Stellate Macroporous Silica Nanospheres in
Bio-Macromolecules Encapsulation
and Delivery

By

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A THESIS

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Dover, Delaware

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ABSTRACT

This project focused on using mesoporous silica as a solid support to encapsulate enzymes for operating a highly economic, and recyclable biomass processing system. The main objective is to turn non-food biomass sources into food products.

Enzymes are macromolecules with the structural backbone of proteins or ribonucleic acid sequences (RNAs) which work as catalysts in living organisms. Enzymes have the advantage of being the least contaminating catalyst due to normal catalyst might generate toxic by-product, and preferable to organic and inorganic catalysts, especially when used for product related to human used, which require biocompatibility of final product.

However, there are several disadvantages in enzyme utilization. Their fabrication is time-consuming and requires elaborated molecular biology processes. Most of the enzymes need well-defined reaction conditions to be functional and operate at high yield. Unfortunately, although they are reusable as normal catalysts, it proves difficult to extract or reuse the enzymes from a reaction. Also, enzyme molecules are easily degradable and demand proper storage.

To overcome some of the disadvantages, especially regarding stability to degradation, recovery, and reusability, immobilization of enzyme on solid support has become a thriving methodology. In recent years, mesoporous silica nanomaterials(MSN) have been at the forefront of enzyme immobilization given their extensive surface area, which provides capability to increase

enzyme loading and for their demonstrate ability to protect enzyme from degradation, thus enabling high recyclability. Mesoporous silica is biocompatible and has already been used for several applications included. Catalysis, drug delivery, and Bio-imaging. Previously published research utilized mesoporous silica to deliver drugs, DNAs, RNAs or encapsulate single enzyme.

The objective of this research is completed to develop a new porous silica platform that is unique in its porosity structure and develop it into a dual-enzyme platform with the scope of demonstrating a multi-reaction bio nanocatalyst. In regard to the further applications, the stellate MSN can be used as drug delivery or become a package of the biomacromolecule delivery system kit.

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LIST OF ABBREVIATIONS

BET   Brunauer-Emmett-Teller

BG   β-glucosidase

CHO cell   Chinese hamster ovary cell

CTATos   Cetyltrimethylammonium tosylate

DNA   Deoxyribonucleic acid

EDTA   Ethylenediaminetetraacetic acid

MSN   Mesoporous Silica Nanomaterial (Nanosphere)

GI   Glucose Isomerase

HFCS   High-fructose corn syrup

IPTG   Isopropyl β-D-1-thiogalactopyranoside

MOPS   3-(N-morpholino)propanesulfonic acid

RhB   Rhodamine B

RNA   Ribonucleic acid

SEM   Scanning Electron Microscope

SDS-PAGE   Sodium dodecyl sulfate polyacrylamide gel electrophoresis

TEA   Triethanolamine

TEM   Transmission Electron Microscope

TEOS   Tetraethyl orthosilicate

UHPLC   Ultra-High-Performance Liquid Chromatography

CHAPTER 1. INTRODUCTION

The focus of my thesis is on enzymes and proteins encapsulation, and their applications, including enzyme immobilization and protein delivery. Several concepts were used as the foundation for this research, and a brief description of these concepts is presented as follows.

1.1 Host-Guest Chemistry

Compared to traditional chemistry, supramolecular chemistry focuses on the reversible and weaker noncovalent bonding (intermolecular forces, electrostatic or hydrogen bonding), which involves discrete structure provided by assembled molecular subunits. Supramolecular chemistry is critical to study biochemical systems due to the complexity of noncovalent interaction occurring in macromolecules. Host-guest chemistry represents one of the important subdivisions of molecular chemistry and describes the relation of the molecules complexes made by multiple
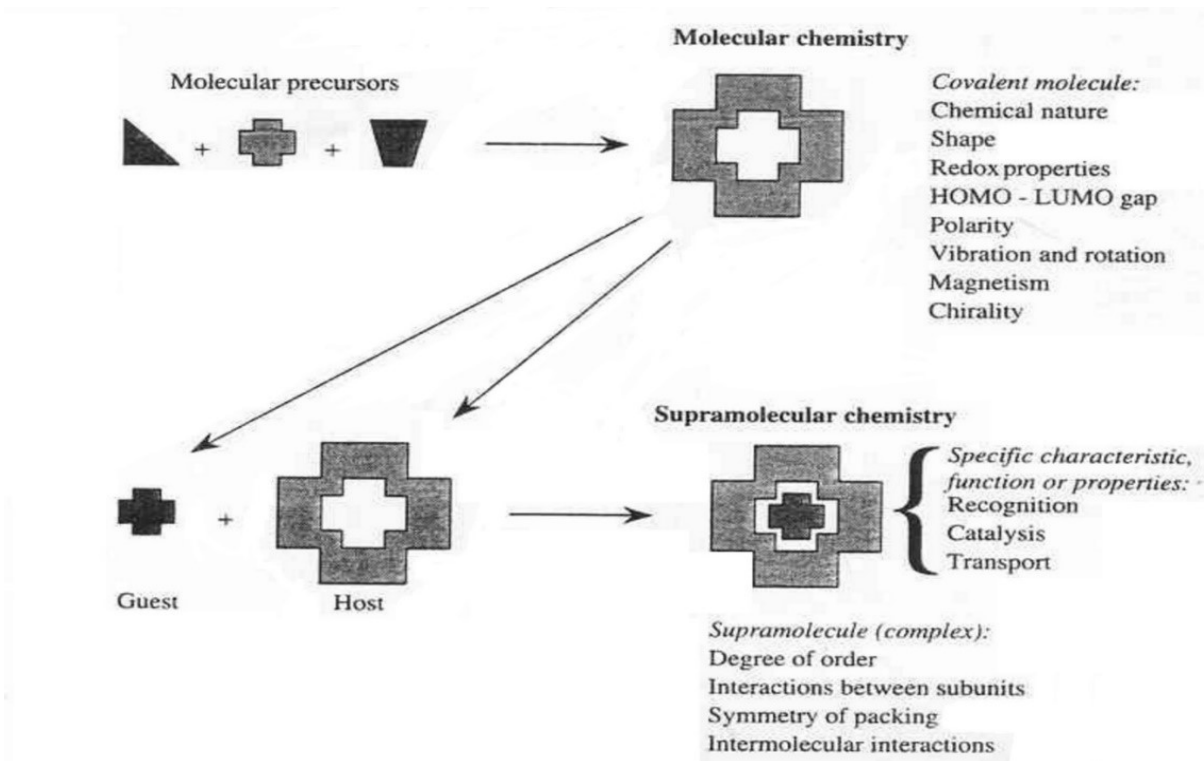
Figure 1.1. Molecular chemistry vs. Supramolecular chemistry [2]

molecules, ions or both, as illustrated in Figure 1.1. The interactions in host-guest chemistry are mostly attributed to hydrogen bonds, Van der Waals forces, hydrophobic/hydrophilic interactions and ionic bonds. An important application is enzyme immobilization, where the host-guest chemistry concept is applied to bind enzymes to a solid support, such as a mesoporous silica nanoparticle (MSN).[1-4]

1.2 Porous Materials

Since the beginning of the human history, materials' innovations have always been related to the progress of human civilization. Material science applications are versatile, and many applications, from consumer goods to industrial machinery components involve materials developments.

For the past few decades, porous materials have impacted many research fields. According to IUPAC's definition, porous materials with pore diameters smaller than 2 nm are classified as "microporous" materials with pores larger than 50 nm are classified as "macroporous" materials. The category with the intermediate pore size distribution, between 2~50 nm, are distinguished as "mesoporous", and their pores can be either ordered or disordered.[5-7] Mesoporous materials can be made up of metal oxides, silica or both. Silica (SiO_2), in smaller scale, might be known as sand. It is used as glass precursor, industrial casting mold or food additive.

Specifically, mesoporous silica nanoparticles (MSN) are widely applied in many fields, such as catalyst chemistry, drug delivery, gas absorption, etc.[8-12]

MSN is produced through a sol-gel process involving the transformation from liquid (sol) phase into a solid (gel) phase by hydrolysis and condensation of the metal oxide around a template.

Attachment of organic functional groups onto the particle is possible due to the process by addition of precursors.

Enzymes or therapeutic proteins can interact with the organic functional groups on the material via host-guest chemistry, enabling the transport of enzymes into cells.[13-17]

1.3 Enzyme Immobilization

In the chemical or pharmaceutical industries, it is critical to have economic, consistent and stable manufacture procedures, and to reduce the usage of energy and reactants, toxic by-products and waste.

Catalysts, chemical substances that increase reactions rate by decreasing the activation energy, often involve expensive transitional metals, with difficult recovery, and potential toxic by-products generation. In order to have more efficient and clean chemical synthesis procedures, chemical catalysts could be replaced by bio-derived catalysts, enzymes.[18-20] Enzymes are proteins or RNAs, which work as catalysts in living organisms. Most of the enzymes used in research are proteins.[21-24] With different three-dimension structures, they are able to provide three significant advantages in synthesis reactions: high substrate specificity, high selectivity and high catalytic efficiency with mild reaction conditions.[25] On the other hand, the production cost of enzymes is high due to the time-consuming preparation and purifications steps. In addition, enzymes require proper storage and usage condition. Various techniques, including immobilization, protein engineering and recombinant DNA technologies. are widely utilized to improve the use of enzymes.

Protein engineering and recombinant DNA technology have been proven to perform the aimed characteristic (e.g., chemoselectivity, regioselectivity, stereoselectivity long-term stability, activity in the presence of high substrate concentrations, and tolerance towards organic solvents).[27]

To harness all the above properties and add convenient handling, easier product separation by eliminating protein contaminants, high reusability, higher stability under extreme physical and chemical conditions, easier shipment of enzyme from one place to another, making the complete process more viable economically, and providing easier process control,[26] enzyme immobilization allows optimized processes in versatile enzyme-immobilization applications.[28]

Several ways to immobilize enzymes have been studied.[29-31] As shown in figure 1.2, there are two major approaches: physical method (adsorption, entrapment, and encapsulation) as well as
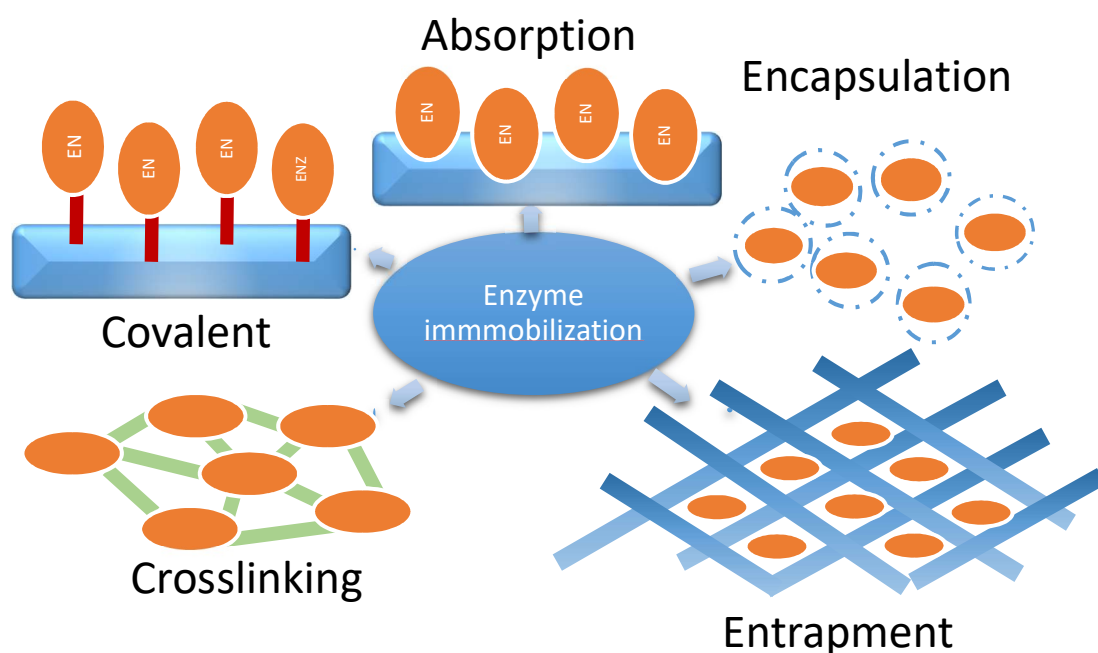
Figure 1.2. Enzyme immobilization methods

the chemical method (cross-linking, and covalent attachment) on substrates such as natural polymers, synthetic polymers or inorganic materials.[26,32,33]

1.3.1 Absorption

There are three kind of forces facilitating enzyme immobilization : ionic interactions, hydrogen bonds and Van der Waals. The material used can be either organic or inorganic in nature. Examples are: activated carbon, ceramic, porous glass, cellulose, starch, chitosan, and more. The method is a possible strategy for preventing enzyme degradation, but it involves extensive optimization due to the numerous factors such as pH, temperature, solvent, ionic strength and high substrate concentrations that play a significant role in enzyme micro-environment.

1.3.2 Entrapment and Encapsulation

Entrapment and encapsulation center on the similar concept of enzyme containment. Entrapment implies that the carrier is covering almost every side chain present on the surface of the enzyme by physical entrapment within the polymer lattice. However, this method would only contain limited number of enzymes. Encapsulation involves wrapping up enzymes within semi-permeable polymers or membranes with controlled porosity (1–100 μm). Encapsulation, giving access to large surface areas of the material, leads to higher catalytic efficiency.

