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Stellate MSN-based Dual-enzyme Nano-Biocatalyst for the Cascade Conversion of Non-Food Feedstocks to Food Products

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Abstract

The successful demonstration of a tandem enzymatic catalyst which utilizes stellate macroporous silica nanospheres (Stellate MSN) platform as dual-enzyme host is reported herein. Upon simultaneous loading of beta-glucosidase and glucose isomerase inside their porous structure, Stellate MSNs-featuring a hierarchical pore arrangement and large surface area, show capability to perform a cascade reaction that converts cellobiose, a cellulosic hydrolysis product, into glucose and further to fructose. The silica platform provides a modality for substrate channelling which involves the transfer of the cascade intermediate, glucose, to the next enzyme without first diffusing to the bulk. A key aspect to this proof-of-concept is the two-enzyme system working in an optimized pH domain to fit the modus operandi for both enzymes. The concept could be extrapolated to other enzyme tandems, with potential to impact dramatically enzymatic processes which require multi-catalyst, one-pot transformations.

Keywords: Catalyst; Macromolecules; Glucose; Enzymatic catalysis

Introduction

Biotechnology is at the core of the environmentally friendly chemical processes of the 21st century. Enzymes, macromolecules capable to accomplish chemical transformation with high selectivity, are key components of the biotechnology toolbox. Applications, such as new industrial processes, creating new functional foods, and contributing to medical treatments are a few areas where innovative approaches toward reducing waste and harmful catalytic processes are required, thus driving the desire to replace existing processes with enzymatic catalysis. However, to fulfill current demands of chemical industry, enzyme productivity, recyclability and increased shelf life are required to enable the replacement of existing processes at scales and in an economical fashion. Food security is one of paramount importance in the wake of increased Globe population. As such, potential sources of food basic ingredients such as glucose and fructose from non-food biomass renewable resources such as switch grass, corn stover, and other lignocellulosic waste, could significantly supplement the ever-growing contemporary food needs. The principal constituent of lignocellulosic biomass is cellulose, a glucose-based polymer. The conversion of cellulose to glucose involves a mixture of enzymes termed cellulase. In microorganisms such as *Trichoderma viride*, the conversion is step-wise process: first, beta-1,4 glucanase breaks the glyosidic linkage to cellobiose—a glucose dimer (Figure 1) which is subsequently converted to glucose by beta-glucosidase (β-glucosidase). However, assuming that the enzymatic catalysis research efforts ultimately aim to product commercialization, high-fructose corn syrup (HFCS) is a more desired outcome, given its sweetness, equating 1.3 times more than sucrose, and the amenability of this product to be used by diabetics. Conversion of glucose to fructose is accomplished by Glucose isomerase (GI) enzyme, catalyzing the reversible isomerization of D-glucose (glucose) to D-fructose (fructose). This enzyme, with high occurrence in prokaryotes, is subjected to research efforts that could improve the catalytic ability and application in industry [1]. The process of converting cellulose to fructose is illustrated in Figure 1.

In the process of converting cellulose to glucose, β-1,4,-glucanase enzyme leads to a glucose dimer, called cellobiose. The enzyme β-glucosidase is necessary to accomplish further conversion to glucose. β-glucosidase, known also as cellobiose, is a ubiquitous component of cellulases [2]. Given that cellobiose inhibits the reaction, cellulose hydrolysis is greatly impaired. Without sufficient β-glucosidase present in the enzymatic process of cellulose, little glucose is formed; the main product being cellobiose. Published work has showed that supplementation of commercially produced cellulases with β-glucosidase increases the rate and extent of glucose production [3,4]. The native level of β-glucosidase activity in cellulase is, therefore, insufficient for the maximum rate and extent of glucose production to be reached [2]. Since the substrate of β-glucosidase, cellobiose, is water soluble, an immobilized (water-insoluble) enzyme preparation could be used to supplement commercial cellulase/cellulose mixtures, and such immobilized enzyme could be subsequently recovered and reused. Enzyme immobilization provides an excellent opportunity increase turnover over a considerable period of time, enzyme availability to the substrate is greater. Several natural and synthetic supports have been assessed for their efficiency for enzyme immobilization [5].

