Immobilization of Enzymes in Sol-Gel Mesoporous Silica, Enzymatic Digestion of Biomass, and Silica-Curcumin Hybrid Materials

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Dedications

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Abstract

Immobilization of Enzymes in Sol-Gel Mesoporous Silica, Enzymatic Digestion of Biomass and Silica-Curcumin Hybrid Materials

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This research describes the immobilization of enzymes by direct encapsulation in porous silica matrix via a nonsurfactant templated sol-gel route pioneered by our group. Nonsurfactant molecules like D-fructose have been used as a template or pore forming agent, to generate silica host materials with pores in the mesoporous range (i.e., 2 to 50 nm). Our research has demonstrated that the activities of the immobilized enzymes were largely dependent on the textural properties of the silica matrix, including the specific surface area, pore volume and pore size. The immobilized enzymes exhibited up to ~80% activity, compared to free native enzyme. The immobilized enzyme samples also enabled easy recovery and subsequent reusability of the enzymes. Since the spatial confinement in nanopores, the encapsulated enzymes exhibited better environmental stability. The stabilization of enzymes in high pH commercial detergent, upon encapsulation in nonsurfactant templated sol-gel silica materials, has also been exploited in this work.
In this work, we have successfully demonstrated the recyclable application of our immobilized enzyme catalyst system in biomass hydrolysis. Hydrolysis efficiencies up to 81% were obtained, and subsequent reuse exhibited negligible loss in the hydrolytic activity of our immobilized enzyme catalyst.

This research also describes the synthesis and characterization of a new class of curcumin-silica organic-inorganic hybrid materials. This work presents the preparation of the hybrid materials via covalent incorporation of the organic curcumin phase into the pre-hydrolyzed sol-gel silicate phase. The degree of incorporation of curcumin phase was verified by thermogravometric analysis (TGA). Materials characterization was done using FTIR, DSC and fluorescence spectroscopic techniques. This new class of inorganic-organic silica hybrid materials may find wide applications in implant and other biomedical materials with reduced inflammatory properties.
Chapter 1: Introduction to sol-gel materials, enzyme immobilization in sol-gel mesoporous silica, enzymatic hydrolysis of biomass and silica hybrids

1.1. Organization of this dissertation

This dissertation contains six chapters and an appendix. Chapter 1 is designed to provide a background of various synthetic concepts and analytical techniques applied in this research. For the readers who are well versed in sol-gel and enzyme chemistry as well as related characterization techniques, you may skip this chapter. Original research results in this doctoral study are described in Chapter 2 through Chapter 6. Since we intend to publish each chapter as an independent article, there will be some minor overlaps and repetitions in these chapters.

Chapter 2 describes immobilization of cellobiase enzyme in non-surfactant templated mesoporous silica. In this chapter, the development of the in-situ immobilization method and various parameters that affect the immobilized enzyme system are described in detail. Chapter 3 presents the enzymatic hydrolysis of cellulose, with the aid of immobilized cellobiase enzyme. In Chapter 4, immobilization of lipase enzyme in non-surfactant templated mesoporous silica is presented. The method used is similar to that in Chapter 2. This work was an attempt to generalize the immobilization method that has been described in Chapter 2. Chapter 5 describes the synthesis and characterization of a novel silica-curcumin inorganic-organic hybrid material with potential applications as an anti-oxidant, anti-inflammatory implant and a biomedical material. Chapter 6 discusses the protection of savinase enzyme against high pH media in a commercial detergent system.
Appendix A is an original research proposal entitled “Flux and fouling resistance improvement in thin film composite (TFC) polyamide reverse osmosis membranes”. The proposal was presented and defended successfully on November 5th, 2008 as a partial fulfillment of the requirement for a Ph.D. degree in the Chemistry Department, Drexel University.

1.2. History and motivation

Materials have had a very profound influence on the history of humankind since the early ages. The ability to access and use advanced materials and technologies has deeply affected the successes of human civilizations either during peace times or during wars, natural disasters and climate changes. The evolution from the stone age to the modern silicon, nanotechnology and plastic age is a result of humankind’s relentless pursuit of advanced materials that would simplify and improve everyday living. Indeed, materials have been defined as “substances that have properties which make them useful in machinery, structures, devices and products” [1].

