentioned.[48] The products of the reaction were analysed directly by injection of a 0.2 μL sample into the GC. The products were analyzed by Shimadzu Gas Chromatograph 14A, Ultra-1 (25 m × 0.3 mm capillary column) equipped with FID and helium as a carrier gas. The major product was *n*-butyl oleate which was compared with the standard. Conversion and selectivity were calculated using following formula.

$$\text{Conversion of oleic acid (\%)} = \frac{\text{moles of oleic acid consumed}}{\text{moles of oleic acid initially added}} \times 100$$

$$\text{Selectivity of } n\text{-butyl oleate (\%)} = \frac{n\text{-butyl oleate produced}}{\text{moles of all products}} \times 100$$

A time-dependent kinetics study was also performed with the interval of 15 minutes. To check the recyclability of the immobilized enzyme, the nanoparticle pellet obtained after centrifugation (containing hollow silica nanocontainers with encapsulated enzyme) was washed twice with 1-butanol and fresh reactants were added for the next reaction cycle, as in the

first cycle. The experiment was continued for 6 cycles and the enzymatic activity obtained in first cycle was considered as 100% for comparison with subsequent cycles. Experiments were conducted in triplicates to minimize experimental error.

Conclusions

In summary, we have shown that ionic liquids can be utilized as non-aqueous self-assembling media for hydrophilic as well as hydrophobic enzymatic proteins. A strategy for structure–function and activity relationship between biomolecules and ionic liquids can be proposed to develop single step procedures for enzyme immobilization while retaining the native activity of biomacromolecule. It is envisaged that various combinations of biomolecules and ionic liquids might lead to the incorporation of interesting properties to biomolecules that have not been explored in past, thus opening doors for various novel applications of such hybrid materials in bio-material science, bio-processes and bio-energy. The present work improves our understanding in lipase self-assembly in ionic liquids, not only for controlling the structure but also for providing a relationship to functionality of inorganic–organic hybrid bio-materials. It would be interesting to elucidate the structure–function relationship of a combination of biomolecules (DNA, protein, lipids) and ionic liquids made up of variable cationic and anionic groups.

Acknowledgements

S. K. S. and SS thanks Australian Government, Department of Education for an Endeavour Research Award and School of Applied Sciences, RMIT University for an ECR-start up grant to SKS. The authors duly acknowledge the RMIT Microscopy and Microanalysis Facility (RMMF) for providing access to their instruments used in this study. The authors would also like to thank the protein data bank in Europe[49] for granting permission to use the *Pseudomonas* sp. MIS38 lipase[50] enzyme that was used to represent lipase enzyme molecules in Scheme 1. Authors are also very grateful to Ms. Ana Maria Martins for proofreading and correcting grammatical errors in this article.

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