The typical approach is one enzyme per support. Several examples of enzyme immobilization onto porous silica have been reported [6-12]. Co-immobilization of enzymes on various platforms is an emerging field, and involves considerations of various factors: spanning from facile access of substrate to the next enzyme in the cascade, optimization of catalytic activity to allow performance of all components, and the stability of enzyme in the solid support [13]. Porous silica materials emerged as scaffolds due to their resilient nature, capacity to protect the encapsulated enzyme, easy access of reactants in a confined space, and large surface area [14]. Among these platforms,

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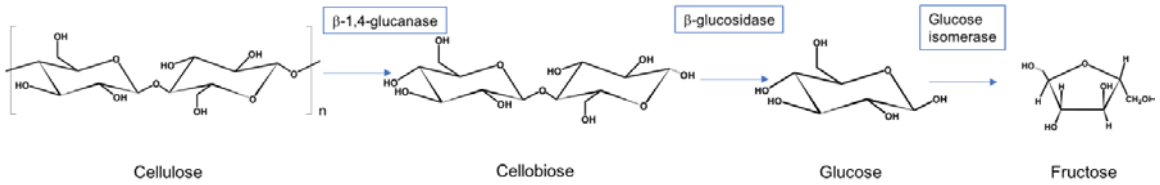

Figure 1: Schematic representation of the step-wise transformation process from cellulose to fructose.

Stellate MSN, a macroporous silica nanospheres platform, presents the advantage of facile and reproducible synthesis, narrow particle size distribution, hierarchical pores and significant surface area. To the best of our knowledge, this is the first report of dual-enzyme immobilization on Stellate MSN showing a functioning cascade enzymatic reaction, in optimized reaction conditions that are suitable for both enzyme activities.

In addition, present work aims to demonstrate that immobilization of two cascade reaction enzymes on a Stellate MSN platform would control the direct transfer of a reactant from one catalytic site to another without first diffusing it to the bulk environment. The process of intermediates control along a pathway is termed substrate channeling, and is key to efficient one-pot multi-step catalysis. In addition to channeling, another aspect of multi-step enzymatic catalysis is the significant overlap of enzymatic reaction conditions. Enzymes optimal function require a defined set of conditions, including reaction pH, and participating ions concentration, and temperature. Present work achieved optimal reaction conditions for β-glucosidase and glucose isomerase tandem system on Stellate MSN platform while demonstrating channelling effect occurring in the conversion of cellobiose to fructose.

Materials and Methods

Materials

Tetraethyl orthosilicate (TEOS), cetyltrimethylammonium tosylate, triethanolamine, anhydrous ethanol, D-(+)-Cellobiose, D-(+)-glucose, D-(-)-fructose, Tris (hydroxymethyl) aminomethane hydrochloride, magnesium sulphate, 4-Morpholinepropanesulfonic acid, Cobalt(II) chloride hexahydrate, acetonitrile (HPLC grade), Immobilized Glucose Isomerase from *Streptomyces murinus* (Sweetzyme T, Novazyme) and β-glucosidase from almonds lyophilized powder were purchased from Sigma Aldrich without further purification. *E. coli* strains DH5α, IPTG (isopropylthio-β-galactoside), B-PER™ Bacterial Protein Extraction Reagent, and Ni-NTA Agarose and Kanamycin were purchased from Thermo-Fisher. BL21 (DE3) Chemically Competent *E. coli* was purchased from Agilent. Nanopure water was used in all experiments.

Methods

The experiments carried out in this work involve the synthesis of the silica platform, preparation of enzymes, and the fabrication and validation of the enzyme catalyst platforms (both dual-enzyme and single enzyme immobilization were prepared for the control experiments).