1.3.3 Cross-linking

This method uses covalent bonding to bridge the support with the enzyme by connecting functional groups of the two entities. This requires formation of a number of covalent bonds between enzyme and the matrix; sometimes this could lead to the loss of a large amount of enzyme or enzyme functionality due to the difficulty to regulate the reaction.

1.3.4 Covalent Attachment

This immobilization method involves non-essential amino acids, which are the ones not on the active site of enzymes, to binds to the solid support covalently, but sometimes leads to conformational changes of enzymes' structure. It helps to promote the higher resistance of

immobilized enzymes towards extreme physical and chemical conditions, like temperature, denaturants, organic solvents, but this method of immobilization sometimes leads to harsh changes in catalytic properties of the enzyme, due to some amino-groups on the active site might be involved in the interaction between enzyme and the carrier[26,29]

These approaches are amenable to general macromolecule immobilization and delivery and could contribute to advancing research in therapeutic macromolecules delivery, including protein delivery.

1.4   Protein Delivery

Proteins are essential to natural processes in the living organisms and thus, their impairment is closely related to disease. As such, proteins and peptides can serve as effective drugs by restoring homeostasis.

Introduction of proteins into cells requires proteins delivery carriers that are biocompatible and biodegradable. Several useful approaches have been discovered to apply to different therapeutic proteins.[34] Supramolecular delivery systems, which are closely related to protein encapsulation, has become the growing topic to study.[34] Encapsulation, described in the previous section (1.3), is one of several methods to immobilize proteins that researchers have developed for protein delivery

CHAPTER 2. LITERATURE REVIEW

2.1   Biorenewable Resources

Biorenewable resources involve any biomass (plant, animal and any residues of animal/plant origin) that does not involve fossil resources. These resources contain bio-based polymers, which could become source for valuable chemicals upon a series of treatments and proper refinery, when they are not used as food. Cellulose, derived from plants, is one of the predominant biopolymers found on Earth. Several processes reported to date enable industrial conversion of cellulosic biomass to biofuels.[35,39-41] Similar to the chitins and several marine polysaccharides, cellulose is largely made up by primary feedstocks such as saccharides.[36-38] The primary component of the structure of cellulose is cellobiose, which is a disaccharide and a reducing sugar consist of two glucoses by $\beta(1{\rightarrow}4)$ glycosidic bond. Furthermore, the crystalline structure of the cellulose, constructed by hydrogen bonding between cellulose chains makes it rigid and hard to breakdown. Two significant processes, cellulose breakdown and glucose fermentation, are involve in converting cellulose to biorenewable fuels.

Cellulose breakdown could be accomplished though chemical or enzymatic processes, where the latter is preferable as it provides a cleaner and easier-to-modified process conditions such as pH, and temperature.[35] The latest research on biomass conversion focuses on using immobilized enzymes to increase the reusability or the efficiency of enzymes. Recent reports from C.-W. Wu's group[45], show that enzyme encapsulation in silica nanomaterials allow enzyme protection and reusability.

2.2   Enzyme Immobilization

The usage of enzymes for synthetic purposes has grasped the researchers' attention for long time. In nature, organisms adjust the conditions of their enzymes to produce an optimum rate of

reaction, where necessary, or they may have enzymes which are adapted to function to extreme conditions. Most enzymes have limitation to their reaction conditions due to their sensitivity to physical and chemical properties such as narrowly defined temperature, pH, and ion concentration, which might cause self-degradation and denaturation. Changing these factors will affect the rate and efficiency of reaction caused by the enzyme. Meanwhile, the process of recycling enzyme is extremely costly and the enzymes separation from the complete reaction is another challenge. As a result, researchers have already discovered several approaches to increase the reusability of
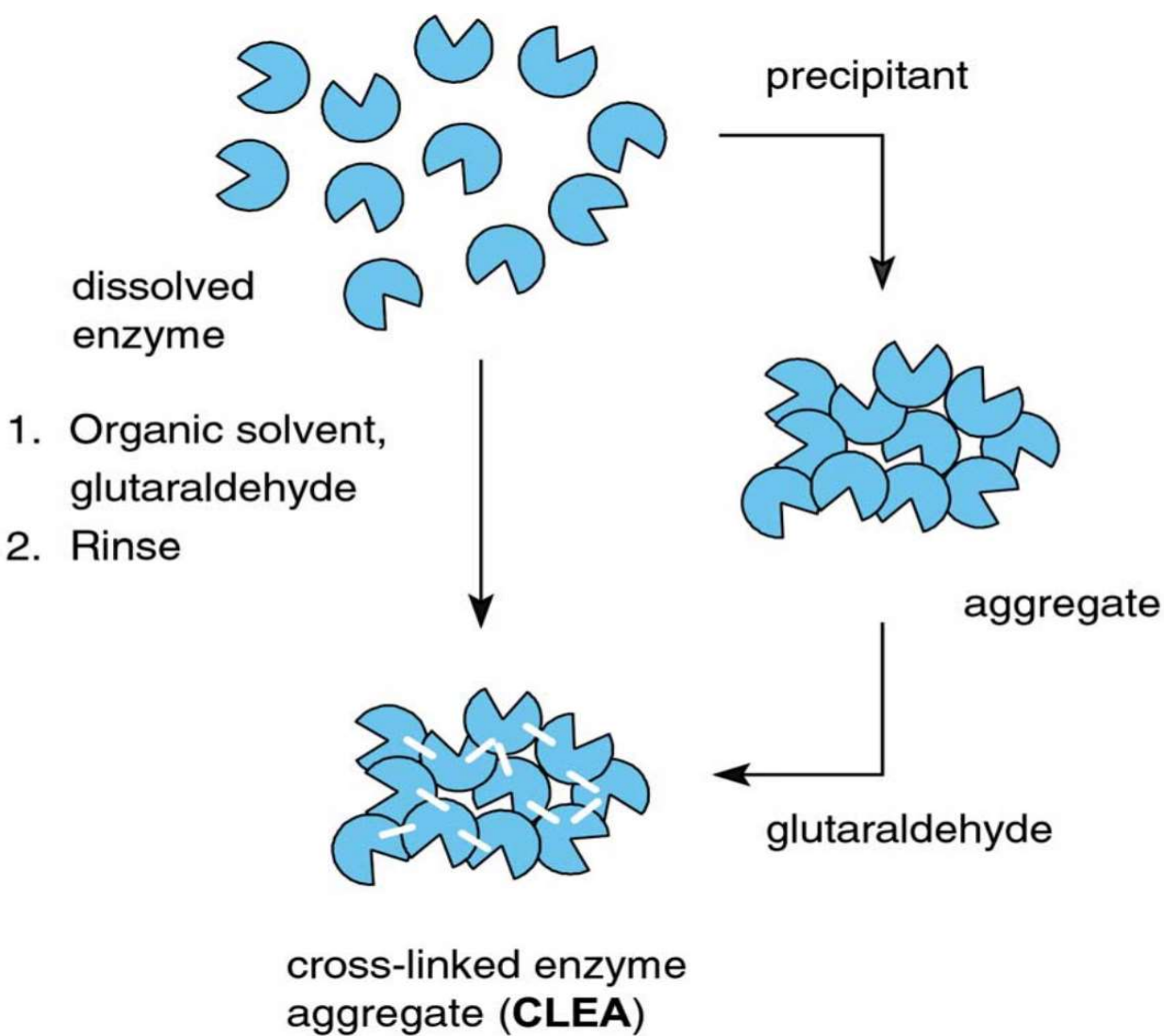
Figure 2.1. Simplified procedure for the synthesis of lipase cross-linked enzyme aggregate in organic solvent[75]

enzymes. One of the most efficient method is to immobilized the enzyme onto or into a solid support.[42,44]

Enzyme immobilization provides a very durable platform for increasing availability of enzyme to the substrate with greater turnover over times. Several natural and synthetic supports have been assessed for their efficiency for enzyme immobilization.[43,44] The major technic to immobilize enzymes are found as adsorption, covalent binding, affinity immobilization, and entrapment. Moreover, these techniques had been tried on many kinds of enzymes, and some of them are immobilized on natural or artificial polymers.[44] From the work of R.A. Sheldon *et al.*,
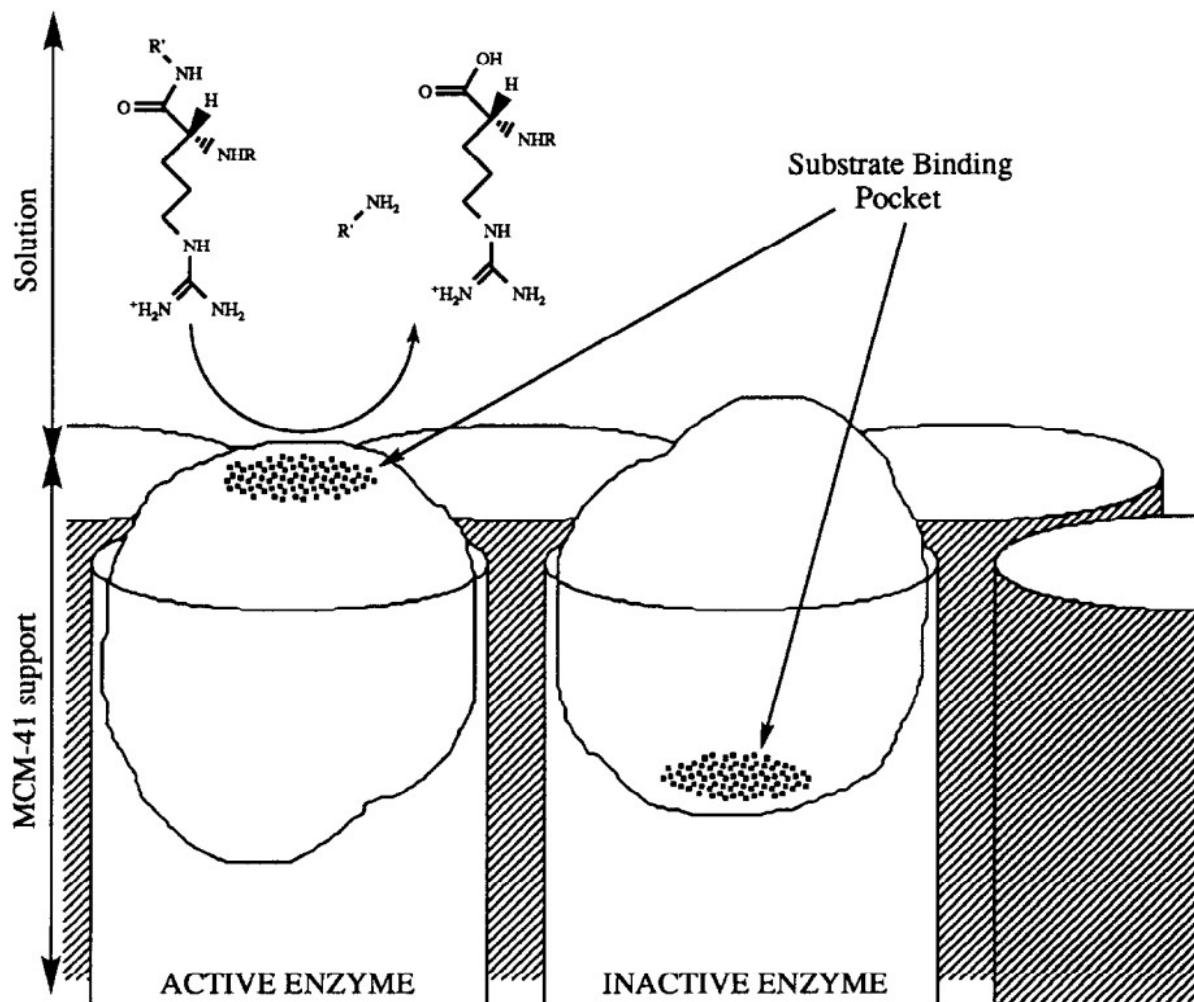
Figure 2.2. Schematic diagram illustrating the effect of adsorbed enzyme orientation on the activity of MCM-41 immobilized trypsin.[69]

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lipase was immobilized by crosslinking between the enzymes themselves, and present a higher activity than single form.[75] The schematic of crosslinking is showed in figure 2.1. Madamwar *et al.*, reported on amyloglucosidase entrapment into reverse micelles of Triton X-100–xylene–hexanol, and studied in aqueous conditions.[76] Balkus and co-workers reported the immobilization of cytochrome C, papain and trypsin, studied by physical adsorption in the mesoporous molecular sieve MCM-41.[69] The schematic of the absorption effect on enzyme is shown in figure 2.2.

