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Self-Assembled Enzyme Capsules in Ionic Liquid [BMIM][BF_4] as Templating Nanoreactors for Hollow Silica Nanocontainers

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Most of the self-assembly studies have hitherto explored the aqueous media as fluid phase for self-assembly of amphiphilic biomacromolecules, wherein architectural modification of biomolecules is generally a prerequisite for self-assembly of modified biomolecules. We demonstrate for the first time that ionic liquids can act as nonaqueous designer solvents to self-assemble amphiphilic biomacromolecules without requiring their prior modification. To this end, we show that enzyme (phytase) molecules self-assembled in the presence of an appropriate ionic liquid, resulting in the formation of enzyme capsules. Phytase capsules synthesized using this approach were further used as templating nanoreactors for the synthesis of enzyme-containing hollow silica nanocontainers. In situ immobilized phytase enzyme in the silica nanocontainers, when subjected to enzyme-reusability application, establishes them as excellent reusable biocatalysts.

Self-assembly is one of the most important strategies used within biological systems for the development of complex and functional macromolecular superstructures such as nucleic acids, proteins, and lipopolysaccharides. Taking inspiration from naturally occurring self-assembled structures, there have been a myriad of efforts toward controlled self-assembly under laboratory conditions.[1−7] Significantly, these investigations have led to a variety of nanostructured materials including silica/organoclay casing of single polysaccharide molecule,[1,2] DNA,[2] proteins,[2−4] and enzymes.[3−5] Additionally, a diverse range of synthetic organic molecules has been used to synthesize functional nanostructure composites that has advanced the template-directed assembly of organic−inorganic hybrid nanoscale objects under equilibrium conditions.[8−15] Some representative examples include self-assembled peptides with sequences programmed to have appropriate polar or charged surface amino acid residues[8−10] that induce either β-sheet (amyloid) formation[11,12] or initiate coil−coil intermolecular interactions.[13] Similarly, peptide-based surfactants have also been investigated for self-assembly in aqueous solutions.[14,15] In general, the above amphiphiles and peptides show certain key characteristics that are designed into their molecular structure to facilitate self-assembly and their further use as effective templates for nanoscale deposition of inorganic materials.[16−21]

Among inorganic materials, silica is one of the extensively used technologically important materials for a wide range of potential commercial applications.[16] Various kinds of inorganic silica structures synthesized by molecular self-assembly have been previously reported, including chiral diaminocyclohexane derivatives,[17] sugar-appended porphyrin,[18] collagen fibres,[19] β-1,3-glucan polysaccharide,[1] J-aggregates of tetrakis(4-sulfonatophenyl)porphine (TPPS),[20] dextran,[21] amylopectin,[21] horse heart cytochrome,[21] and self-assembled peptide fibres.[13] It is noteworthy that most studies including the aforementioned ones have hitherto explored aqueous solvents as the fluid phase for self-assembly of organic species.[22] Because the molecular self-assembly process can be significantly influenced by the surrounding medium,[23] it is highly possible that if regularly employed aqueous solvents are replaced with nonconventional solvents (such as ionic liquids (ILs)), then it might lead to another level of control over the self-assembly processes. Therefore, in addition to the cumbersome programmed amino acid sequences-mediated self-assembly, solvents can also provide a facile handle for self-assembly of nanomaterials. To this end, we have recently demonstrated the formation of novel dendritic metal nanostructures via a self-organization process in ILs, which suggests that ILs can act as designer solvents to promote self-assembly processes.[24]