Since ancient times, humans designed materials based on their understanding of fundamental chemistry. The Mesopotamians developed lime mortars by calcining limestone [2]. Indian iron’s excellent resistance to atmospheric corrosion, as exhibited by the “Kutub Minar” iron pillars and the “Konarak” iron beams, has been well documented [3-5]. Such examples of science and technology of designing and developing new materials exemplifies what is known as materials chemistry.

The development of synthetic polymers in the early 1800’s was a major step taken by chemistry in the development of new materials. The fundamental understandings of
the chemistry of polymers or macromolecules led to the development of *thermoset* and *thermoplastic* polymeric materials. Wallace Carothers applied the well-known chemistry of condensation of small organic molecules (i.e., monomers) to generate the first mass produced synthetic polymer, Nylon-66™.

In the present age, materials chemistry is widely used in development of materials for medical purposes. The synthetic polymeric materials could replace malfunctioning human hearts, as resulted in extensive research in biocompatible materials. Additionally, Ultra High Molecular Weight Polyethylene, a grade of the very commonly used polymer polyethylene (a popular plastic), which has a very high molecular weight, is common in medical devices. Silicone is a polymer that is widely used as a medical implant material. It is actually poly(dimethyl siloxane), a polymer with inorganic backbone having organic side chains.

Glass is perhaps the most ancient and widely used inorganic materials known to man. Likewise, silica (SiO₂) and alumina (Al₂O₃) are two very common metal oxide ceramic materials in daily lives. The chemical inertness and rigid structure of these materials have promoted their extensive usage over a very long period of time. In more recent times, extensive research in ceramic materials have led to the development of biocompatible ceramic materials, which are fit to replace human bones [6]. Bioglass® [7] is a kind of newly developed ceramic materials, which has the ability to bond with bones and tissues. The silica, alumina and zirconia (ZrO₂) ceramic materials are now being extensively used in various applications, which include corrosion protection [8] and chromatographic applications. The porosity of the metal oxide ceramic materials also
allows wide applications of these materials in enzyme immobilization [9-12] and catalysis [13].

The following sections will provide a brief background on the different aspects of materials chemistry that this dissertation addresses. These include: sol-gel chemistry, mesoporous materials, enzyme immobilization, and organic-inorganic hybrid materials as well as the related characterization techniques for these materials. A brief introduction of enzymatic hydrolysis of biomass will also be given in the following sections.

1.3. General methodologies and concepts

1.3.1. The sol-gel chemistry

Sol-gel chemistry is a process of transformation of liquid (colloidal) sol phase to a solid gel phase. The process typically involves sequential hydrolysis and polycondensation of a metal alkoxide precursor to yield a metal oxide material [14, 15]. The hydrolysis and condensation of the metal alkoxide leads to the formation of a colloidal phase, consisting of relatively higher molecular weight polymeric intermediates. This is what is known as the sol phase. These intermediates undergo further polycondensation reactions, which lead to the formation of a crosslinked macroscopic three dimensional gel material. The role of the sol-gel method in inorganic ceramic and glass materials has been studied since the mid-1800’s. While studying silica gels, Ebelman and Graham observed that tetraethyl orthosilicate, Si(OC2H5)4, underwent hydrolysis in acidic conditions to form SiO2, in a glass-like form [16]. The method enabled a significantly low temperature processing of glasses and ceramics [17].
In the first step of the sol-gel process, a metal alkoxide precursor, represented as [M(OR)\(_x\)], where M is a network forming element (typically Si, Ti, Zr, Al, B) and R is an alkyl group (typically CH\(_3\) and C\(_2\)H\(_5\)), undergoes hydrolysis to form M – OH groups. The hydrolyzed precursor undergoes condensation to form a metal oxide network (M–O–M). Metal alkoxide silica precursors like tetraethyl orthosilicate (TEOS) and tetramethyl orthosilicate (TMOS) are most commonly used. Non-siliceous metal oxide precursors like tetraisopropoxy zirconium and tetrabutoxy titanium are also widely used. The hydrolysis step of the silica alkoxide is usually either acid catalyzed or base catalyzed. The sol-gel hydrolysis of non-silicate alkoxides proceeds without any catalyst, owing to their high reactivity. Scheme 1.1 shows the sol-gel synthesis of SiO\(_2\) from alkoxy silane.
Scheme 1.1. Hydrolysis and condensation of alkoxy precursors [18].