2.2.1   Nanomaterials in bio-nanotechnology

Nanomaterials have been broadly studied in variety of fields, such as electrochemistry, biochemistry, biosensors, pharmaceuticals, drug delivery, electronics and more.[52-57] Our research utilizes nanomaterials in biotechnology applications. Various inorganic nanoparticles, typically made of metal oxides or metals, and including silica (SiO_2), gold (Au) and silver (Ag) have demonstrated biocompatibility. Such materials have been employed as nanoparticle carriers for drugs or therapeutic DNA, RNA or peptides. It was demonstrated by J. I. Zink et al. and J. Brinker et al., that both DNA and proteins can be successfully delivered into cells through engineered
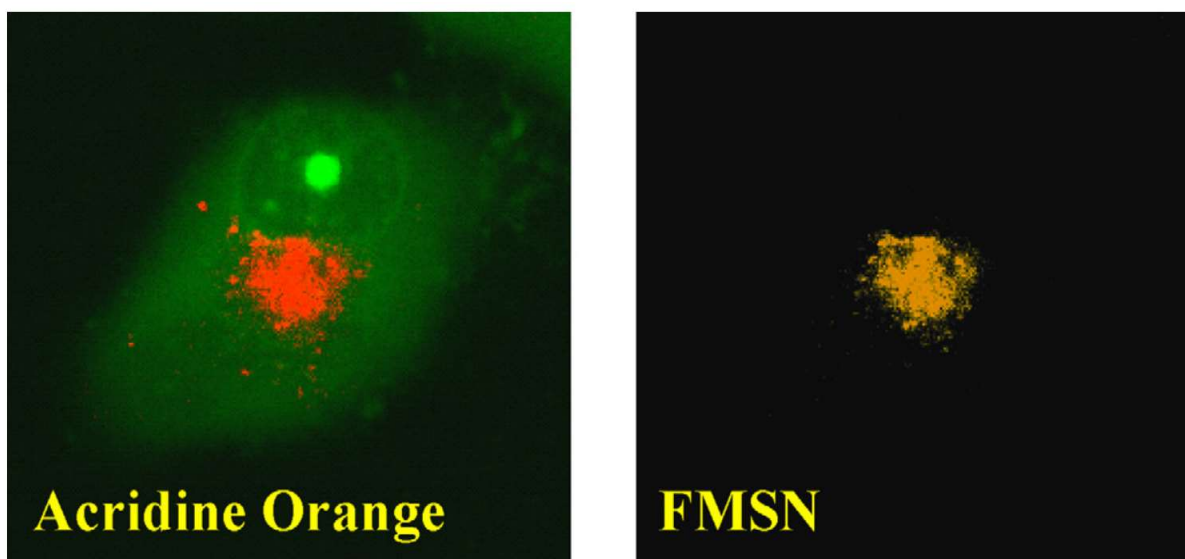
Figure 2.3. Uptake of the nanoparticles by cancer cells. PANC-1 cells stained with Acridine Orange (AO, left) and the fluorescence of the nanoparticles within the same[57]

nanomaterials, and retain their activity. Biocompatibility was proven by cells viability, and the optical properties of the materials allowed their visualization. (Figure 2.3)[57,58]

C. Kato *et al*. successfully synthesized porous silica in 1990. Templated synthesis resulted into a material with porosity and calcination allowed surfactant elimination leading 3-dimentional SiO$_2$ network with micrpores (Figure 2.4).[59] Following this breakthrough, a series of methods to
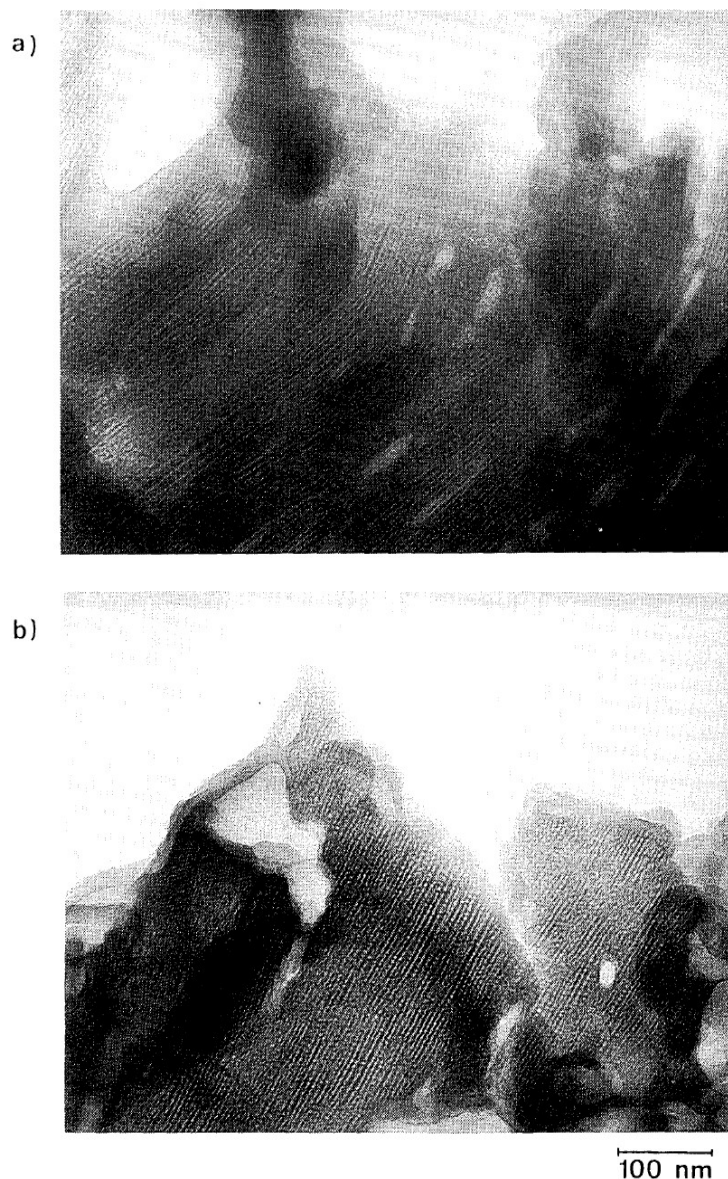
Figure 2.4. Transmission Electronic Microscopic(TEM) image of a) octadecyltrimethylammoniu-kanemite complexes and b) calcined (700°C) ctadecyltrimethylammoniu-kanemite complexes [59]

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synthesize different pore shapes, sizes and functional groups have been explored to optimize the characteristics for different uses.[48] MCM-41 pororosity was fine-tuned by modifications in synthesis conditions or starting materials used.[43,60,62] (Figure 2.5). A variation of mesoporous silica structure with larger pores, SBA-15, was synthesized by G. D. Stucky et al. The group also reported on a co-condensation method for functionalization, which allowed retention of the high surface areas, and long-range order in contrast to post-grafting methods.[43,61]   There are other synthetic conditions that could be altered to modified the morphology of mesoporous materials. The first mesoporous silica nanosphere (MSN) has been demonstrated and functionalized with
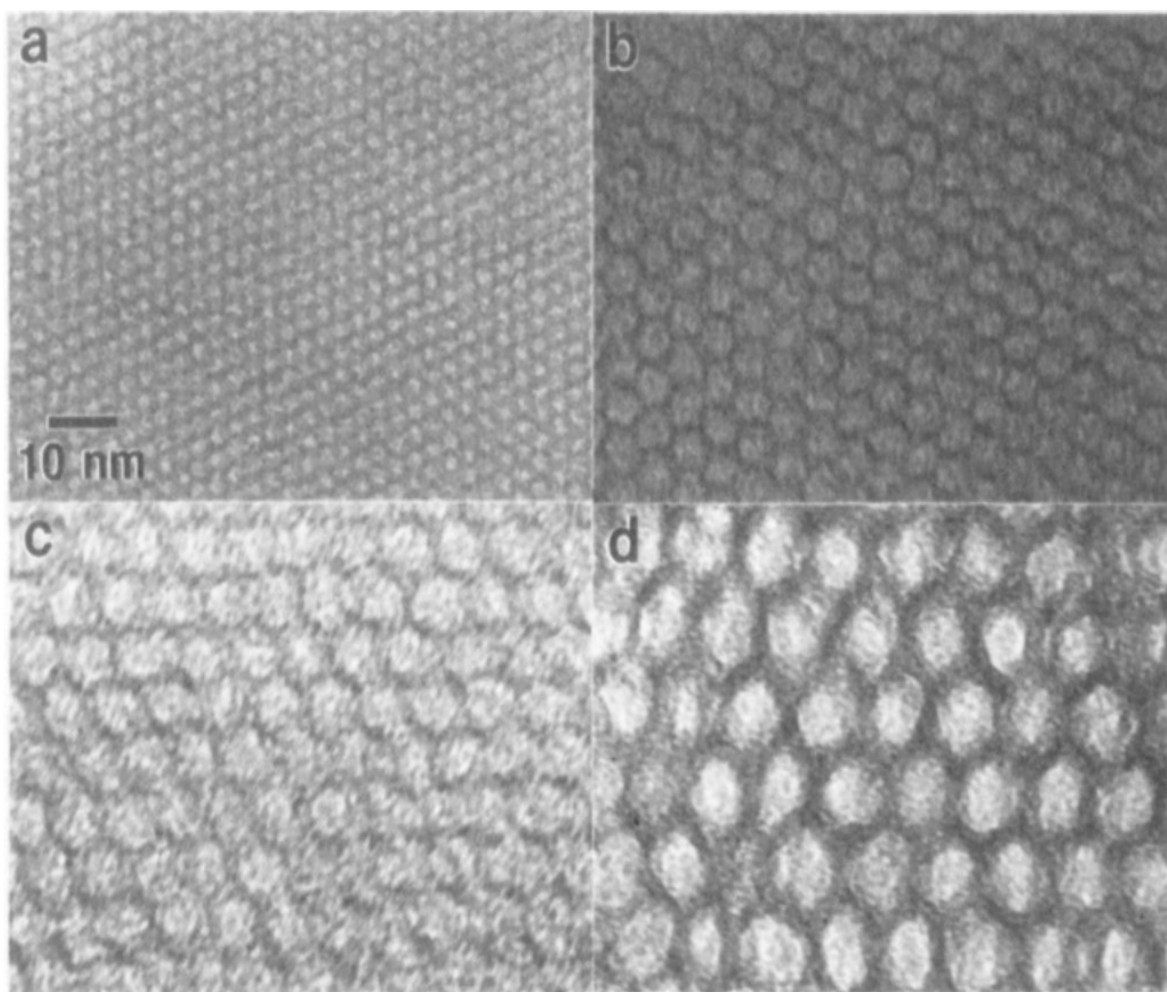
Figure 2.5. Transmission electron micrographs of several materials having approximate sizes of (a) 20, (b) 40, (c) 65, and (d) 100 A.[60]

amine groups by Lin et al.[73] The procedure to synthesize MSNs involves a sol-gel reaction. To synthesize MSNs, surfactant serves as a template in the reaction; this is required to form mesopores and to restrict mesopores to small sizes. When the synthesis is complete, the surfactant is removed by either acid wash or calcination. Base on different application of the MSN, functional groups are attached through two methods: post-grafting and co-condensation. For post-grafting, the reaction is performed on MSN with or without surfactant-removal in nonpolar anhydrous solvents to avoid a reaction of the organo group with anything but the silica material. On the other hand, it has been found that materials functionalized through post-grafting method might contain an inhomogeneous surface coverage of organic functional groups. For co-condensation, this functionalization method is a direct synthesis method, in which the organoalkoxysilane is introduced to the basic, aqueous surfactant solution during the frabication. With this synthetic approach, it is possible to control the morphology of the particles by the addition of functional co-condensing reagents.[74] Mesoporous silica is a well-developed platform for biotechnology applications.

2.2.2   MSN uses for Host-Guest Chemistry

Three approaches of introducing the functional units into the MSN host–guest systems through silica-surfactant mesostructures are reported. In a literature review published by Ogawa *et al,* the three methods are defined as: "(1) solidification from precursor solution containing soluble silica source and surfactant with dissolved functional guest species; (2) complexation of silica source with amphiphilic molecules working as supramolecular template and functional unit; and (3) polymerization of organosilanes bearing covalently bound functional units with surfactant."[66]

Some others entered and were carried by the MSN have the mechanism of   absorption and diffusion.[51,68] The patterns are showed in figure 2.6 and 2.7.