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ILs (commonly referred to as room-temperature molten salts or room-temperature ILs) have recently become attractive reaction media for the "green" synthesis of nanomaterials due to their unique physicochemical properties such as high viscosity, high ionic conductivity, high thermal and chemical stability, and negligible volatility.[24−27] Bioscience is among the most interesting areas where ILs are just beginning to play an important role, with demonstrated applications in enzyme stabilization, protein crystallization, and biofuel cells.[27] Recent studies show that the enzyme/biocatalyst activity may be influenced by the solvent properties of ILs, resulting in increased enzyme activity, stability, or selectivity and an extensive solubility of substrates and enzymes.[28] This is an interesting property of ILs in the context of potential protein self-assembly-mediated synthesis of hybrid inorganic−organic nanomaterials in ILs because in a hybrid nanoarchitecture the functionality of the organic component should ideally be retained after assimilation of the inorganic component, which is not always easy to achieve.[22,23] Although the unique solvent properties of ILs have led to the exploration of different biocatalytic reactions in ILs[28−30] as well as self-assembly of nonbiological amphiphiles (e.g., surfactants, lipids, block copolymers, etc.),[25] to the best of our knowledge, no attempt has been made to capitalize the potential of ILs as designer solvents for self-assembly of amphiphilic biomacromolecules (e.g., peptides, proteins, or enzymes).

In this Article, we report for the first time controlled self-assembly of myo-inositol hexakis phosphohydrolase (hereafter referred as "phytase") enzyme in the IL 1-butyl-3-methylimidazolium tetrafluoroborate ([BMIM][BF_4]), leading to the formation of phytase capsules. Phosphohydrolase (phytase) has been chosen as a model enzyme in this study because of its significant biomedical importance associated with bone regeneration and growth[31] as well as its industrial applications.[32] Phytase capsules obtained via self-assembly in [BMIM][BF_4] have been further utilized as functional templates for the spontaneous growth of hollow silica nanocontainers. These silica nanocontainers are found to immobilize phytase enzyme in situ during their synthesis while retaining enzymatic activity. The mechanism for self-assembly of phytase capsules has been proposed by comparing self-assembly in a different IL [BMIM][PF_6], and phytase-immobilized silica nanocontainers have been further tested for enzyme reusability studies.

Results and Discussion

In brief, phytase enzyme was initially purified to homogeneity from the submerged fermentation broth of a fungus *Aspergillus niger*, as was recently reported.[33] In a typical reaction, phytase enzyme (20 μg mL^{-1}) was added to IL [BMIM][BF_4] and left at room temperature for 24 h under shaking conditions. Figure 1A shows the transmission electron microscopy (TEM) micrograph of phytase capsules obtained after self-assembly of phytase molecules in the IL [BMIM][BF_4]. Interestingly, the self-assembled phytase capsules do not disintegrate and retain their morphology during drying for TEM sample preparation. The protein capsules are quasi-spherical in morphology with an apparently rough surface and an average diameter of 150−200 nm. Notably, because of their organic nature, on exposure to the electron beam of a TEM, these capsules begin to burst during TEM imaging. This is evident from a higher magnification TEM micrograph shown in Figure 1B, wherein a phytase capsule popped open from the center while showing its interior is shown. In a control experiment in water, no phytase self-assembly was observed.

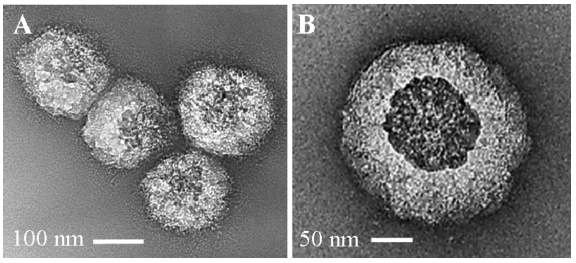

Figure 1. (A) Lower and (B) higher magnification TEM images of self-assembled phytase (20 μg mL^{-1}) capsules synthesized in ionic liquid [BMIM][BF_4].

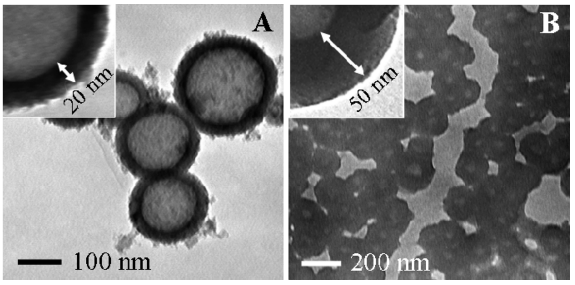

Figure 2. TEM images of hollow silica nanoparticles synthesized in ionic liquid [BMIM][BF_4] using self-assembled phytase capsules as catalytic templates. Phytase template capsules were self-assembled using (A) 20 and (B) 40 μg mL^{-1} phytase enzyme in [BMIM][BF_4]. Insets show the thickness of hollow silica nanoparticles in a higher magnification image of one of the particles shown in the respective main figures.