Synthesis and characterization of stellate MSN platform

The synthetic procedure of MSNs with stellate morphology was modified from previous literature reports [15,16]. In a typical experiment, a mixture of 1.920 g of cetyl-trimethylammonium tosylate (CTATos), 0.4 g of triethanolamine (TEA) and 100 mL of nanopure water was stirred at 80°C for 1 h, and then 14.58 g of tetraethyl-orthosilicate (TEOS, 6.998 mol) was quickly added into the surfactant solution. The molar composition of the precursors was 1.0 SiO_2: 0.06, CTATos: 0.026, TEA: 80.0 H_2O. The mixture was stirred at 80°C for another 2 h. The solid product resulting upon reaction was filtered, washed copiously with nanopure water and methanol, and dried in under vacuum at 60°C for 12 h. To remove the surfactant template (CTATos), 1.50 g of as-synthesized Stellate MSN was refluxed for 24 h in a methanolic solution of hydrochloric acid (9.00 mL of HCl (37.4%) in 160.00 mL of methanol) followed by extensive washes with nanopure water and methanol. The resulting surfactant-free Stellate MSN material was placed under high vacuum to remove the remaining solvent in pores of the material. Nitrogen adsorption-desorption isotherms of the sorbents were obtained on a Nova 4200e (Quantachrome, Boynton Beach, FL) surface area and porosity analyzer) in the relative pressure range of 0.05-0.95 at 77K. Stellate MSN was degassed at 110°C for 3 h under high vacuum. The total pore volume was calculated as the adsorbed volume of liquid nitrogen at the relative pressure of 0.95. Specific surface areas were calculated with the Brunauer-Emmett-Teller (BET) method. Pore volume was determined by Non-Local Density Functional Theory model. High-resolution TEM images were obtained on a JEM-2100F model operated at 200 kV. SEM images were obtained on a Hitachi S4700 instrument.

Glucose isomerase enzyme: plasmid construction and bacterial expression in *E. coli*.: The Glucose Isomerase enzyme is not commercially available in pure form and therefore it was produced in-house by gene cloning. The gene encoding hyperthermostable D-glucose-isomerase from *Thermotoga neapolitana*-5068 (*xlyA* gene) was chemically synthesized (GenScript, Piscataway, NJ). The synthetic gene was cloned as an NdeI-XhoI insert into plasmid pET28a (+), thereby creating pTNXI [17]. The pTNXI plasmid was transformed into chemically competent *E. coli* DH5α according to the manufacturer's instructions.

Recombinant protein purification: The plasmid carrying the Thermotoganeapolitana glucose/xylose isomerase gene (PET28a-TNXI) was transformed into BL21 (DE3) bacteria for protein expression. Bacterial cells were grown in 50 mL of LB media with 50 μg/mL kanamycin to an A600 of 0.6-0.8 prior to induction with isopropyl-β-D-thiogalactopyranoside (IPTG, 0.5 mM). After 12 h, the cells were collected and cells pellets resuspended in 20 mL of Bugbuster (Novagen) protein extraction reagent supplemented with Benzonase (20 U/mL, Novagen), 1.0 mg/mL of lysozyme (Sigma), and one tablet of EDTA free protease inhibitor cocktail (Roche). After 60 min rocking at 4°C, the suspension was subjected to centrifugation at 7000 rpm for 30 min at 4°C to remove cell debris. Recombinant proteins were purified on nitrilotriacetic acid-Ni^{2+} agarose columns (Thermo-Fisher). After extensive washing, the recombinant proteins were eluted with 10 mL of 50 mM Tris, 300 mM NaCl, 500 mM imidazole, pH 8 and then immediately dialyzed at 4°C into M Buffer, consisting of 50 mM MOPS, 5 mM $MgSO_4$, 0.5 mM $CoCl_2$, with pH 7.