In the hydrolysis step, the alkoxy silane is hydrolyzed to form silanol and alcohol (byproduct). The condensation step proceeds via condensation between two silanol molecules, or between a silanol and an unreacted alkoxy silane, to form the Si–O–Si network as illustrated in Scheme 1.1. The hydrolysis and condensation of the precursor has been reported to proceed simultaneously after the initiation of hydrolysis [19]. Finally, through extensive crosslinking, the molecular weight of the network becomes large enough or approaches practically infinite to form a gel.
The rate of hydrolysis of the alkoxy silane is high at acidic as well as basic pH [15, 20], as shown in Figure 1.1. Under both reaction conditions, hydrolysis proceeds via nucleophilic substitution reactions [21]. In acid catalyzed reaction, entangled linear or randomly branched chains are generated in the silicate sol [15]. Upon gelation (i.e., when the reaction system lost its fluidity), silicate gel or eventually silica monolith is produced. Base catalyzed hydrolysis results in the formation of a network consisting of discrete, highly branched clusters [22]. At basic pH, colloidal silica or weakly crosslinked clusters are formed. The different morphologies of the silica obtained from acid and base catalyzed reactions are shown in Figure 1.2.

The metal alkoxide precursors provide a very efficient and advantageous route to sol-gel synthesis due to the following factors:

i) The reactions proceed under very mild conditions of temperature.

ii) The byproducts (alcohol and water) formed in this route can be easily separated by simple vacuum extraction.

iii) Different morphologies of silica can be achieved by altering the catalyst during synthesis.

iv) A wide range of metal alkoxides is commercially available.

Apart from metal alkoxide precursors, various inorganic metal salt precursors are also used in the sol-gel process. Sodium silicate (Na$_2$SiO$_3$) and potassium silicate (K$_2$SiO$_3$) are two very widely used metal salt precursors used for preparing porous silica gels. Scheme 1.2 illustrates the hydrolysis and condensation sodium silicate precursor to
form SiO₂ network. However, during the synthesis, byproducts like NaCl or KCl are formed, which are difficult to remove after from the reaction [23]. This severely affects the purity of the SiO₂ formed.

Scheme 1.2. Hydrolysis and condensation of metal salt precursors in sol-gel process [18].

1.3.2. Mesoporous Materials

Porous materials have been the focus of extensive research, in regards to their applications as adsorbents, catalysts, as well as catalyst support materials. Based on their pore sizes, the IUPAC has divided the porous materials into three distinct classes [24, 25]: (1) Microporous materials (pore diameter < 2 nm); (2) Mesoporous materials (pore diameter 2-50 nm) and (3) Macroporous materials (pore diameter > 50 nm).

In microporous materials, zeolites are the most well known and extensively studied [24, 26]. Owing to their crystallographically defined pore system, zeolites are known to have a narrow pore size distribution, and provide excellent catalytic and adsorbent properties [26]. However, due to the smaller pore size, their application is
severely restricted in processes that involves large reactant molecules [24]. Hence, extensive research is being done to generate mesoporous materials, which would minimize the restricted accessibility of the pores to the larger molecules.

The synthesis of the first mesoporous material dates back to 1969, but its immense potential was not recognized due to lack of analysis [27]. In 1992, scientists at Mobil Oil Corporation developed MCM-41 (Mobil Composition of Matter No. 41), a silicate mesoporous material that exhibited an ordered pore structure and a very narrow pore-size distribution [24, 26, 28]. Since then, there has been a tremendous amount of research in generating new mesoporous materials based on templating mechanism.

1.3.2.1. Synthesis of mesoporous materials by surfactant templated method

In this method, cationic surfactants like cetyltrimethylammonium bromide (CTAB), anionic surfactants like sodiumdodecylsulfate (SDS), nonionic surfactants like polyethylene oxide (PEG) and block copolymers like Pluronic F-127 are used as template or pore-forming material.