The self-assembled phytase capsules obtained in IL [BMIM]-[BF_4] were further utilized as templating nanoreactors for the synthesis of hollow silica nanoparticles. To obtain hollow silica nanoparticles, a silica precursor, tetraethyl orthosilicate (TEOS), was added to IL [BMIM][BF_4] containing phytase capsules (preassembled using 20 μg mL^{-1} phytase). Illustrated in Figure 2A is the TEM image of hollow silica nanoparticles synthesized in [BMIM][BF_4]. The hollow silica nanoparticles are 120−150 nm in diameter with a rough surface and quasi-spherical morphology. The higher magnification TEM micrograph of one of the hollow silica nanoparticles indicates that these hollow silica nanoparticles are ~20 nm thick (inset, Figure 2A). When the phytase concentration in [BMIM][BF_4] is doubled to 40 μg mL^{-1} during capsule synthesis, thick-walled hollow silica nanoparticles (~50 nm wall thickness) without any significant difference in capsule size were obtained (Figure 2B and inset).

The self-assembly of phytase molecules in IL [BMIM][BF_4] leading to the formation of enzyme capsules and their utilization as self-templating nanoreactors for the synthesis of hollow silica nanoparticles with controllable wall thickness is quite interesting and has not been previously demonstrated. Particularly notable is the formation of hollow silica spheres in the absence of any

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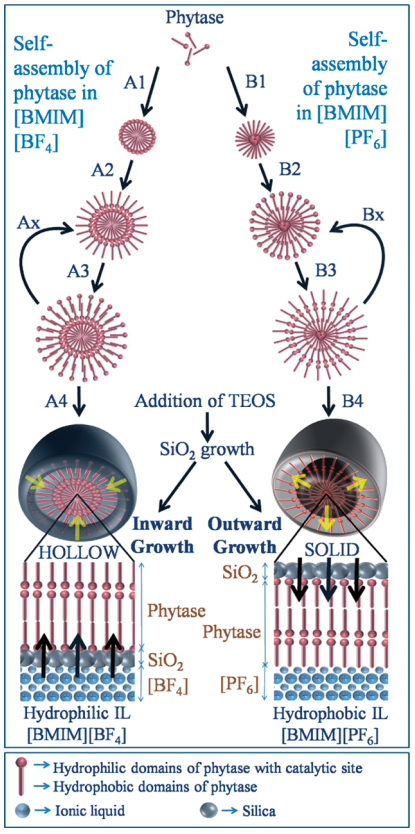
Scheme 1. Schematic Illustration for Self-Assembly of Phytase Enzyme in Hydrophilic versus Hydrophobic Ionic Liquids viz. [BMIM][BF_4] and [BMIM][PF_6], Respectively[a]



[a] Phytase molecules are shown in the form of a "head and tail" model with "head" representing the hydrophilic catalytic domains of phytase and "tail" representing the hydrophobic non-active domains. Self-assembly in hydrophilic [BMIM][BF_4] (A1-Ax) leads to phytase nanospheres with catalytically-active hydrophilic domains facing ionic liquid, whereas in hydrophobic [BMIM][PF_6] (B1-Bx), catalytically-active hydrophilic domains are sequestered towards the center. Addition of TEOS to the self-assembled phytase (A4 and B4) leads to the formation of a hollow silica nanosphere in [BMIM][BF_4] but results in a solid core silica structure in [BMIM][PF_6].