Protein quantification, electrophoresis, and molecular mass determination: Protein concentration was determined according to the Bradford method using a BSA standard curve and a prefabricated assay solution (Bio-Rad). SDS-PAGE was carried out using WedgeWell Tris-Glycine precast gradient gels (4–12%, ThermoFisher) and Tri-color Prestained Protein Marker II (Bioland Scientific). SimplyBlue™ safe stain was used for visualization of the protein bands (Invitrogen) (The gel images are included in Supporting Information). The theoretical mass was derived from the ExPASy ProtParam tool (http://web.expasy.org/protparam/).

Enzyme immobilization on stellate MSN

Dual-enzyme immobilization: A volume of 2 mL recombinant glucose isomerase (0.2 mg/mL in in M buffer, 0.4 mg) was mixed with 2mL β-glucosidase solution (1 mg/mL in M buffer, 2 mg). Enzyme immobilization was performed by mixing the enzymes solution with 50 mg of the mesoporous supports in 4mL M buffer, at 4°C under stirring for 12 h at 200 rpm. The suspension was then centrifuged, and the enzyme immobilized on Stellate MSN was washed with buffer. To quantify the immobilization efficiency, the remaining protein in supernatant was measured by a previously described method, showing that enzyme was completely immobilized. The resulting dual-enzyme Stellate MSN was resuspended in 4mL buffer and stored at 4°C.

Single-enzyme immobilization: Single-enzyme immobilization of recombinant GI enzyme on Stellate MSN, and separately, of the β-glucosidase on Stellate MSN were also performed for control experiments in the same manner as described for the dual enzyme immobilization.

Enzymatic assays

Specific substrate conversion assay by HPLC analysis: The products of enzymatic analyses such as glucose, fructose or mixtures were performed on a Shimadzu Nexera UHPLC/HPLC System with a Restek Ultra Amino column. After enzyme conversion reaction, the reaction products were purified by syringe filtration, possible products were identified, and their calibration curves were developed. An isocratic run at 20% water and 80% acetonitrile was used for the quantitative analyses of all possible analytes. Flow rate of mobile phase was 1.5 mL/min. The column temperature was set at 35°C and the injection volume of samples was 25 µL for all analyses.

Dual-enzyme MSN enzymatic conversion of cellobiose to fructose: A volume of 2 mL of immobilized dual-enzyme platform was mixed with 1 mL of 2 % Cellobiose in the M buffer and maintained at 20 h at 55°C to obtain cellobiose to fructose conversion.

Conversion of cellobiose to glucose catalysed by free β-glucosidase enzyme: For cellobiose to glucose conversion, 2.0 mL of 1 % Cellobiose (0.02 g of cellobiose) in the M buffer was mixed with β-glucosidase solution in the M buffer (1 mg/mL, 1 mL) and the mixture was converted to glucose for 20 h at 55°C.

Conversion of glucose to fructose catalysed by free recombinant GI enzyme: For glucose to fructose conversion, 2 mL of 1 % Glucose (0.02 g of glucose) in the M buffer was mixed with the recombinant glucose isomerase 1mL (0.2 mg/mL, 0.2 mg) and the mixture was converted to D-Fructose for 20 h at 55°C.

Conversion of glucose to fructose catalysed by commercially available immobilized GI enzyme (sigma-aldrich): Immobilized Glucose isomerase from *Streptomyces murinus* (50 mg/1 mL) was used for enzyme activity comparison. In a typical experiment, 2 mL of Sigma-Aldrich GI, as provided by vendor, was mixed with 2 mL of 1% Glucose and the experiment was conducted for 20 h at 55°C.

Conversion of cellobiose to fructose catalysed by a mixture of β-glucosidase and recombinant GI: For cellobiose to fructose conversion, 1 mL of 2% cellobiose in the M buffer was mixed with β-glucosidase solution in the M buffer (1 mg/mL, 1 mL) and recombinant glucose isomerase 1mL (0.2 mg/mL). Same experiment was conducted for a mixture of β-glucosidase and Immobilized Glucose Isomerase from Streptomyces murinus (Sigma-Aldrich GI, 50 mg/1mL) and whole mixture was converted to glucose for 20 h at 55°C.