Due to electrostatic and hydrophobic interactions, ionic surfactants undergo aggregation to form micelles. The hydrolysis and condensation of precursors around the regularly aligned micelle assembly leads to the entrapment of surfactant within the silica network. The post synthesis removal of the surfactant from within the silica network leaves large voids or pores within the silica network. The process is called liquid crystal templating (LCT) method, and the pore size of the material could be controlled by varying the alkyl chain length of the surfactant used [24, 29]. Figure 1.3 shows a pictorial illustration of surfactant templated pathway for formation of ordered mesoporous
materials. Highly ordered porous structures such as MCM-41 (hexagonal), SBA-15 (hexagonal), SBA-1 (cubic) and MCM-48 (cubic) could be prepared, depending on the shape of the supramolecular template (ordered micelle) formed [29]. For nonionic surfactant templating, the formation of the ordered template shapes or mesophases is mostly driven by weak hydrogen bonding [30], rather than electrostatic interactions. The nonionic surfactant templating method has shown to generate rather worm-like and disordered pore system, than long-range ordered arrays as generated by ionic surfactant templating [24].

The removal of template is a critical issue in the surfactant templated method for mesoporous material synthesis. In the ionic surfactant method, strong electrostatic interactions lead to the formation of the aligned micelle assembly. Hence, surfactant removal requires high temperature calcination. However, in nonionic surfactant templating method, weak hydrogen bonds form the mesophases. Hence, surfactant removal is possible by simple extraction with ethanol or acidified water [31, 32].

1.3.2.2. Non-surfactant templated synthesis of mesoporous materials

As discussed in the previous section, surfactant removal is a major issue in surfactant templated mesoporous material synthesis. The high temperature calcination, as required in ionic surfactant method, leads to possible collapse of the pore structures [33-35]. In case of nonionic surfactant template, surfactant removal requires extraction with organic solvents like ethanol. Furthermore, the synthesis involving these templates often requires highly acidic conditions that are detrimental to bioactive substances such as
DNAs, RNAs, proteins, etc. The primary objective of using mesoporous materials in this dissertation is for in-situ encapsulation of enzymes within the pores of the material. However, high temperature treatment, solvent extraction and/or high acidity are both very detrimental to biological molecules (e.g., enzymes) entrapped within the pores. Hence, surfactant templated method for mesoporous materials is not a viable pathway for application of these materials in encapsulation of biological molecules. Besides, the toxicity of various ionic surfactants poses an environmental threat to the application of these materials [36, 37].

Our group has pioneered a non-surfactant templated route to synthesis of mesoporous materials [38, 39]. In this approach, the silica network was allowed to grow around non-surfactant organic molecules like D-glucose and D-maltose or their assemblies and aggregates. Hydrogen bonding between the aggregated templates led to the formation of mesophases in this route. The non-surfactant D-glucose and D-maltose templates were highly water soluble, and could be extracted out of the pores by simple washing with water, thus eliminating the need for high temperature calcination and organic solvent extraction. Besides, the availability of a very large array of organic molecules as template, and their low cost, biocompatibility and environmental friendliness compared to surfactants added to the versatility of the process.

The non-surfactant method usually generated mesoporous silica with disordered pore structure, but with good thermal stability [38], similar to the nonionic surfactant template generated materials, like MSU-1 [30, 40]. The non-surfactant templated method thus provided a very economical, environment friendly and highly biocompatible route to mesoporous material synthesis. In this dissertation, the non-surfactant templated route has
been very extensively used for generating mesoporous silica materials, which were applied in the direct (or in-situ or one-pot) process for immobilization of biological molecules.

1.3.3. Immobilization of enzymes

    Biocatalysts have a very deep impact on the global drive towards green, environment friendly methodologies for chemical manufacturing [41-43]. The use of biocatalysts in chemical processes promotes relatively milder reaction conditions as well as high chemo-, regio- and stero-selectivities [41]. Enzymes are a class of biocatalysts that promote high activity, selectivity and specificity in complex chemical reactions under mild experimental and environmental conditions [44-47]. Hence, engineering of enzymes to industrial reactors from their biological entities is a vast current field of research. Modern biotechnological developments have permitted the widespread application of these biocatalysts in industrial organic synthesis [48-57]. However, the industrial applications are largely plagued by long-term operational instability and most importantly, the difficulty in recovery and re-use of the enzymes in chemical reactions [41]. Immobilization of enzymes offers a considerable solution to these drawbacks [58-62].