external hydrolyzing agent, suggesting that self-assembled phytase molecules can enzymatically catalyze the hydrolysis of TEOS, in addition to acting as a template for the synthesis of hollow silica nanoparticles. Phytase enzyme used in this study has been isolated from the fungus *Aspergillus niger* and belongs to the histidine acid phosphatase (HAP) family of proteins. A common catalytically active site motif, RHGXRXP (Arg-His-Gly-X-Arg-X-Pro), is shared by all HAPs, wherein the catalytic histidine in this sequence is known to initiate a two-step reaction that results in the hydrolysis of substrate.[34] Such catalytically active motifs that are present in phytase seem to be responsible for TEOS hydrolysis, leading to hollow silica nanoparticles.[35]

A cartoon illustrating the plausible mechanism for growth of hollow silica nanoparticles in IL [BMIM][BF_4] has been elucidated in Scheme 1. We propose that when a small amount of an amphiphilic biomacromolecule (phytase enzyme in this study) is added to the hydrophilic IL [BMIM][BF_4],[25] the hydrophilic domains of phytase orient themselves toward hydrophilic [BMIM][BF_4], whereas its hydrophobic domains would tend to orient away from [BMIM][BF_4] molecules (Scheme 1). To maintain the energy equilibrium of the system, this would result in self-assembly of phytase molecules in [BMIM][BF_4] in a spherical form (Scheme 1). It is expected that the additional phytase molecules will further keep assembling on pre-self-assembled phytase vesicles in a multilamellar (head-to-head/tail-to-tail) fashion, as commonly observed in the self-assembly of biological membranes.[8] In addition to the hydrophobic tail packing between hydrophobic domains of phytase, the amino acid residues of different phytase units may also interact with each other through intermolecular hydrogen bonding, thus forming a phytase nanocapsule structure. This self-assembly of phytase in hydrophilic [BMIM][BF_4] should, however, always result in a hydrophobic core sequestered away from [BMIM][BF_4] and its hydrophilic groups facing toward [BMIM][BF_4]. Considering the hydrophilic nature of the catalytically active site of phytase,[34] these active sites are expected to be present in close proximity to IL [BMIM][BF_4]. When TEOS is added to the reaction, the zwitterionic nature of the catalytically active hydrophilic domains of phytase provides flexibility in the interfacial binding of silicate anions to the exterior of phytase capsules.[36] Therefore, hydrolysis of TEOS starts on the surface of the self-assembled phytase template (20 μg mL^{-1}) in an inward growth pattern, resulting in a hollow silica nanosphere with a thin silica layer (Figure 2A). An increased concentration of phytase (40 μg mL^{-1}) results in thick-walled hollow silica nanospheres (Figure 2B), predominantly because of an increase in the number of phytase lamellar layers formed in capsules and the presence of more active sites on the template surface, thus further enhancing hydrolysis of TEOS on phytase capsules surface. This also suggests that phytase capsules obtained by self-assembly in IL [BMIM][BF_4] not only act as a template for silica growth but also play the key role in enzymatically catalyzing the hydrolysis of silica precursor.

To validate the above proposed mechanism, additional experiments were performed wherein similar reaction conditions were used; however, hydrophilic IL 1-butyl-3-methylimidazolium tetrafluoroborate ([BMIM][BF_4]) was replaced with a hydrophobic IL 1-butyl-3-methylimidazolium hexafluorophosphate ([BMIM][PF_6]). As per the aforementioned mechanism, when phytase is added to a hydrophobic IL, it is expected that the self-assembly of amphiphilic phytase molecules should occur conversely to that observed in the hydrophilic IL. Therefore, in a spherical phytase nanostructure self-assembled in [BMIM][PF_6], the hydrophobic domains of phytase should orient themselves facing outward toward the hydrophobic IL [BMIM][PF_6], whereas its hydrophilic domains should be sequestered in the center, away from [BMIM][PF_6]. Because catalytically active phytase sites are present in the hydrophilic region of phytase (which are sequestered toward the center in hydrophobic IL), silica hydrolysis on self-assembled phytase templates in [BMIM][PF_6] should occur in an inside-outwardly fashion (Scheme 1). Figure 3 shows the TEM micrographs of silica nanostructures obtained after phytase self-assembly and TEOS hydrolysis in the hydrophobic IL [BMIM][PF_6]. In comparison with the hollow silica nanoparticles obtained in [BMIM][BF_4], solid silica nanoparticles with a quasi-spherical morphology and a broad particle size distribution (200−600 nm) were obtained in [BMIM][PF_6]. The higher magnification TEM image of silica structures in [BMIM][PF_6] shows a dense particle core that fades away in intensity toward the