Effects of the temperature, ph, and metal ions on recombinant enzyme activities: The optimum pH for β-glucosidase is 5 whereas the commercially available GI operates at pH 7 (courtesy of Sigma Aldrich website). To optimize the operating conditions toward enabling the one-pot reaction, the effects of temperature, pH, and metal ion were investigated for the dual system. The effect of the temperature on enzyme activity was analyzed by assaying the enzymatic conversion of D-Glucose to D-Fructose catalyzed by the recombinant GI over a temperature range of 4–65°C. To determine the influence of metal ions on enzyme activities, the recombinant enzyme was dialyzed separately in two different customized buffers: M buffer (50 mM MOPS, 5 mM MgSO4, 0.5 mM Co^{2+}, pH 7.0) and C buffer (50 mM citric acid, 5 mM MgSO4, 0.5 mM Co^{2+}, pH 5.0) (Table 1) [17]. The optimal pH for both β-glucosidase and recombinant GI were determined using the aforementioned assay conditions for the conversion of cellobiose to fructose in the two buffer systems. The working conditions for the recombinant GI enzyme and the GI-BG couple are illustrated in Table 2.

Catalyst reusability

Following the completion of the enzymatic reaction, the dual-enzyme Stellate MSN, termed Stellate (BG+GI) was separated from the reaction mixture by centrifugation, washed copiously with water and stored in M buffer. The enzymatic reaction was repeated within 24 h. This sequence was repeated four consecutive times.

Results

The preparation of Stellate MSN, as previously reported by us and others, [16] renders materials with average surface area of 580 m^2/g, as illustrated in Table 3. The macroporous Stellate materials, as illustrated by SEM and TEM images (Figure 2), present spherical morphology and hierarchical porosity, suitable for encapsulation of large macromolecules. The GI in pure form is not commercially available. As such, this product could not be used for immobilization experiments. GI in pure form was produced in-house by genetic engineering and

Buffer Components and Conditions	C Buffer (citric)	M Buffer (MOPS)
Dialysis Buffer pH	5	7
50 mM Buffer Salt	Citric	MOPS
5 mM $MgSO_4$	+	+
0.5 mM $CoCl_2$	+	+

Table 1: Buffer Components.

Enzyme	C Buffer	M Buffer
Recombinant Enzyme GI*	pH Too close to enzyme's pI no reaction	Optimal conditions
BG+GI Cellobiose to D-Fructose	Trace amount of fructose	optimal conditions

Table 2: Optimal reaction conditions.

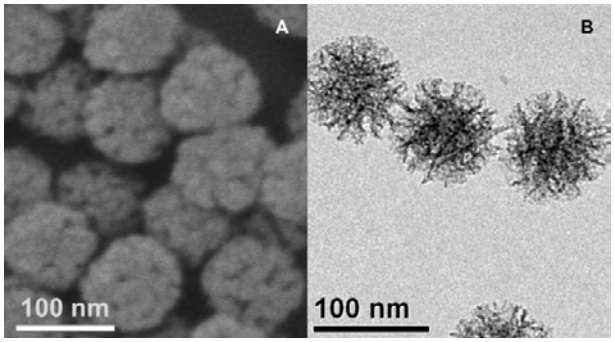

Figure 2: SEM (A) and TEM (B) of Stellate MSN.

Material	Average BET (m^2/g)	Average Pore Size Distribution (nm)
Surfactant–free Stellate MSN	580.0	12.6

Table 3: Surface Area (BET) and Average Pore Size.