2.2.3   Progress of enzyme encapsulation

Drug delivery and biosensor development have been accomplished by taking advantage of the high surface area, pore volume and excellent biocompatibility exhibited by MSN.[51,57,58,63-65]
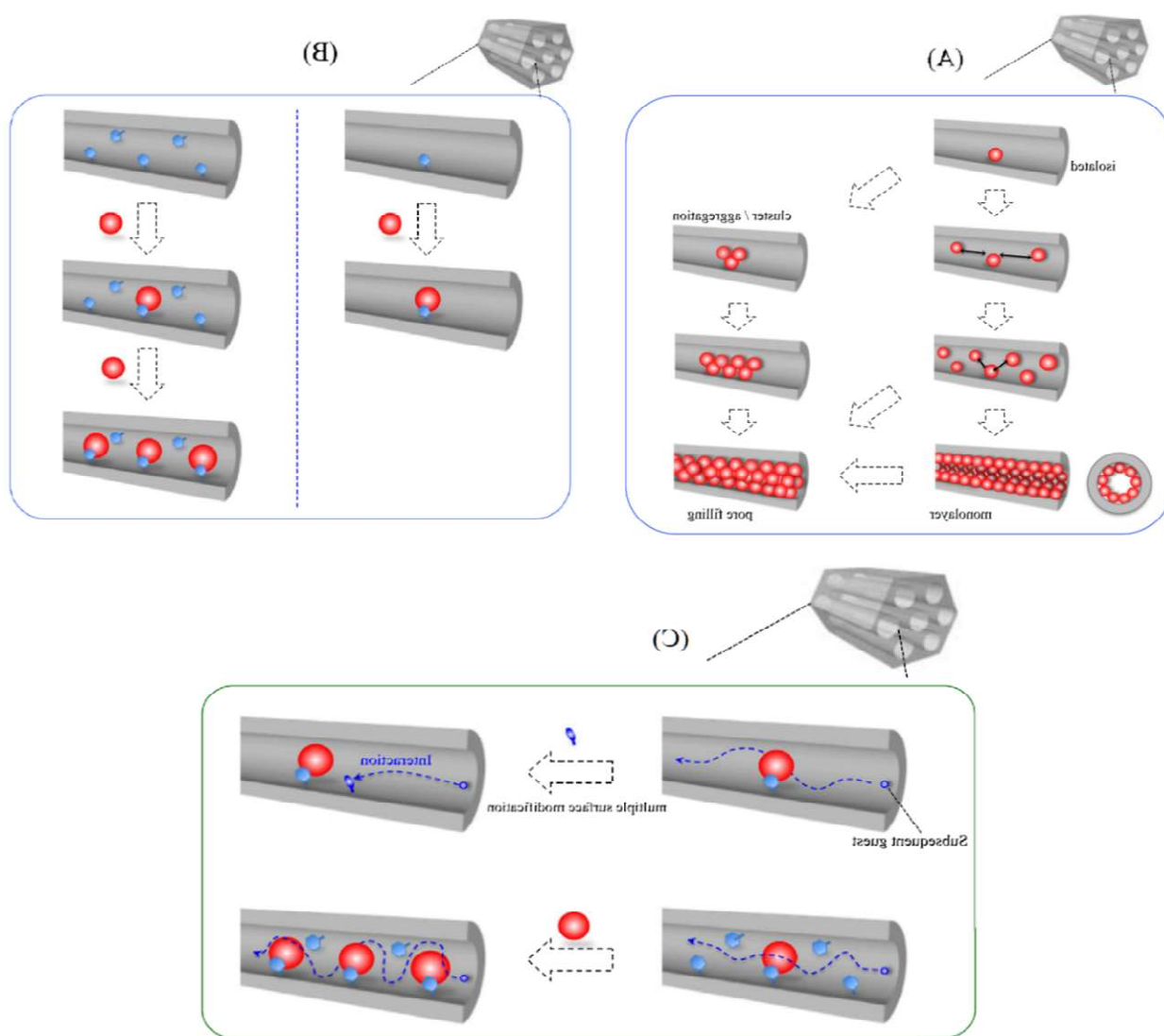
Figure 2.6. (a)Schematic drawings of the variation in the guest species included in a mesopore (b) Schematic drawings of the variation of the remaining nanospaces after guest immobilization in surface-modified mesoporous materials. (c) Schematic drawings of the diffusion of subsequent guest (adsorbate, reactant or products) in the remaining space[66]

SBA-15 and MCM-41 have been initially used as the most basic model to screen out the conditions for MSN as enzymes carriers.[69,70] Balkus Jr. et al. demonstrated that by physical adsorption in the hexagonal 40 Å pore of MCM-41, the efficiency of papain and trypsin immobilization was found favorable at pH values < 7, while immobilization of cytochrome c was found most efficient at pH > 7. The entrapped trypsin enzyme was fully active, while enzyme

(A) Post-synthetic grafting

organosilane
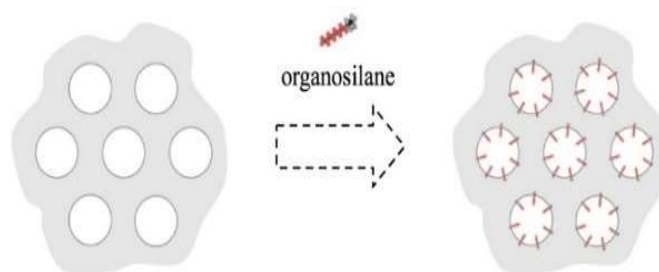
(B) Direct (One-pot) synthesis

organosilane

silica source

surfactant

extraction of template

organosilane

silica source

surfactant

extraction of template

Periodic mesoporous organosilica (PMO)
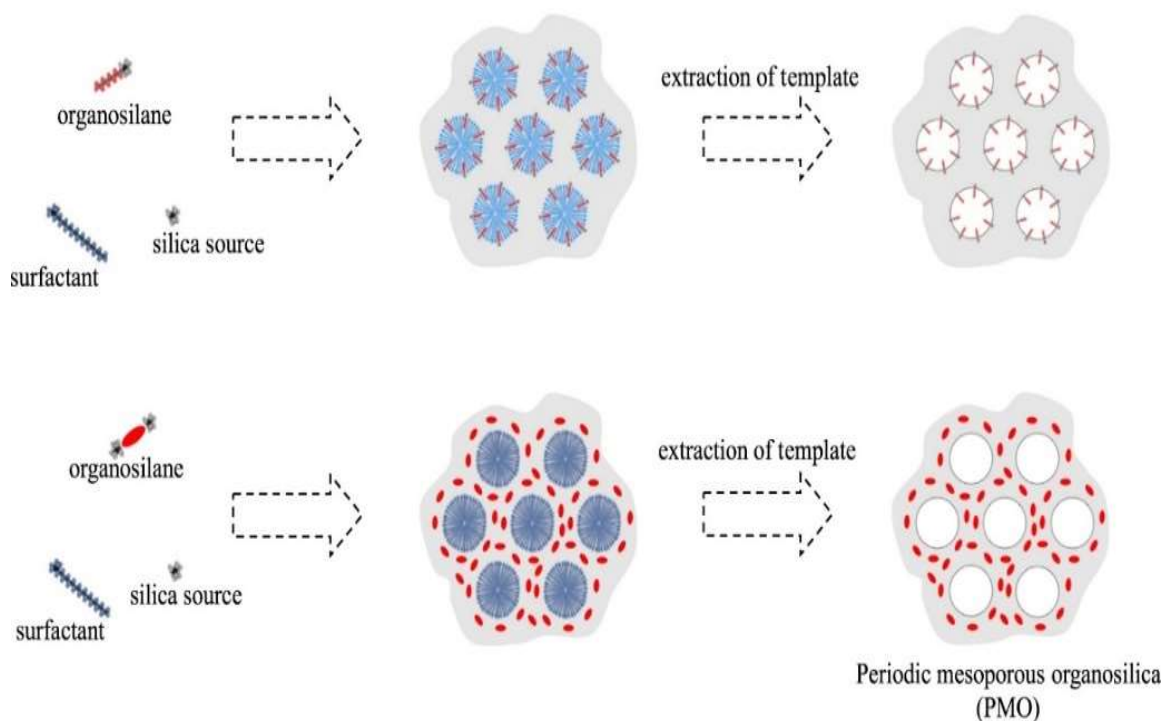
Figure 2.7. Covalent attachment of functional units onto mesostructures by (a) post-synthetic grafting and (b) direct (or one-pot) synthesis by condensation[66]

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stability was also enhanced in MCM-41.[69] Further, trypsin in different types of MSN had been studied. N. P. Botting et al. reported that large pore mesoporous silica like SBA-15 with similar hexagonal pore structure have a better potential in immobilizing enzymes than MCM-41 materials. However, the leaching of enzyme during applications became a problem. It was assumed that surface modification or covalent attachment of the enzyme will strengthen the encapsulation.[70] Therefore, functionalized material become one of the option to find out the better condition for enzymes.

In recent work done by the same group (N. P. Botting et al.), SBA-15 was functionalized with several organo group such as -SH, -Cl, -NH₂, -COOH and -phenyl group by either post-grafting or co-condensation, and proven having better encapsulation of enzymes on -SH and -COOH than non-functionalized one. Meanwhile, the efficiency of immobilized trypsin is maintained at 80% compared with free enzyme.[71] The correlation between pore size and more biomolecules immobilized in mesoporous silica has been summarized in a recent review(Table 2.1). According to enzyme and material prerequisite, the condition is better optimized for each application.

2.3   Biomass Conversion

The typical approach in biomass conversion is the use of immobilized enzymes, with one enzyme per support. Several examples of enzyme immobilization onto mesoporous silica have been discussed in the former sections.[48,49] In previous work, different size (150nm, 600nm) and pore size (2-15nm, 20-40nm) of MSN have been examined as enzyme encapsulation supports for biomass conversion.[46,50] However, even though the large pore MSN was found effective, the correlations among material size, pore sizes and enzymes' sizes are not linear. Meanwhile, some other work is on using different functional groups to alter the properties of MSN by adding acid

(SO$_3$H), base (NH$_2$) or both functionality to generate fully inorganic based catalysts for biomass conversion.[47]

In sum, MSN is a promising material to accomplish protein (including enzymes) encapsulation.[47,51] Modified MSN materials, typically by surface-functionalization, enabled this material to become a frontrunner in host-guest chemistry.

adsorbate	adsorbent
small biological molecules	
lysine, phenylalanine, histidine, glutamic acid, asparaginin, etc.	MCM-41
chorophyll a	FSM-16
vitamin E	CMK-1, CMK-3
vitamin B2	MCM-41, MCM-48
Purified Proteins	
catalase	SBA-15
conalbumin	SBA-15, thiol-functionalized SBA-15
conalbumin	APTS-modified MCF
cytochrome c	MCM-41, MCM-48, SBA-15, Nb-TMS-1, Nb-TMS-4
cytochrome c	MCM-41, SBA-15
cyctochrome c	SBA-15, thiol-functionalized SBA-15
cytochrome c	MCM-41, SBA-15, AlMCM-41, AlSBA-15
cytochrome c	CMK-3
HRP	FSM-16, MCM-41, SBA-15
β-lactoglobulin	SBA-15, thiol-functionalized SBA-15
lysozyme	MCM-41, SBA-15
lysozyme	modified SBA-15
lysozyme	MCM-41, SBA-15, AlMCM-41, AlSBA-15
lysozyme	CMK-1, CMK-3
myoglobin	SBA-15, thiol-functionalized SBA-15
ovalbumin	SBA-15, thiol-functionalized SBA-15
papain	MCM-41
penicilin acylase	MCM-41
RNase A	MCM-48
trypsin	MCM-41/SBA-15
trypsin	SBA-15, thiol-functionalized SBA-15
trypsin	MCM-41
trypsin	MCM-41, MCF
subtilisin Carlsberg	FSM-16, MCM-41, SBA-15
bovine serum albumin	SBA-15, thiol-functionalized SBA-15
bovine serum albumin	AlMCM-41
Whole Cells	
Arthobacter sp., *Bacillus subtilis*, and *Micrococcus luteus*	MCM-41, AlMCM-41

Table 2.1. Adsorption of Biologically Interesting Compounds on Ordered Mesoporous Materials[72]

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My thesis will be focused on demonstrating immobilization of a cascade reaction system obtained by immobilization of two enzymes on a Stellate porous silica platform. (Macroporous Silica Nanospheres Stellate –MSN)

CHAPTER 3. EXPERIMENTAL METHODS

3.1 Materials

3.1.1 Synthesis and Characterization of Stellate MSN

1. Tetraethyl orthosilicate (TEOS)

2. Cetyltrimethylammonium tosylate(CTATos)

3. Triethanolamine(TAE)

4. Anhydrous ethanol

3.1.2 Glucose Isomerase Enzyme: Plasmid Construction and Bacterial Expression in E. coli

5. E. coli strains DH5α

6. BL21 (DE3) Chemically Competent E. coli was purchased from Agilent

3.1.3 Recombinant Protein Purification

7. IPTG (isopropylthio-β-galactoside)

8. B-PER™ Bacterial Protein Extraction Reagent from Thermo-Fisher

9. Ni-NTA Agarose and Kanamycin were purchased from Thermo-Fisher

10. Tris(hydroxymethyl) aminomethane hydrochloride(TRIS-HCl)

11. Magnesium sulphate

12. 4-Morpholinepropanesulfonic acid(MOPS)

13. Cobalt(II) chloride hexahydrate, acetonitrile (HPLC grade)

3.1.4 Specific Substrate Conversion Assay by HPLC analysis

14. D-(+)-Cellobiose

15. D-(+)-glucose

16. D-(-)-fructose

17. Immobilized Glucose Isomerase from Streptomyces murinus (Sweetzyme T, Novazyme) from Sigma Aldrich without further purification

18. β-Glucosidase from almonds lyophilized powder were purchased from Sigma Aldrich without further purification

3.2 Characterization Methods

3.2.1 Characterization of MSN physical properties

1. Surface area and porosity analyzer (Quantachrome, Nova 4200e) is used to characterize surface area and porosity of MSN

2. Transmission electron microscopy (JEM-2100F) is used to characterize pore structure of MSN

3. Scanning electron microscope (Hitachi S4700) is used to characterize morphology of MSN

3.2.2 Characterization of Enzyme Product

4. Xcell surelock® mini-cell (electrophoresis tank)(Invitrogen) is used to characterize the purity and size of enzyme

5. Plate reader (Eppendorf AF2200) is used to characterize the concentration of enzyme before and after immobilization

6. Biospectrometer (Eppendorf) is used to characterize the concentration of DNA for plasmid construction

3.2.3 Analysis of Specific Substrate Conversion Assay

7. Ultra-High Performance Liquid Chromatographs (Shimazu Lc-2040c) is use to analyze the efficiency of the home-made or commercial enzyme under different condition

3.3 Synthetic and Production Methods (are described in detail in next chapter)

1. Synthesis and characterization of Stellate MSN platform

2. Plasmid construction and bacterial expression in E. coli for Glucose Isomerase(GI) Enzyme.

3. Recombinant protein purification

4. Protein quantification, electrophoresis, and molecular mass determination

5. Enzyme immobilization on Stellate MSN

6. Specific substrate conversion assay by HPLC analysis

7. Dual-Enzyme MSN enzymatic conversion of cellobiose to fructose

8. Conversion of cellobiose to glucose catalysed by free β-Glucosidase(BG) enzyme

9. Conversion of glucose to fructose catalysed by free recombinant GI Enzyme

10. Conversion of glucose to fructose catalysed by commercially available Immobilized GI Enzyme (Sigma-Aldrich)

11. Conversion of cellobiose to fructose catalysed by a mixture of β-Glucosidase and recombinant GI

12. Effects of the temperature, pH, and metal ions on recombinant enzyme activities

13. Catalyst reusability

CHAPTER 4. DUAL-ENZYME NANO-BIOCATALYST FOR THE CASCADE CONVERSION

4.1 Introduction

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.