    In terms of industrial usage, there are several beneficial factors for immobilizing enzymes. Apart from easy and convenient handling of enzyme preparations, the main targeted benefits for immobilizing enzyme are [59, 63]:

(1) Easy separation of the enzyme from the products in the reaction mixture

(2) Re-use of the enzyme in multiple batches of biocatalyzed reactions.
The ability to easily separate the enzyme from the reaction mixture minimizes protein contamination of the products [41], thus enhancing reliable and efficient reaction technology. Moreover, reusability of the enzyme actively provides cost advantages, which is an extremely important factor for the economic viability of the enzyme-catalyzed processes [59].

In addition to easy separation and reusability, another benefit of enzyme immobilization is the enhanced storage stability and often enhanced stability toward denaturation by heat, organic solvents and other harsh operational conditions [64]. Also, in multienzyme and chemoenzymatic processes, immobilization promotes compartmentalization of different enzymes, which minimizes enzyme inhibition (and also deactivation) due to mutual interactions [65, 66].

1.3.3.1. Types of immobilization

Enzyme immobilization is usually done through three general methodologies: binding or attaching the enzyme to a solid support material; crosslinking of enzyme aggregates using bifunctional reagents; entrapment or encapsulation of enzymes.

1.3.3.1.1. Attachment of enzyme to support

In this method, the enzyme is attached to a support material via ionic, covalent or physical adsorption bonding. Figure 1.4a shows a pictorial representation of the different modes of attachment. The type of support material used for attachment can be categorized into: (1) biopolymers, which are based on hydrophilic natural polysaccharides (e.g. cellulose); (2) synthetic lipophilic polymers (e.g. polyacrylamide, polystyrene); (3) Inorganic support materials (e.g. controlled pore glass).
Among the different modes of attachment, physical adsorption is often too weak to keep the enzyme binded to the support material [41]. The ionic and covalent bondings are strong enough to overcome detachment of the enzyme. However, these two types of attachments are highly dependant on the structure of the enzyme and the support material, and often require chemical manipulation of both. Besides, chemical bonding with enzymes often alters enzymatic activities [67].

1.3.3.1.2. Crosslinking of enzyme aggregates

In this method, enzyme molecules are binded together through intermolecular crosslinking, using bifunctional reagents. The enzymes are also crosslinked onto functional groups on a support. A common crosslinking agent that is used in this methodology is glutaraldehyde [68]. Similar to ionic or covalent attachment, the crosslinking method is also highly dependant on the chemical nature of the enzyme. Figure 1.4b provides a pictorial representation of the crosslinking of enzyme molecules.

1.3.3.1.3. Immobilization through entrapment of enzymes

This method for immobilization involves entrapment or encapsulation of the enzyme within the lattice, interstice or pores of a crosslinked polymer. Figure 1.4c shows a pictorial illustration of the method. Various synthetic polymers like polyvinylalcohol, polyacrylamide and polyurethane are used as the entrapping polymeric matrix. Among inorganic metal oxide networks, microporous sol-gel silica is widely used as the immobilization matrix.

Unlike attachment to support material, the enzyme is physically entrapped within the pores of the crosslinked polymeric material, without any primary bonding between
the enzyme and the matrix. Hence, the natural or native state of the enzyme is preserved in this method. Therefore, in terms of enzyme types, this is a highly versatile method, as it allows immobilization of virtually any kind of enzyme, independent of its chemical nature [69]. Figure 1.4c provides a pictorial illustration of immobilization through entrapment.

This dissertation deals extensively with the immobilization of enzymes through entrapment in mesoporous sol-gel silica materials. A more detailed discussion of the enzyme entrapment in sol-gel materials is presented in the introduction section of Chapter 2 of this dissertation.