molecular cloning, followed by protein expression and purification techniques. Preparation of GI along with characterization is described in detail in supporting information. Enzyme macromolecules loading in Stellate MSN was performed by an impregnation method which typically does not differentiate between external and internal surface of the nanospheres. According to the protein assay used upon the immobilization of two enzymes, β-glucosidase and GI on Stellate-MSN, the residual amount of protein remaining in solution after encapsulation by physical entrapment was negligible. We assumed therefore complete immobilization of the two enzymes in the Stellate MSN. The experimental evidence of complete disappearance of the enzyme in the loading solution upon impregnation, corroborated with the enzyme sizes (Table 4) suggest that the enzymes are primarily located in the pores of Stellate MSN, given the amount of material used and the reported porosity and surface area. Optimization of enzymatic reaction conditions resulted in choosing the M buffer for all experiments performed, while the time and temperature were chosen as 20 h and 55°C, respectively. The recombinant GI activity was assessed in comparison with the commercially available GI. Figure 3 shows the performance of the free recombinant enzyme in the time interval reported active for the Sigma GI. The performance of the immobilized dual-enzyme catalyst in M buffer was compared with the activity exhibited by the free enzymes in solution. It is important to note that commercially available GI represents a cellular extract containing GI which is immobilized by encapsulation (Sigma GI). Upon characterization of enzymatic activity, it was determined that the activity of recombinant GI is similar with the commercially available GI and the pure form could be used for the immobilization experiments. Figure 4 shows the relative activity of the free and immobilized enzymes (Stellate MSN and controls) used in conversion of cellobiose to fructose. The controls used included direct conversion of cellulose to fructose using the Stellate-MSN-encapsulated GI and the free GI respectively. Controls for the dual system included the free enzymes, as follows: (BG+recombinant GI) and (BG+commercially available GI from Sigma Aldrich). Enzyme recyclability, critical to economic feasibility of the dual-enzyme immobilization process, has been evaluated. The experiments were conducted four consecutive times, showing gradual decrease in activity as illustrated in Figure 5.

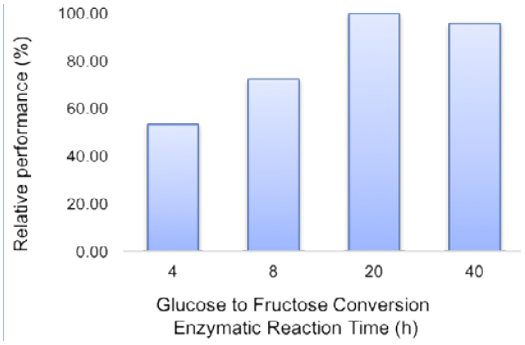

Figure 3: Timeline optimization of recombinant GI activity.

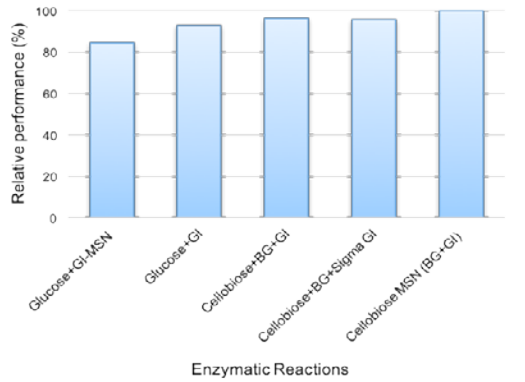

Figure 4: Relative performance of free and immobilized enzymes.

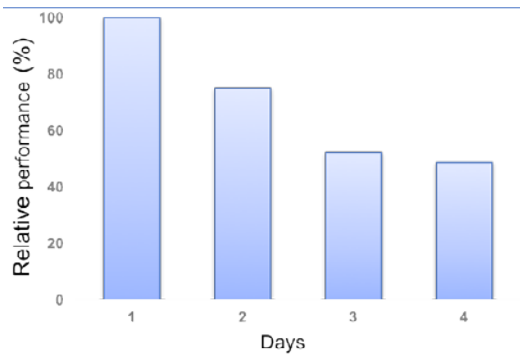

Figure 5: Enzyme recyclability.

Enzyme	Size (kDa)
β-Glucosidase	135
Recombinant Glucose Isomerase	52.7

Table 4: Size of Loaded Enzymes.