4.2 Background and Significance

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 a step-wise process: first, beta-1,4 glucanase breaks the glyosidic linkage to *cellobiose*—a glucose dimer (Figure 4.1) 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.[77]

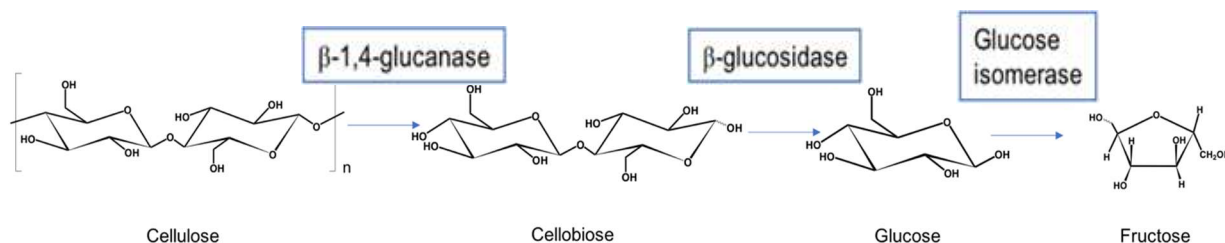The process of converting cellulose to fructose is illustrated in Figure 4.1.

Figure 4.1. Schematic representation of the step-wise transformation process from cellulose to fructose

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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 cellobiase, is a ubiquitous component of cellulases.[77] 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.[78,79] The native level of β-glucosidase activity in cellulase is, therefore, insufficient for the maximum rate and extent of glucose production to be reached.[77]

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 to increase turnover over a considerable period of time, since enzyme availability to the substrate is greater. Several natural and synthetic supports have been assessed for their efficiency for enzyme immobilization.[44]

The typical approach is one enzyme per support. Several examples of enzyme immobilization onto porous silica have been reported.[45,47-50,70,80]

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.

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.[82]

Among these platforms, 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 activity.

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.

4.3 Materials and Methods

4.3.1 *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.

4.3.2 *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 prepared for the control experiments).

4.3.2.1 *Synthesis and Characterization of Stellate MSN Platform*

The synthetic procedure of MSNs with stellate morphology was modified from previous literature reports.[81,82] 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 hour, 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 hours. 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 hours 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.

4.3.2.1.1 *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.[84] The pTNXI plasmid was transformed into chemically competent E. coli DH5α according to the manufacturer's instructions.

4.3.2.1.2 *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 hours, 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 minutes 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.

4.3.2.1.3 *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 theoretical mass was derived from the ExPASy ProtParam tool (http://web.expasy.org/protparam/).

4.3.2.2 _Enzyme Immobilization on Stellate MSN_

4.3.2.2.1 *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 2 mL 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 M 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.

4.3.2.2.2 _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.

4.3.2.3 _**Enzymatic Assays**_

4.3.2.3.1 _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(JAPAN) 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.

4.3.2.3.2 _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 for 20 h at 55 °C to obtain cellobiose-to-fructose conversion.

4.3.2.3.3 _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.

4.3.2.3.4 _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.

4.3.2.3.5 *Conversion of Glucose to Fructose catalysed by Commercially Available Immobilized GI Enzyme (Sigma-Aldrich)*

Immobilized Glucose Isomerase from *Streptomyces murinus* (50 mg/1ml) 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.

4.3.2.3.6 *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.

4.3.2.3.7 *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 MgSO$_4$, 0.5 mM Co^{2+}, pH 7.0) and C buffer (50 mM citric acid, 5 mM MgSO$_4$, 0.5 mM Co^{2+}, pH 5.0). As shown in Table 4.1.

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 4.2.

Table 4.1. Buffer Components

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 4.2. Optimal reaction conditions

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

4.3.2.4 *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.

4.4 Results

The preparation of Stellate MSN, as previously reported by us and others,[81] renders

materials with average surface area of 580 m^2/g, as illustrated in Table 4.3.

<div align="center">

Table 4.3. Surface Area (BET) and Average Pore Size

Material	Average BET (m²/g)	Average Pore Size Distribution (nm)
Surfactant–free Stellate MSN	580.0	12.6

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The macroporous Stellate materials, as illustrated by SEM and TEM images (Figure 4.2), 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 molecular cloning, followed by protein expression and purification techniques.(Figure 4.3) Enzyme macromolecules loading in Stellate MSN was performed by an impregnation method
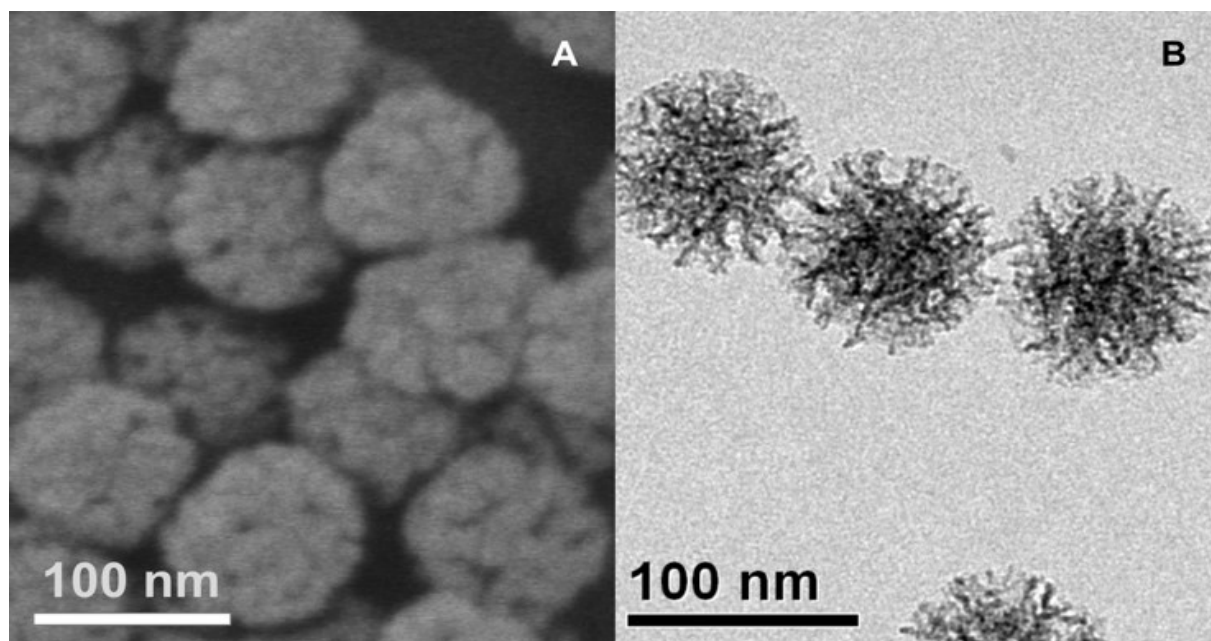

Figure 10. Stellate MSN in (A) SEM to show the morphology and (B)TEM to show the pore structure

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 (indicated in Table 4.4) 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.

Table 4.4. Size of Loaded Enzymes

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

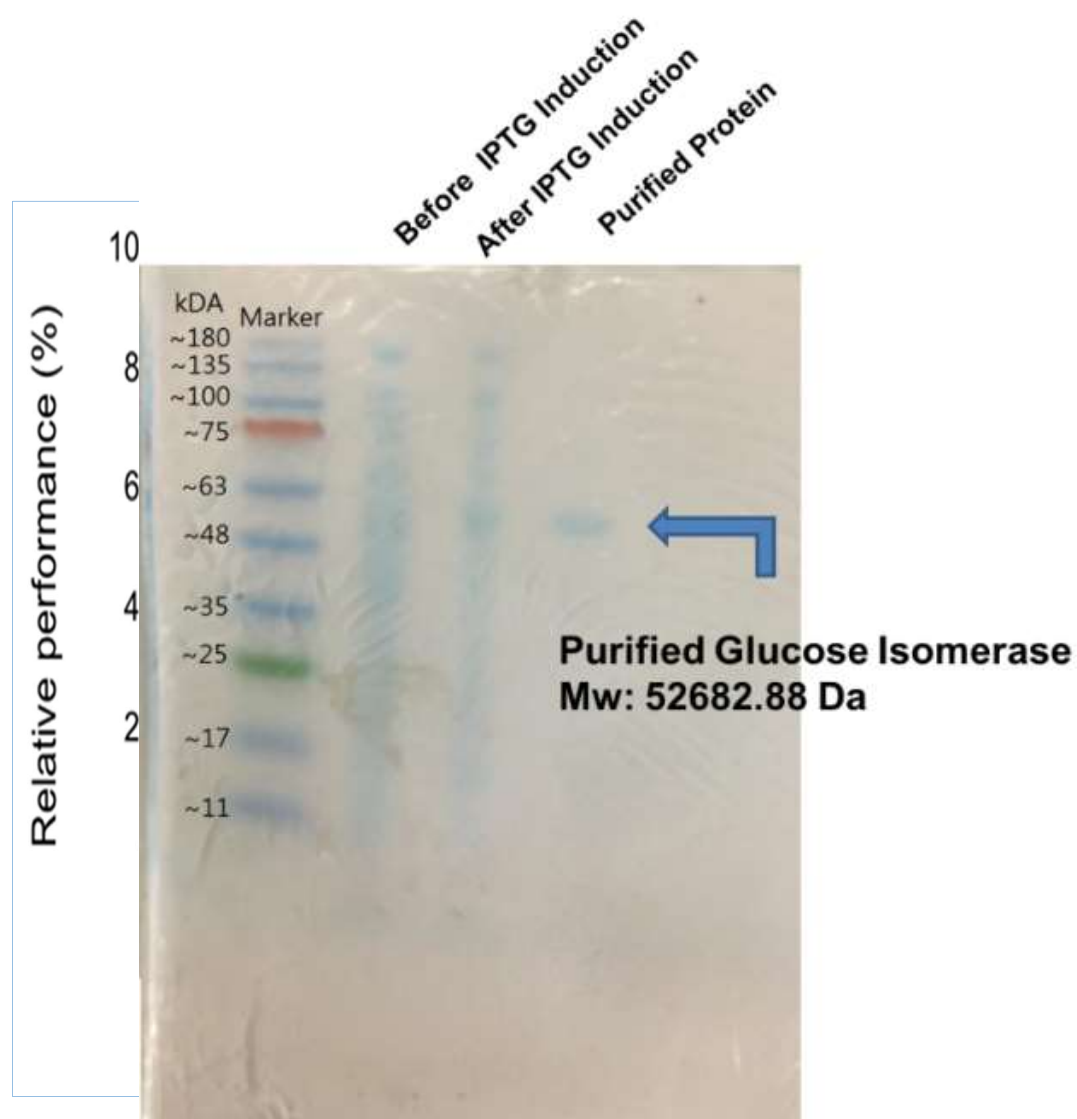Optimization of enzymatic reaction conditions resulted in choosing the M buffer for all

Figure 4.3. GI enzyme purity by SDS-PAGE

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 4.4 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 4.5 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
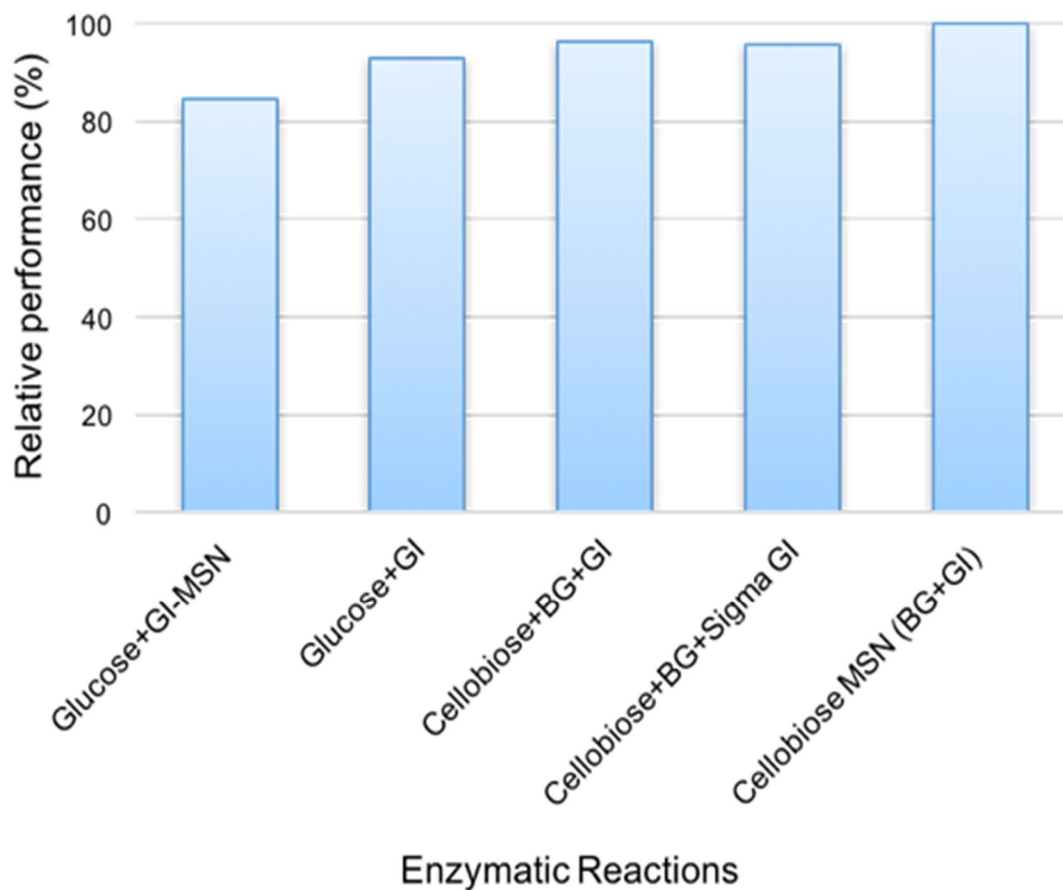