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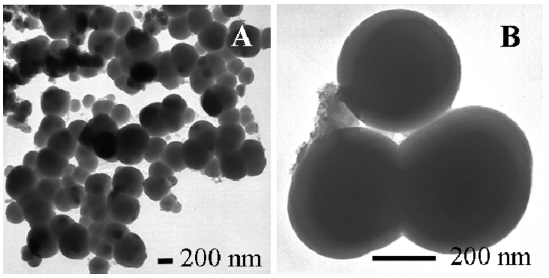

Figure 3. (A) Lower and (B) higher magnification TEM images of solid silica nanoparticles synthesized in ionic liquid [BMIM][PF_6] using 20 μg mL^{-1} self-assembled phytase.

periphery. This further suggests that in hydrophobic IL [BMIM]-[PF_6], TEOS hydrolysis and silica nucleation begin from the hydrophilic protein domains sequestered toward the center and result in solid silica nanoparticles with a dense core (Figure 3B). It is interesting to observe that the same protein phytase results in the formation of hollow silica nanoparticles in the hydrophilic IL [BMIM][BF_4], whereas it results in solid silica particles in the hydrophobic IL [BMIM][PF_6]. This clearly shows for the first time that a control over self-assembly of biomacromolecules can be achieved by tuning the solvent properties. This provides a complementary advantage over previous approaches of biomacromolecular self-assembly, wherein smart architectural modifications of protein are generally a prerequisite before their controlled self-assembly can be achieved.[8]

The immobilization of technologically important enzymes onto solid supports is an area of extensive research because of their widespread applications in biomedicine as therapeutic peptide-loaded nanovehicles and in various industries as reusable biocatalysts.[37] However, one of the major challenges associated with enzyme immobilization is the retention of enzymatic activity after their immobilization onto a substrate. Previous studies have indicated that the enzyme activity can be preserved in ILs because of their unique solvent properties.[28] Phytase enzyme used in this study belongs to the phosphohydrolase group of enzymes that have significant biomedical importance[31] as well as industrial importance for the synthesis of bioactive compounds such as myo-inositol.[32,33] Because self-assembled phytase capsules act as templates for in situ growth of silica nanostructures in ILs, it is highly possible that enzyme molecules are embedded in the silica nanostructures (hollow and solid silica nanoparticles in [BMIM]-[BF_4] and [BMIM][PF_6], respectively) during their enzyme-mediated synthesis. Therefore, hollow and solid silica nanoparticles synthesized in ILs were subjected to enzyme reusability studies (Figure 4). When tested for phytase activity (see Methods section for details), hollow silica nanoparticles formed in [BMIM][BF_4] were found to show significant enzyme activity that was fully retained for at least up to four cycles of testing (Figure 4). The hollow silica nanoparticle synthesized by in situ self-assembly and templating of phytase capsule in [BMIM][BF_4] thus acts as an enzyme nanocontainer, in which phytase molecules are encapsulated during its synthesis while retaining the native activity of enzyme molecules for at least up to four cycles. Retention of phytase activity in silica nanocontainers is particularly interesting considering that enzyme activity, in general, is highly sensitive to external environments such as pH, temperature, and solvent system, and a variation in any of these factors

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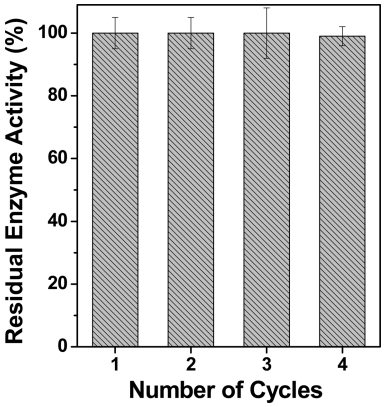

Figure 4. Reusability study of phytase enzyme (20 μg mL^{-1}) in situ encapsulated within hollow silica nanocontainers during their synthesis in ionic liquid [BMIM][BF_4].