Discussion

Prior work reporting enzyme encapsulation in porous silica materials with application to the cellulose to glucose to fructose conversion sequence, has been accomplished by two single enzyme nanospheres, where the two separate platforms were utilized sequentially [8,18]. Therefore, reaction conditions were not unified but changed for each sequential step to match the enzyme working

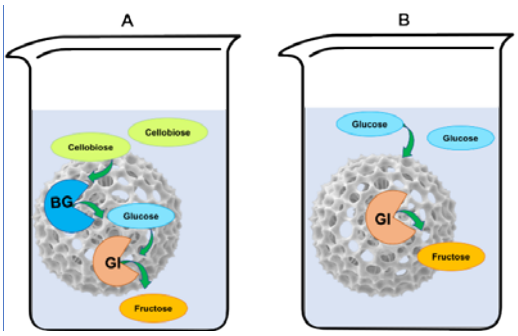

Figure 6: Schematic of channelling effect in the dual enzyme system: A. Glucose proximal access to the enzymatic site *vs*. B. Bulk diffusion to the enzymatic site.

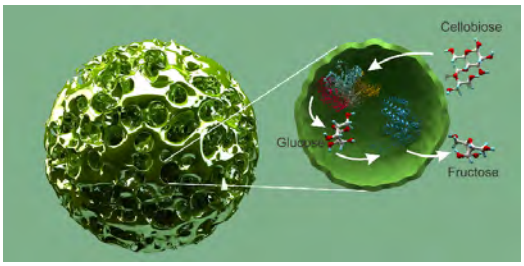

Figure 7: Schematic representation of the cascade reaction occurring in the Stellate MSN pore.

conditions. The significant contribution of the dual-enzyme system accomplished in this work resides with finding a set of conditions that enables working condition adequate for both enzymes function. While the common reaction conditions need to be identified and optimized for any other tandem enzyme potentially explored in the future, this work established the proof-of-concept for the specific GI-β-glucosidase (BG) tandem.

In addition, the encapsulation of two enzymes on the same nanosphere provides ground for demonstrating the channelling effect derived from easy access of the first product of the cascade, glucose, to the next catalytic site, without diffusion in solution. When cellobiose is reacted with Stellate MSN (BG+GI), the two-step cascade reaction enables direct access of Glucose produced by β-glucosidase to GI immobilized on the material, as schematically showed in Figure 6. In contrast, Stellate-MSN immobilized GI (single enzyme) is accessed by glucose from bulk solution, involving a longer diffusion path. The conversion data showed in Figure 4, in the same reaction conditions, the enzymatic reaction in the cascade case is more efficient then in the single enzyme case and support this hypothesis. Further investigations will be directed to understand the effect of the enzymes location in respect to each other and how the relative location impacts the reaction rates and the channelling effect. An important advantage of enzyme immobilization is represented by the stability typically conferred to the encapsulated enzymes; thus, it is expected that the enzyme would be recyclable. To demonstrate that the Stellate MSN host structure provides stability to degradation, we have performed four cycles of the cascade reaction. While a gradual decrease of enzyme activity was observed, potentially due to extensive washing and centrifugation, the enzymes are still active upon the four cycles. Further work aims to mitigate the potential detachment of the enzyme from the support by host functionalization and covalent attachment of the enzymes to the silica support.

Conclusion

Stellate MSN proves to be an excellent platform for multi-site cascade reactions as demonstrated here for the dual-enzyme catalyst produced by immobilization of β-glucosidase and glucose isomerase on the silica nanospheres (Figure 7). This is the first demonstration of biomacromolecules encapsulation in Stellate MSN and while this application was targeted to enzymatic catalysis, a plethora of other applications are envisioned. Future work will undertake mechanistic studies of to prove the channelling hypothesis. Additional focus will be on elucidating the exact location of the enzymes in the Stellate MSN, by enzyme labelling with chromophores toward enabling localization of enzymes in the porous platform and in respect with each other. Stellate MSN proves to be an excellent platform for multi-site cascade reactions as demonstrated here for the dual-enzyme catalyst produced by immobilization of β-glucosidase and glucose isomerase on the silica nanospheres.

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