Figure 4.5. Relative performance of free and immobilized enzymes

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 4.6.
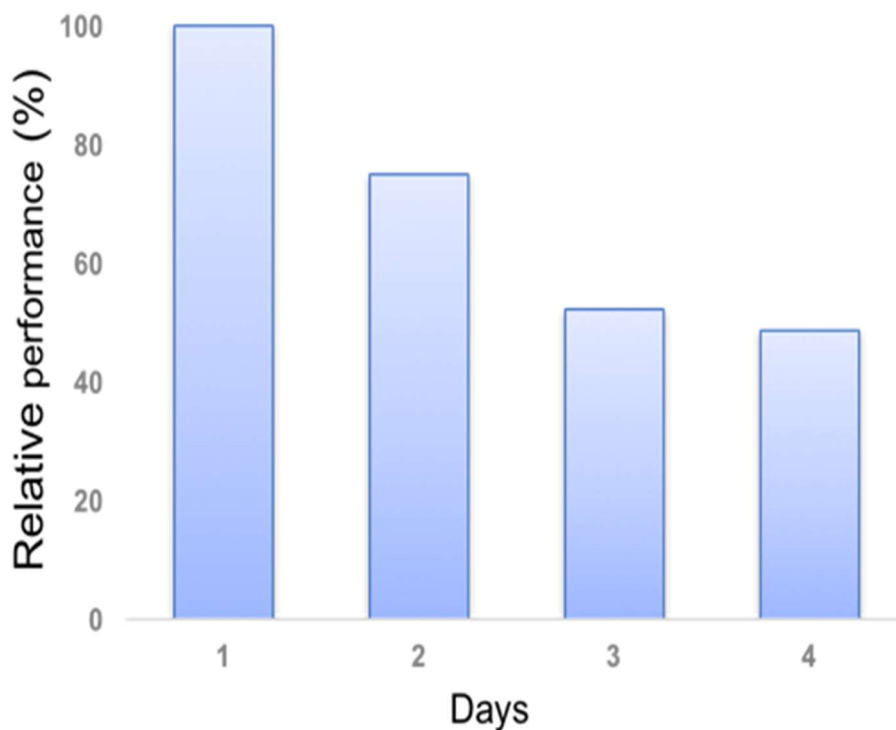
Figure 4.6. Enzyme recyclability

4.5 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.[80,84] Therefore reaction conditions were not unified but changed for each sequential step to match the enzyme working 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 4.7.
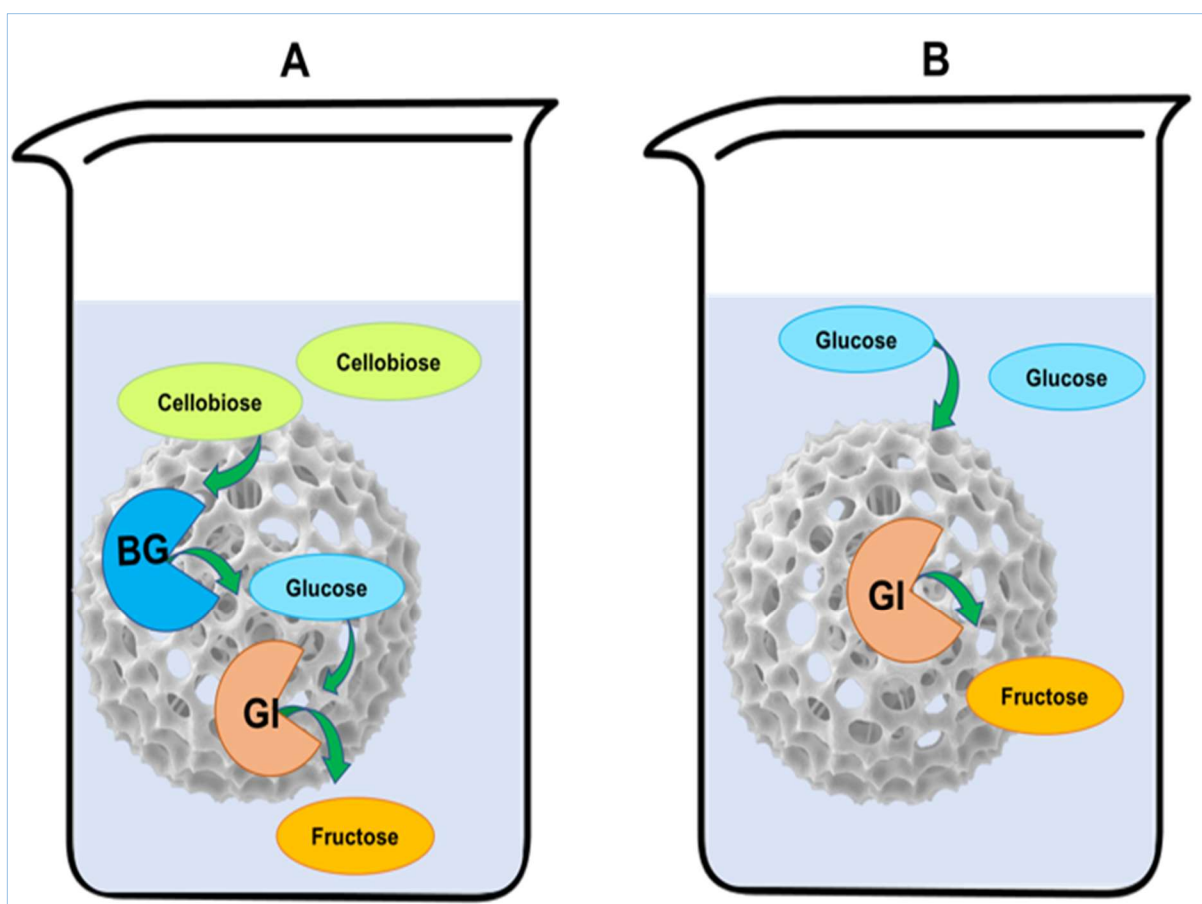

Figure 4.7. Schematic of channelling effect in the dual enzyme system: A). Glucose proximal access to the enzymatic site vs. B). Bulk diffusion to the enzymatic site.

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 4.5, 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.

Chapter 5 CONCLUSON AND FUTURE RECOMMENDATIONS

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 5.1). 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.
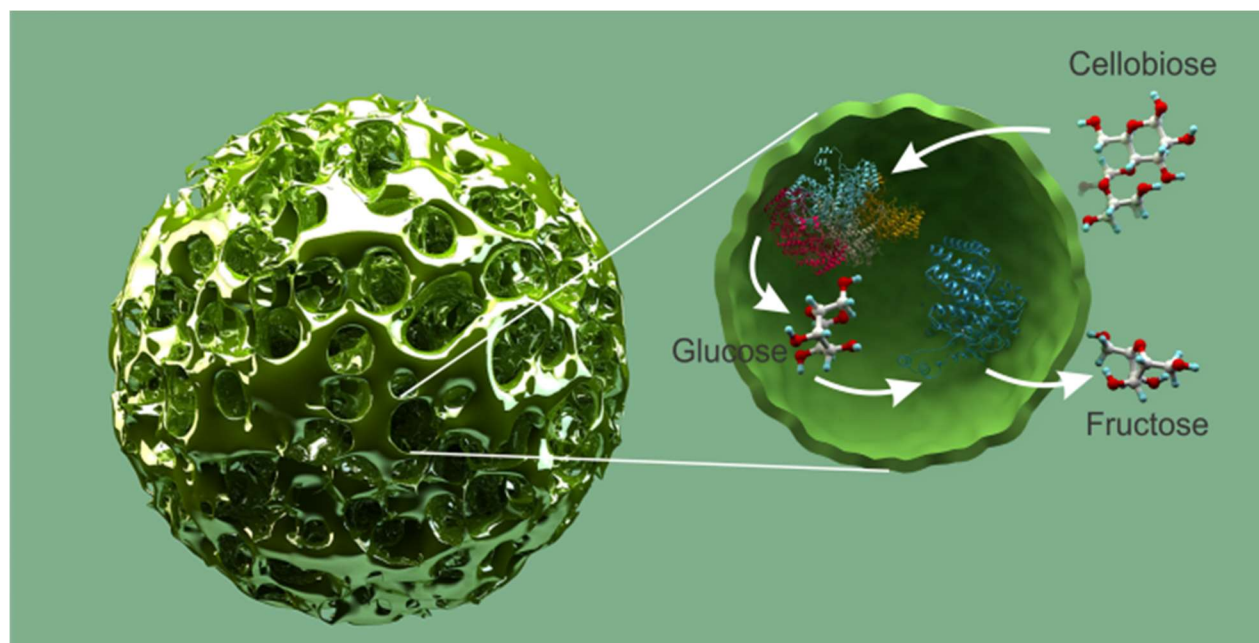
Figure 5.1. Schematic representation of the cascade reaction occurring in the Stellate MSN pore

Future work will undertake mechanistic studies 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.

Further applications of the encapsulation methodology is the extrapolation of this new knowledge for protein encapsulation and delivery. In addition to encapsulation, this endeavor involves the addition of a controlled-release delivery mechanism.

The interest in controlled-release drug and biomacromoelcules delivery is motivated by the fact that most of the conventional drugs and biomacromolecules present obstacles in regard to delivery to desired organs, including: poor solubility, tissue damage on extravasation, rapid degradation in vivo, unfavorable pharmacokinetics, poor biodistribution, and lack of selectivity for target tissues, which might cause the drugs ineffective. While poor solubility would cause precipitation in aqueous circumstance leads to failure; extravasation from some cytotoxic drug might harm the close tissue; degradation in short term loss the availability before drug reach the target; bad pharmacokinetics cases the drug to be eliminated from the body prior any activity; poor biodistribution could cause harm due to spread to other than the targeted tissue; lack of selectivity would cause the similar harm as extravasation and poor biodistribution.

In regard to conventional drugs, lack of selectivity is an important cause of harmful side-effects. Proteins such as insulin, antibody and some viral, bacterial proteins, etc. have high target specificity, and have been used as effective therapeutics.

From former chapter, the enzymes immobilized in MSN is proven effective The result not only demonstrate the availability of two different enzymes immobilizing in one support with maintaining enzyme activity, but also the stellate MSN has the ability to encapsulate protein and can used for transfer with minimal loss.

With the similar approach, stellate MSN could be used to carry proteins. Herein, it is presented that the material is available to carry the green fluorescence protein with Rhodamine B (RhB), the red fluorescent dye as a labelling dye, on stellate MSN to show the possibility for using MSN as a protein carrier. In figure 5.2, CHO cells were treated with RhB-MSN carrying a green-fluorescent protein (GFP), as model protein. The top-left image displays green fluorescence which is due to the GFP. In the top-center, there is red fluorescence of RhB on MSN, showing where the material settled. In the top-right image, the cell shape and distribution can be visualized. In the bottom-left image, there is blue fluorescence from DAPI staining dye, which interacts with the nucleus of the cells, and ultimately indicates that the cells are still alive before being fixed on slides. The last image presented is a composite of all previous images overlapped for better comparison.
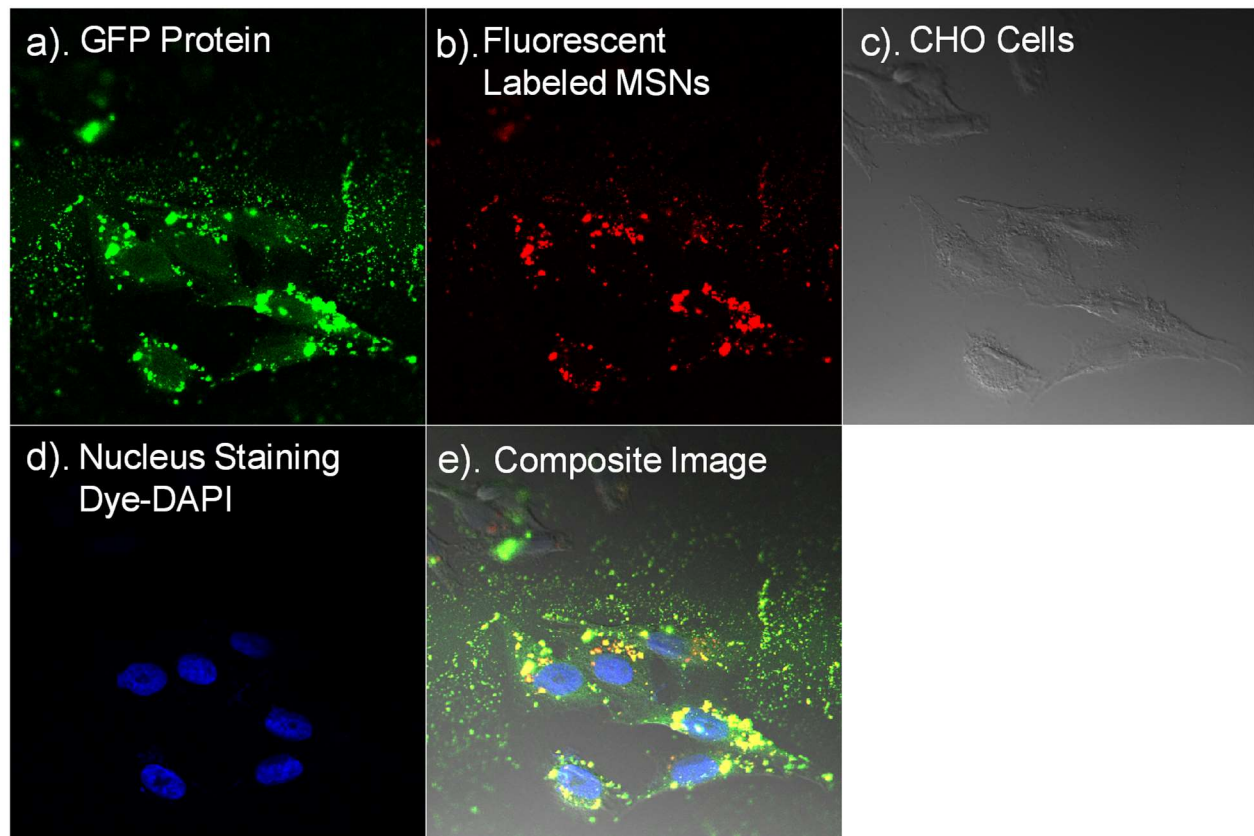

Figure 5.2. Cho stellate-RhB-GFP 4hr 63X Cho cells were treated with RhB-MSN carrying GFP for 4 hours. The GFP was successfully introduced into cells then released. Picture was taken from Zeiss 710 Confocal in Delaware Biotechnology i nstitute

From here, it can be concluded that the GFP was successfully introduced into live cells and then released without causing cell fatality. This conclusion can be made based on the fact that the GFP is presented where the materials were and the fluorescence can be visualized.