1.3.4. Enzymatic hydrolysis of biomass

Over the last century, there has been a steady increase in energy consumption owing to the growing world population and industrialization. In fulfilling this growing demand, crude oil has been the primary source of energy [70]. However, due to this large-scale consumption, a decline in the crude oil reserves and production has already begun [71]. Hence, to meet the ever-growing energy demands, extensive research is being done worldwide to explore alternative energy resources. In the United States, ethanol has been used as an alternative fuel resource since the 1980’s [70]. Currently, the total consumption of ethanol in the US transportation sector accounts for almost 1% of the total gasoline consumption [72].

Ethanol is a renewable energy source, which is typically generated from the fermentation of saccharides. Currently in USA, ethanol is produced mostly using
cornstarch as the saccharide source. However, the corn production for ethanol generation competes directly with corn production for food and feed. Hence, the current corn-starch technology may not be the most practical approach [70]. Lignocellulosic materials, which include crop residues, wood chips, saw dust and solid animal waste, pose a potential economic ethanol production source [70, 73, 74]. In the last couple of decades, extensive research has been done to study the production of ethanol from lignocellulosic materials [75-81].

Lignocellulosic biomass is plant matter, which consists of cellulose, hemicellulose and lignin. Cellulose is a linear polysaccharide, with two D-glucose units linked through a $\beta$-1, 4-glycosidic linkage, as the repeating unit. The structure of cellulose is shown in Scheme 1.3. Two D-glucose units linked by the $\beta$-1, 4-glycosidic linkage is what is known as cellobiose. Hence, essentially, cellulose is a polysaccharide consisting of cellobiose repeating units.

Scheme 1.3. The chemical structure of cellulose.

In biomass, the cellulose and hemicellulose are tightly attached to lignin by hydrogen and covalent binding. The cellulose itself is highly crystalline, owing to strong
intermolecular and intramolecular hydrogen bonding. This high crystallinity, along with the tight binding to the lignin and hemicelluloses makes cellulose insoluble [82].

The production of ethanol from lignocellulosic biomass is a two step process [70]. Firstly, the cellulose in the biomass is hydrolyzed to fermentable reducing sugars. In the second step, ethanol is produced by fermentation of the sugars by yeast or bacteria. The hydrolysis step is usually an enzymatic process, catalyzed by cellulase enzymes. Scheme 1.4 demonstrates the enzymatic hydrolysis of cellulose.

Scheme 1.4. Hydrolysis of cellulose by cellulase enzymes [83].

The presence of lignin and hemicellulose is a very important factor in the enzymatic hydrolysis of cellulose [84]. These materials severely hinder the accessibility
of the cellulose to the enzymes. Hence, pretreatment of the lignocellulosic biomass is essential to remove the lignin and hemicellulose [70, 84].

In the enzymatic hydrolysis of lignocellulosic materials, enzyme cost is a critical issue [70, 85, 86]. Enzyme recovery and re-use of the enzymes are very critical for lowering the enzyme costs. In this dissertation, the application of a newly designed novel immobilized enzyme system in the biomass hydrolysis has been demonstrated.

1.3.5. Inorganic-organic hybrid materials

Inorganic materials are quite different to organic polymeric materials, in terms of material properties. The combination of inorganic materials with organic materials in molecular level results in inorganic-organic hybrid materials. The hybrid materials exhibit organic material properties combined with properties of inorganic materials, like inertness, hardness and heat resistance.

The sol-gel method is a very efficient method for the synthesis of inorganic-organic hybrid materials, as it involves very mild reaction conditions and is compatible with a wide variety of solvents [87]. The mild processing temperatures and wide solvent compatibility enables the incorporation of a wide range organic phases within the inorganic network via the sol-gel method [19, 87, 88]. A detailed discussion on sol-gel synthesis of inorganic-organic hybrid materials is provided in Chapter 5 of this dissertation.
1.4. Analytical characterization techniques

1.4.1. Thermogravimetric analysis (TGA)

Thermogravimetric analysis or TGA is a technique in which the weight loss of a sample is studied with increasing temperature. It is done by placing the sample in a weighing balance and heating the sample at a controlled rate. The weight loss is studied in various ambient atmospheres like nitrogen or air. It is a highly sensitive technique, which provides information about the weight percentage of moisture, volatiles, solvent, organic /inorganic contents, etc in a samples. TGA is widely used for studying thermal and thermo-oxidative degradation of high temperature polymers [89]. TGA is also a very effective tool in studying the weight percentage of templates in mesoporous material synthesis [90].