can lead to changes in enzyme confirmation, resulting in activity loss. In comparison with hollow silica nanocontainers formed in [BMIM][BF_4], phytase-loaded solid silica nanoparticles formed in [BMIM][PF_6] were found to be enzymatically inactive. Because phytase capsules formed in [BMIM][BF_4] act as in situ templates for TEOS hydrolysis, it is highly possible that the walls of hollow silica capsules are highly porous, which facilitates the diffusion of substrates and products during enzymatic reactions, and thus enzymatic activity is recorded only from enzyme−silica hybrid structures formed in IL [BMIM][BF_4] and not from solid silica structures formed in IL [BMIM][PF_6].

Conclusions

In summary, we have demonstrated for the first time that ILs can be used as nonaqueous designer media for the self-assembly of amphiphilic biomacromolecules (an enzymatic protein in this illustrated case) at ambient temperature, which had hitherto been limited to the surfactant and lipid molecules.[25] In the context of self-assembly of amphiphilic biomacromolecules, changing the properties of a self-assembly system was thus far predominantly dependent on modifying the biological amphiphile, which generally results in an activity loss. However, with the recent developments in the field of ILs, a myriad of ILs can now be created from different combinations of cationic and anionic species,[25] which will allow the possibility of tailoring self-assembly solvents rather than necessarily focusing on tailoring the bioamphiphile molecules to achieve controlled self-assembly. The present work on self-assembly of phytase enzyme in two different ILs can also provide an exciting expansion of our theoretical understanding of nanobio interfacial molecular self-assembly by exploring such processes in nonaqueous media. Moreover, the utilization of self-assembled biomacromolecular capsules as in situ self-catalyzing templates for the synthesis of hollow silica nanocontainers in IL is interesting because these nanostructures retain the enzyme activity for multiple cycles. It is envisaged that this in the future would fuel significant interest toward the controlled templating of bionanomaterials in ILs, wherein a combination of metals and metal oxides with different enzyme encapsulation strategies will be explored in ILs of different properties, thus leading to applications in drug encapsulation and controlled release, biocatalysis, biosensing, and bioelectronics.

Methods

Materials. 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). Tetraethylorthosilicate (TEOS) was purchased from Sigma-Aldrich. All chemicals were used as received, unless specified.

Phytase Purification. Phytase was isolated from a fungus *Aspergillus niger* that was maintained on potato dextrose agar (PDA) slants, as previously described.[33] Fungus was grown in 100 mL of modified fermentation medium (pH 5.5) containing 5 g dextrin, 2.5 g glucose, 0.86 g NaNO3, 0.004 g KH_2PO_4, 0.05 g KCl, 0.05 g $MgSO_4 \cdot 7H_2O$, and 0.01 g $FeSO_4 \cdot 7H_2O$. For fungal growth, fermentation medium (100 mL in 250 mL Erlenmeyer flask) was inoculated with 1% (v/v) of fungal spore suspension (5×10^7 spores per mL) prepared by suspending the spores from 7 day old sporulated slant of *Aspergillus niger* grown on PDA in 10 mL of sterile distilled water containing 0.01% (v/v) Tween 80 and incubated at 30 °C at 200 rpm and allowed to grow for 12 days. After fermentation, fungal mycelium was separated by filtration, followed by centrifugation at 10 000 g for 30 min, and the clear supernatant was collected. The supernatant was further concentrated (50%) by rotavapor at 40 °C under vacuum and applied to hydrophobic column chromatography using Phenyl Sepharose CL-4B (30 mL bed volume), previously equilibrated with 30% ammonium sulfate in 20 mM acetate buffer, pH 2.5. The column was washed thoroughly with 20 bed volumes of the above buffer and eluted with 120 mL of linear decreasing gradient of ammonium sulfate (30−0%) with a flow rate of 20 mL h^{-1}, and ~3 mL fractions were collected. Fractions showing activity at pH 2.5 (Phytase I) were pooled separately, concentrated by rotavapor, and loaded on gel filtration Sephacryl (S-300) column with a flow rate of 12 mL h^{-1}, and 2 mL fractions were collected. Phytase enzyme thus obtained was used further in this study. Unless otherwise mentioned, all purification procedures were carried out at 4 °C. The molecular weight of phytase used in this study is 264 kDa, which is a tetramer composed of two homodimers and a hydrodynamic radius (RH) of 5.21 nm.