The biocompatibility of the material has been proved by viability of cells and the activity of the protein. Future work will employ other type of living cells and therapeutic proteins.

REFERENCES

[1]   Lehn,J. Science. (1993), 260 (5115):1762–1763. DOI: 10.1126/science.8511582.

[2]   Lehn, J.-M. Supramolecular Chemistry. (1995), Wiley-VCH. ISBN 978-3-527-29311-7.

[3]   Lodish, H.; Berk, A.; Kaiser, C. Molecular Cell Biology. (2008), ISBN 978-0-7167-7601-7.

[4]   Anslyn, Eric. Modern Physical Organic Chemistry. (2006), ISBN 978-1-891389-31-3.

[5]   Rouquerol, J., Avnir, D., Fairbridge, C. W., Everett, D. H., Haynes, J. M., Pernicone, N., Ramsay, J. D. F., Sing, K. S. W., Unger, K. K. (1994), Pure and Applied Chemistry. 66 (8). DOI:10.1351/pac199466081739

[6]   K.S.W. Sing, D.H. Everett, R.A.W. Haul, L. Moscou, R.A. Pierotti, J. Rouquerol,T. (1985), Pure and applied chemistry, 57: 603-619.

[7]   U. Ciesla, F. Schuth, (1999), Microporous and Mesoporous Materials. 27(2-3): 131-149.

[8]   T.K. Jain, I. Roy, T.K. De, A. Maitra, (1998), Journal of the American Chemical Society, 120(43): 11092-11095.

[9]   J. -I. Ida, T. Matsuyama, H. Yamamoto, (2000),Biochemical Engineering Journal, 5(3): 179-184.

[10]   T. Shimomura, T. Itoh, T. Sumiya, F. Mizukami, M. Ono, (2009), Talanta, 78(1): 217-220.

[11]   Y. Yang, H. Yang, M. Yang, Y. Liu, G. Shen, R. Yu, (2004), Analytica Chimica Acta, 525(2): 213-220.

[12]   K. Koch, K. Roos, M. Stocker, W. Reschetlowski, (1998), Chemical Engineering & Technology, 21(5): 401-403.

[13]   A.M. Siouffi, (2003), Journal of Chromatography A, 1000(1-2): 801-818.

[14]   L.L. Hench, J.K. West, (1990), Chemical Reviews, 90(1): 33-72.

[15] I. Mukherjee, Mesoporous materials for dental and biotechnological applications, Polymerized Curcumin and Enzymatic Saccharification of Biomass. (2009), Ph.D. Dissertation, Drexel University: Philadelphia.

[16] J. Wen, G.L. Wilkes, O. (1996), Chemistry of Materials, 8(8): 1667-1681.

[17] R.K. Iler, Chemistry of Silica-Solubility, Polymerization, Colloid and Surface Properties and Biochemistry. (1979), New York: John Wiley & Sons.

[18] R.A. Sheldon, (2007), Advanced Synthesis & Catalysis, 349(8-9): 1289-1307.

[19] R.A. Sheldon, (2000), Pure and Applied Chemistry, 72(7): 1233-1246.

[20] R.A. Sheldon, F.V. Rantwijk, (2004), Australian Journal of Chemistry, 57(4): 281-289.

[21] Stryer L, Berg JM, Tymoczko JL. Biochemistry(5th ed.). (2002), San Francisco: W.H. Freeman. ISBN 0-7167-4955-6

[22] Anfinsen CB. (1973), Science, 181(4096): 223–30.DOI: 10.1126/ science.181. 4096.223.

[23] Dunaway-Mariano D. (2008), Structure. 12;16(11):1599-600.
DOI: 10.1016/j.str.2008.10.001.

[24] Petsko GA, Ringe D. Protein structure and function. (2003), London: New Science. ISBN 978-1405119221.

[25] Nagayasu, T., Miyanaga, M., Tanaka, T., Sakiyama, T. & Nakanishi, K. (1994), Biotechnol. Bioeng, 43: 1118.

[26] Dwevedi, A. Enzyme immobilization. 1st ed. (2016). Springer. ISBN:978-3-319-41418-8

[27] Singh RK, Tiwari MK, Singh R, Lee J-K. From protein engineering to immobilization: promising strategies for the upgrade of industrial enzymes. (2013), Int J Mol Sci, 14: 1232–1277.

[28] Redeker ES, Ta DT, Cortens D, Billen B, Guedens W, Adriaensens P. (2013), Bioconjugate

Chem. 24:1761–1777.

[29] Ahmad Abolpour Homaei, Reyhaneh Sariri, Fabio Vianello, and Roberto Stevanato, (2013), J Chem Biol. 6(4): p. 185–205

[30] Engelmark Cassimjee, K., Kadow, M., Wikmark, Y., Svedendahl Humble, M., Rothstein, M. L., Rothstein, D. M., Bäckvall, J. -E., (2014), Chemical Communications, 50 (65): 9134. DOI: 10.1039/C4CC02605E.

[31] Zucca, Paolo; Sanjust, Enrico. Molecules. (2014), 19 (9): 14139–14194.

[32] W. Hartimer, Immobilized Biocatalysts; An Introduction. (1988), Berlin: Springer Verlag.

[33] M.D. Trevan, Enzyme Immobilization by Entrapment, in New Protein Techniques, J.M. Walker, Editor. (1988), Humana Press: Clifton, NJ. p. 491-494.

[34] Ailing Fu, Rui Tang, Joseph Hardie, Michelle E. Farkas, and Vincent M. Rotello, (2014), Bioconjug Chem. 25(9): 1602–1608. DOI: 10.1021/bc500320j

[35] Dezhi Zhang, Hisham E. Hegab, Yuri Lvov, L. Dale Snow, and James, (2016), Springerplus, 5(1): 48. DOI: 10.1186/s40064-016-1682-y

[36] Kitaoka, M.; Taniguchi, H.; Sasaki, T. J. Ferment. (1989), Bioeng. 67: 182–185.

[37] Bhat, M. K.; Bhat, S. Biotechnol. AdV. (1997), 15: 583–620.

[38] Bhat, M. K. (2000), Biotechnol. AdV. 18: 355–383.

[39] Arthur T. Johnson (2016) Biology for Engineers, Boca Raton, FL: CRC Press

[40] S.-Y. Ding, Science, (2012), 338: 1055–1060.

[41] S. P. Chundawat, G. T. Beckham, M. E. Himmel and B. E. Dale, (2011), Annu. Rev. Chem. Biomol. Eng., 2:121–145.

[42] I. Gelfand, R. Sahajpal, X. Zhang, R. C. Izaurralde, K. L. Gross and G. P. Robertson, (2013), Nature, 493: 514–517

[43] Chia-Hung Lee, Tien-Sung Lin, Chung-Yuan Mou. (2009), Nano Today, 4: 165-179

[44] Datta, S., Christena, L.R., Rajaram, Y.R.S. (2013), 3 Biotech, 3(1): 1–9 DOI:10.1007/s13205-012-0071-7

[45] Saikat Duttaa and Kevin C.-W. Wu, (2014), Green Chem., 16: 4615-4626 Doi:10.1039/c4gc01405g

[46] S-B Hartono, S-Z Qiao, J Liu, K Jack, B P. Ladewig, Z-P Hao, G-Q Max Lu, (2010), J. Phys. Chem. C, 114: 8353–8362

[47] W-H Peng, Y-Y Lee, C Wu, KCW Wu. (2012), Journal of Materials Chemistry, 22: 23181. Doi:10.1039/c2jm35391a

[48] C Ispas, I Sokolov, S Andreescu. (2009), Anal Bioanal Chem, 393: 543. DOI:10.1007/s00216-008-2250-2

[49] M Hartmann, X Kostrov. (2013), Chem. Soc. Rev. 42: 6277. Doi:10.1039/c3cs60021a

[50] RH-Y Chang, J Jang, KCW Wu, (2011), Green Chemistry, 13: 2844. Doi:10.1039/C1GC15563F

[51] Radu DR, Lai CY, Jeftinija K, Rowe EW, Jeftinija S, Lin VS. (2004), J Am Chem Soc. 126(41): 13216-13217

[52] Minhua Cao , Xiaoyan He , Jun Chen , and Changwen Hu, (2007), Crystal Growth & Design, 7 (1): 170–174. DOI: 10.1021/cg060524w

[53] Motao Zhu, Guangjun Nie, Huan Meng, Tian Xia, Andre Nel, and Yuliang Zhao, (2013), Acc. Chem. Res., 46 (3): 622–631. DOI: 10.1021/ar300031y

[54] Mehmet V. Yigit, Leyun Zhu, Marytheresa A. Ifediba, Yong Zhang, Kevin Carr, (2011), Anna Moore, and Zdravka Medarova, ACS Nano, 5 (2): 1056–1066. DOI: 10.1021/nn102587h

[55] Sarah A. Engelberth, Nadine Hempel, and Magnus Bergkvist, (2015), Bioconjugate Chem., 26 (8): 1766–1774.

DOI: 10.1021/acs.bioconjchem.5b00313

[56] J. Kim, J.W. Grate, P. Wang, (2008), Trends Biotechnol, 26: 639.

[57] Sarah Angelos, Monty Liong, Eunshil Choi, Jeffrey I. Zink, (2008), Chemical Engineering Journal, 137(1): 4–13. DOI: 10.1016/j.cej.2007.07.074

[58] D. Tarn, C. E. Ashley, M-X, ERIC C. Carnes, Jeffrey I. Zink, C. Jeffrey Brinker, (2013), Acc Chem Res. 46(3): 792–801. DOI: 10.1021/ar3000986

[59] Tsuneo Yanagisawa , Toshio Shimizu , Kazuyuki Kuroda , Chuzo Kato, (1990), Bulletin of the Chemical Society of Japan, 63: 4. DOI: 10.1246/bcsj.63.988

[60] J. S. Beck, J. C. Vartuli, W. J. Roth, M. E. Leonowicz, C. T. Kresge, K. D. Schmitt, C. T. W. Chu, D. H. Olson and E. W. Sheppard, (1992), J. Am. Chem. Soc. , 114: 10834–10843.

[61] D. Margolese, J. A. Melero, S. C. Christiansen, B. F. Chmelka and G. D. Stucky, (2000), Chem. Mater. , 12: 2448–2459.

[62] A. Sayari, Y. Yang, M. Kruk and M. Jaroniec, (1999), J. Phys. Chem. B , 103: 3651–3658.

[63] Brian G. Trewyn, Supratim Giri, Igor I. Slowing , Victor S.-Y. Lin, (2007), Chem. Commun., p. 3236-3245. DOI:10.1039/B701744H

[64] Igor I. Slowing, Juan L. Vivero-Escoto, Chia-Wen Wu, Victor S.-Y. Lin, (2008), Advanced Drug Delivery Reviews, 60(11): 1278–1288.

DOI: 10.1016/j.addr.2008.03.012

[65] T. Heikkiläa, 1, J. Salonena, J. Tuuraa, M.S. Hamdyb, G. Mulb, N. Kumarc, T. Salmic, D.Yu. Murzinc, L. Laitinend, A.M. Kaukonend, J. Hirvonene, V.-P. Lehto, (2007), International Journal of Pharmaceutics, 331(1): 133–138. DOI: 10.1016/j.ijpharm.2006.09.019

[66] Minoru Sohmiya, Kanji Saito & Makoto Ogawa, (2015), Science and Technology of Advanced Materials, 16(5). DOI: 10.1088/1468-6996/16/5/054201

[67] Shioya Y, Ikeue K, Ogawa M and Anpo M, (Appl). Catal. A, 254: 251–259.