1.4.2. Differential scanning calorimetry (DSC)

Differential Scanning Calorimetry or DSC is a technique in which a sample is subject to controlled heating, and the heat flow in and out the sample is measured. The sample is taken in an aluminum or platinum pan, and another identical inert pan is used as a reference. Figure 1.5 shows a pictorial illustration of the DSC sample placement. Only the heat flow for the sample is measured, by maintaining sample and the reference at the same temperature throughout the entire run. Through this technique, different phase transition in a material is studied. These transitions give rise to endothermic or exothermic peak over a temperature range in a DSC scan. A typical DSC curve for a polymeric material is illustrated in Figure 1.7. DSC is a very widely used technique for
material characterization in terms of glass transition temperature ($T_g$), crystallization, melting and curing temperatures for the sample.

1.4.3. Gas adsorption-desorption

Gas adsorption is a very widely used technique for evaluating the surface area, pore size, pore volume and pore size distribution of porous solid materials [91]. In this technique, the amount of gas adsorbed by a solid is measured, which in turn is directly related to the porous properties and pore structure of the material [92]. Depending on the nature of the adsorbent solid and information required, various absorptive gases like Nitrogen ($\text{N}_2$), Carbon dioxide ($\text{CO}_2$) and Argon ($\text{Ar}$) are widely used in this technique. The volume of gas adsorbed by the solid is measured with over a wide range of relative pressures, and plot of the volume adsorbed with varying relative pressure ($p/p_o$) is called the adsorption isotherm. $\text{N}_2$ adsorption isotherm at sub-atmospheric pressures and $-196\degree\text{C}$ is routinely used for determining pore information and pore size distributions in microporous, mesoporous and macroporous range [92].

Surface area is determined from the Brauner-Emmett-Teller (BET) equation [25, 93] (Eq. 1), based on monolayer adsorption of gases.

$$\frac{p}{n^a(p^o - p)} = \frac{1}{n^aC} + \frac{(C - 1)}{n^aC} \frac{p}{p^o} \quad \text{(Eq. 1)}$$

$n^a$ is the amount of gas adsorbed at relative pressure $p/p^o$, $n^a_m$ is the monolayer capacity and $C$ is the BET constant.

The physisorption isotherms have been classified into 6 distinct types by IUPAC [25]:
Type I reversible isotherms exhibited by microporous solids; Type II reversible isotherms exhibited by nonporous and macroporous solids; Type III isotherm, which is rarely seen; Type IV isotherm with hysteresis loop, exhibited by mesoporous solids; Type V isotherm which is rarely seen and arises due to weak adsorbent-adsorbate interaction; Type VI isotherm arising from stepwise multilayer adsorption on a uniform nonporous solid. The different isotherms are shown in Figure 1.7. The hysteresis loop exhibited in the type IV isotherm is classified into 4 distinct types: H1, usually exhibited by pores with regular shape and narrow pore size distribution; H2, usually associated with pores with narrow necks and wide bodies; H3, usually associated with plate-like particles; H4, usually associated with slit-like pores [25]. The shapes of the different hysteresis loops are shown in Figure 1.8.

In summary, this chapter has provided the basic concepts and background on sol-gel chemistry, mesoporous materials, enzyme immobilization, enzymatic hydrolysis of biomass, and organic-inorganic hybrid materials, as well as the related characterization techniques for these materials. The challenges remaining and the motivations for this thesis work have also been described.
Figure 1.1. Variation of the hydrolysis rate of alkoxy silane’s with pH [15].
Figure 1.2. Morphology of SiO$_2$ gel with acid and base catalyzed sol-gel reactions [22].
Figure 1.3. Surfactant templated method for the formation of ordered mesoporous silica material [94].
Figure 1.4. Immobilization methods for enzymes [18].
Figure 1.5. Sample placement inside a DSC sample cell [18].
Figure 1.6. A typical DSC curve for polymeric materials [95].
Figure 1.7. Physisorption isotherms classified by IUPAC [25].
Figure 1.8. Classification of hysteresis loops [25].
1.5. References


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Chapter 2: Immobilization of cellobiase enzyme in nonsurfactant templated mesoporous silica, effect of pore size and porosity on the enzymatic activity and reusability of the immobilized enzyme system.