Ionic-Liquid-Mediated Synthesis of Phytase Capsules. A typical synthesis reaction of phytase capsules in ILs was performed in 0.5 mL volume containing 490 μL of the respective IL ([BMIM][BF_4] or [BMIM][PF_6]) and 10 μL of purified phytase enzyme (1 mg mL^{-1} dissolved in water), thus achieving a final enzyme concentration of 20 μg mL^{-1} in the reaction. The IL−phytase mixture was incubated at 37 ± 0.1 °C for 24 h with gentle reciprocal shaking, after which samples were centrifuged at 14000 rpm, followed by washing with deionized water and acetonitrile to remove the viscous IL. Phytase capsules thus obtained were further analyzed by TEM.

Ionic-Liquid-Mediated Synthesis of Hollow and Solid Silica Nanospheres. To obtain silica nanospheres, 20 mM TEOS stock solution was prepared in the respective ILs viz. [BMIM][BF_4] and [BMIM][PF_6]. TEOS stock solution (500 μL, 20 mM) in ILs was added to 500 μL of the aforementioned reaction mixture containing IL and phytase capsules. The 1 mL reaction contents were incubated at 37 ± 0.1 °C for 24 h under stirring conditions, during which all reactions involving phytase became turbid, indicating TEOS hydrolysis. After 24 h of reaction, samples were centrifuged at 14000 rpm, followed by washing with deionized water and acetonitrile to remove the viscous IL. Silica nanostructures thus obtained were further analyzed by TEM and used for enzyme reusability studies. In a control experiment, 10 μL of deionized water was added to 500 μL of TEOS stock solution and 490 μL of 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][BF_4] and [BMIM][PF_6] and suggesting the role of phytase toward 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 silica nanoparticles formed; however, samples not washed with acetonitrile were difficult to image in TEM because of residual presence of ILs in those samples. The TEM images shown in this article correspond to nanoparticles that were washed with acetonitrile.

Enzyme Activity Assay and Reusability Assay. To determine the enzymatic stability of phytase in hollow silica nanospheres after encapsulation, we dispersed hollow silica nanoparticles synthesized from 20 μg mL^{-1} phytase in IL [BMIM][BF_4] in 1 mL of Gly-HCl buffer (100 mM, pH 2.5). Hollow silica nanoparticles (100 μL) in Gly-HCl buffer were mixed with 300 μL of Gly-HCl buffer, and 100 μL of enzyme substrate (phytic acid, 15 mM, pH 2.5) was added to the reaction. The reaction mixture was then incubated on 55 °C for 30 min at 300 rpm, followed by centrifugation to obtain a clear supernatant and nanoparticles pellet. The inorganic phosphorus released from phytic acid because of enzyme activity was determined in supernatant by a modified ammonium molybdate phytase activity assay.[38] In the supernatant, a freshly prepared solution containing 2 mL of acetone, 1 mL of H_2SO_4 (5 N), 1 mL of ammonium molybdate (10 mM), and 400 μL of citric acid (1 M) were added, and absorbance of the solution was measured at 370 nm after 30 min. One unit of phytase activity (IU) was expressed as the amount of enzyme that liberates 1 μmol phosphorus per minute under standard assay conditions. The nanoparticle pellet obtained after centrifugation (containing hollow silica nanocontainers with encapsulated enzyme) was washed twice with Gly-HCl buffer, and fresh substrate solution was added for the next reaction cycle, as in the first cycle. The experiment was continued for four cycles, and the enzymatic activity obtained in the first cycle was considered to be 100% to compare with subsequent cycles. Experiments were conducted in triplicate to minimize experimental error. No activity was observed in the first cycle when similar experiments were conducted with solid silica nanoparticles.

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