[68] Thomas A, Polarz S and Antonietti M, (2003), J. Phys. Chem. B, 107: 5081–5087.

[69] J.Felipe Díaz, Kenneth J. Balkus Jr. (1996), Journal of Molecular Catalysis B: Enzymatic, 2(2–3):115-126.

[70] Humphrey H.P. Yiu, Paul A. Wright, Nigel P. Botting, (2001), Microporous and Mesoporous Materials, 44–45: 763–768.

[71] Humphrey H.P Yiu, Paul A Wright, , Nigel P Botting, (2001),Journal of Molecular Catalysis B: Enzymatic, 15(1–3): 81–92.

[72] M. Hartmann, (2005), Chem. Mater., 17 (18): 4577–4593. DOI: 10.1021/cm0485658

[73] Cheng-Yu Lai , Brian G. Trewyn , Dusan M. Jeftinija , Ksenija Jeftinija , Shu Xu , Srdija Jeftinija , and Victor S.-Y. Lin, (2003), J. Am. Chem. Soc., 125 (15): 4451–4459. DOI: 10.1021/ja028650l

[74] Brian G. Trewyn, Igor I. Slowing, Supratim Giri, Hung-Ting Chen and Victor S.-Y. Lin, (2007), Acc. Chem. Res., 40 (9): 846–853 DOI: 10.1021/ar600032u

[75] P. Lopez-Serrano, L. Cao, F. van Rantwijk & R.A. Sheldon, (2002), Biotechnology Letters, 24: 1379–1383

[76] Claudia Shah, Subramani Sellappan, Datta Madamwar, (2000), Process Biochemistry, 35(9): 971–975.

[77] J Woodward, LJ Koran, LJ Hernandez, LM Stephan (1993) ß-Glucosidases, American Chemical Society,

[78] J Woodward, JC Bales (1989): 87. Doi:10.1007/978-3-642-74227-9_8

[79] D Sternberg, GR Mandels (1980) Journal of Bacteriology, 144: 1197.

[80] Y-C Lee, C-T Chen, Y-T Chiu, KCW Wu (2013) ChemCatChem, 5: 2153. Doi:10.1002/cctc.201300219

[81] K Zhang, L-L Xu, J-G Jiang, et al. (2013) Journal of the American Chemical Society, 135: 2427. Doi:10.1021/ja3116873

[82] DR Radu, NA Pizzi, C-Y Lai (2016) Journal of Materials Science, 51: 10632. Doi:10.1007/s10853-016-0284-y

[83] D Sriprapundh, C Vieille, JG Zeikus (2003) Protein Engineering, Design and Selection, 16: 683. Doi:10.1093/protein/gzg082

[84] DN Tran, KJ Balkus (2011) ACS Catalysis, 1: 956. Doi:10.1021/cs200124a

APPENDIX

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].
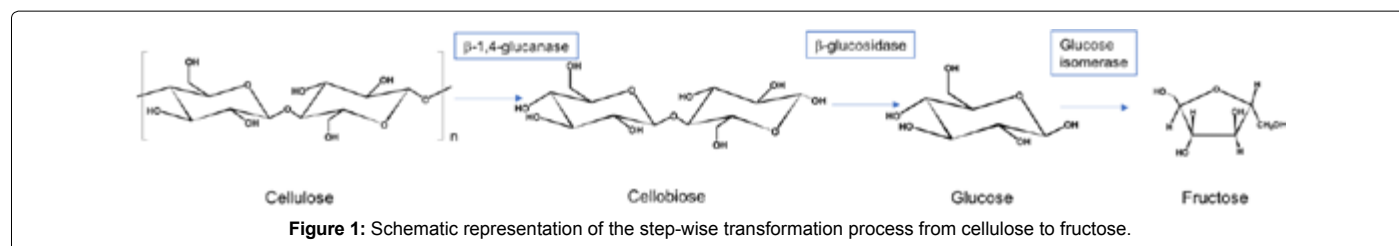
The typical approach is one enzyme per support. Several examples of enzyme immobilization onto porous silica have been reported [6-12]. Co-immobilization of enzymes on various platforms is an emerging field, and involves considerations of various factors: spanning from facile access of substrate to the 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 $MgSO_4$, 0.5 mM Co^{2+}, pH 7.0) and C buffer (50 mM citric acid, 5 mM $MgSO_4$, 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.

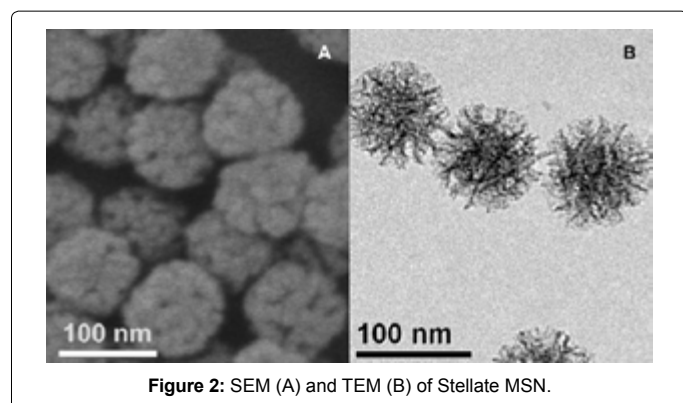| Enzyme	C Buffer	M Buffer
Recombinant Enzyme GI*	pH Too close to enzyme's pI no reaction	Optimal conditions
BG+GI Cellobiose to D-Fructose	Trace amount of fructose	optimal conditions

Table 2: Optimal reaction conditions.

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

Material	Average BET (m²/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.
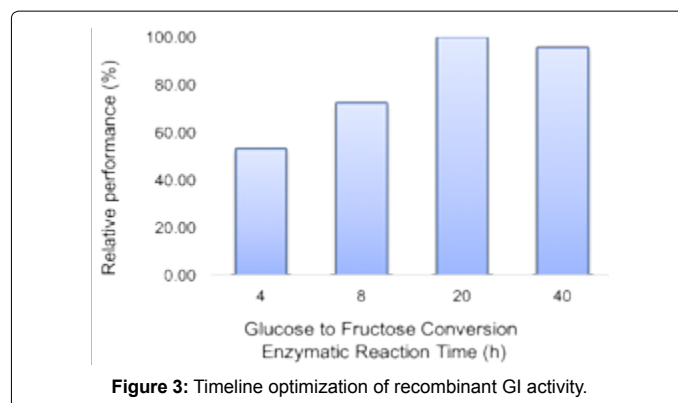

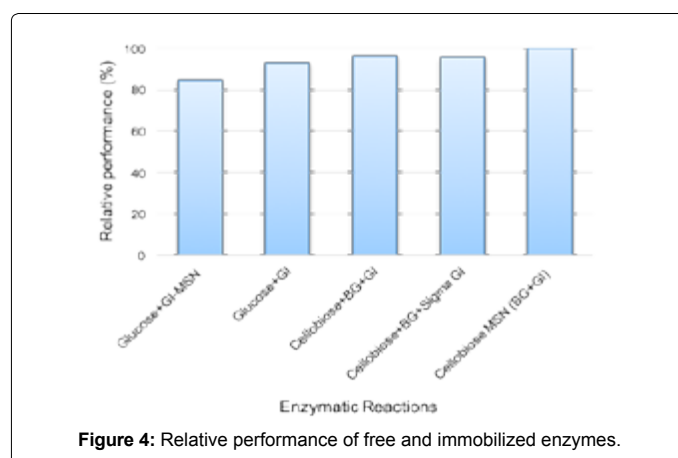**Figure 3:** Timeline optimization of recombinant GI activity.

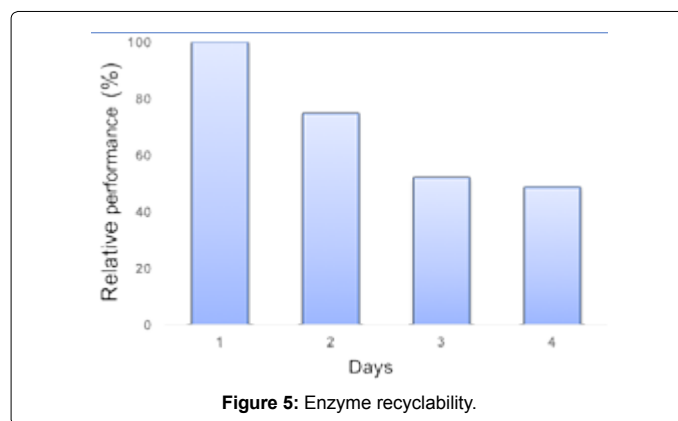**Figure 4:** Relative performance of free and immobilized enzymes.

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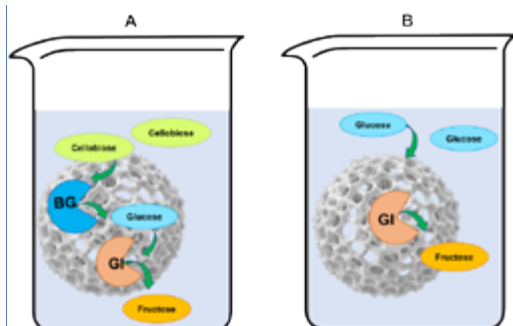
Figure 6: Schematic of channelling effect in the dual enzyme system: A. Glucose proximal access to the enzymatic site *vs.* B. Bulk diffusion to the enzymatic site.
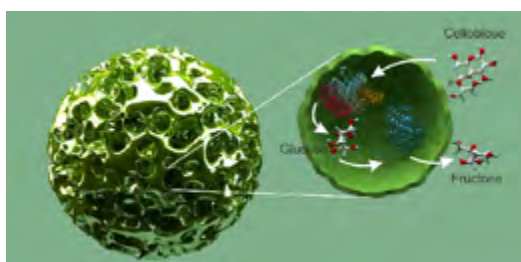

Figure 7: Schematic representation of the cascade reaction occurring in the Stellate MSN pore.

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.

Acknowledgements

References

1. Bhosale SH, Rao MB, Deshpande VV (1996) Molecular and industrial aspects of glucose isomerase. Microbiol Rev 60: 280-300.

2. Woodward, JKoran Jr LJ, Hernandez LJ, Stephan LM (1993) Use of Immobilized β-Glucosidase in the Hydrolysis of Cellulose, in β-Glucosidases. Am Chem Soc pp: 240-250.

3. Woodward J, Bales JC (1989) The Adsorption of Trichoderma reesei C30 Cellulase Protein to Protein Adsorbents: Application to Cellulase Recovery and Reuse. pp: 87-101.

4. Sternberg D, Mandels GR (1980) Regulation of the cellulolytic system in Trichoderma reesei by sophorose: induction of cellulase and repression of beta-glucosidase. J Bacteriol 144: 1197-1199.

5. Datta S, Christena LR, Rajaram YR (2013) Enzyme immobilization: an overview on techniques and support materials. 3 Biotech 3: 1-9.

6. Yiu HH, Wright PA, Botting NP (2001) Enzyme immobilisation using siliceous mesoporous molecular sieves. Microporous Mesoporous Mater 44: 763-768.

7. Peng WH, Lee YY, Wu C, Wu KC (2012) Acid–base bi-functionalized, large-pored mesoporous silica nanoparticles for cooperative catalysis of one-pot cellulose-to-HMF conversion. J Mater Chem 22: 23181-23185.

8. Lee YC, Chen CT, Chiu YT, Wu KC (2013) An Effective Cellulose-to-Glucose-to-Fructose Conversion Sequence by Using Enzyme Immobilized Fe3O4-Loaded Mesoporous Silica Nanoparticles as Recyclable Biocatalysts. Chem Cat Chem 5: 2153-2157.

9. Ispas C, Sokolov I, Andreescu S (2009) Enzyme-functionalized mesoporous silica for bioanalytical applications. Anal Bioanal Chem 393: 543-554.

10. Hartmann M, Kostrov X (2013) Immobilization of enzymes on porous silicas-benefits and challenges. Chem Soc Rev 42: 6277-6289.

11. Dutta S, Wu KC (2014) Enzymatic breakdown of biomass: enzyme active sites, immobilization, and biofuel production. Green Chem 16: 4615-4626.

12. Chang RH, Jang J, Wu KC (2011) Cellulase immobilized mesoporous silica nanocatalysts for efficient cellulose-to-glucose conversion. Green Chem 13: 2844-2850.

13. Storey KB, Chakrabarti AC (1990) One-step conversion of cellulose to fructose using coimmobilized cellulase, β-glucosidase, and glucose isomerase. Appl Biochem Biotechnol 23: 139-154.

14. Betancor L, Luckarift HR (2010) Co-immobilized coupled enzyme systems in biotechnology. Biotechnol Genetic Eng Rev 27: 95-114.

15. Zhang K, Xu LL, Jiang JG, Calin N, Lam KF, et al. (2013) Facile Large-Scale Synthesis of Monodisperse Mesoporous Silica Nanospheres with Tunable Pore Structure. J Am Chem Soc 135: 2427-2430.

16. Radu DR, Pizzi NA, Lai CY (2016) Functionalized stellate macroporous silica nanospheres for CO_2 mitigation. J Mater Sci 51: 10632-10640.

17. Sriprapundh D, Vieille C, Zeikus JG (2003) Directed evolution of Thermotoga neapolitana xylose isomerase: high activity on glucose at low temperature and low pH. Protein Eng Design Selec 16: 683-690.

18. Tran DN, Balkus Jr KJ (2011) Perspective of Recent Progress in Immobilization of Enzymes. ACS Catal 1: 956-968.