2.1. Introduction

The field of enzyme immobilization has attracted a great deal of research efforts, as it offers immense technological potential [1-4]. Enzyme immobilization is a key step for the wide range of applications of these natural biocatalysts, because it enables easy separation and recovery of the enzyme catalyst from the reaction mixture [5]. Utilization of solid supports has been the most exploited approach for the immobilization [6]. Such an immobilization of enzymes is often achieved by the fixation of the enzyme to or within the solid support leading to heterogeneous enzymatic catalytic systems. This heterogeneity of the immobilized enzyme system is very important with respect to easy recovery of the enzyme and products, reutilization of enzymes, continuous operation of enzymatic processes, as well as versatility in bioreactor designs [7].

For enzyme immobilization in solid support materials, the most widely used techniques include: adsorption of enzyme on the solid surface; covalent anchorage of the enzyme to the support material; electrostatic binding; and enzyme entrapment within inorganic or organic inert matrices [8-14].

Physical adsorption of enzyme is an easy technique. However, the process involves a weak bonding of the enzyme to the support, which often leads to enzyme leaching [11, 15-17]. Covalent bonding of the enzyme to the solid support offers a very
strong binding and leads to better stabilization of the enzyme on the support [10, 18]. But this technique often involves the chemical manipulation of the enzyme, which may adversely alter the enzymatic activities [1, 6]. In this respect, the immobilization technique using enzyme-support electrostatic interactions provide high enzyme stability and also lead to easy regeneration of the support material [11, 19, 20]. However, a critical consideration in this technique is the operating pH conditions [8]. The operating environment should have a pH value that must be compatible with the isoelectric point and activity of the enzyme.

Utilization of various polymeric materials has been reported as solid support for enzyme immobilization [7, 21, 22]. As examples, poly(vinyl alcohol) nanofibrous membranes have been utilized for immobilizing lipase enzyme [6]. Crosslinked gelatin particles have also been used for surface immobilization and for entrapment of enzymes [23].

In addition to the above-mentioned methods, enzyme immobilization by entrapment in silica matrix via sol-gel process has been extensively studied in the recent years [1, 13, 24-32]. This method provides good compromise between the heterogeneous biocatalyst and the activity of the enzyme [8].

2.1.1. Immobilization of enzyme in sol-gel silica materials

Sol-gel silica materials are widely used in the encapsulation of enzymes and biomolecules. They are an attractive class of support materials for enzyme immobilization chiefly due to the following properties: (a) These materials are able to
entrap any desired amount of enzyme molecules in-situ; (b) Sol-gel silica materials are relatively inert and exhibit high chemical and thermal stability; (c) Sol-gel silica materials can be prepared via very mild reaction and processing conditions [28, 33].

2.1.1.1. Encapsulation of enzymes into the pores of pre-formed mesoporous silica materials

Mesoporous silica materials have been widely used as a host material for enzyme immobilization. Different porous silica materials with different pore sizes have been used to entrap the enzyme into the pores of the host material. In this process, enzyme solutions were allowed to diffuse through the pores of porous silica materials like MCM-41, SBA-15 and MCF mesocellular foam [9, 34-36]. After the diffusion, the pores were occluded by silanation to prevent the enzyme from leaching out [34]. The pore size of the host material, as well as the molecular size of the enzyme played a key role in allowing the diffusion of enzymes inside the pores. Materials with larger pore size, like SBA-15 (pore size between 5 to 13 nm) and MCF mesocellular foam (pore size between 15 to 40 nm) showed better adsorption of enzymes, compared to smaller pore sized materials like MCM-41 (pore size ~ 4 nm) [35]. However, in this method of enzyme entrapment into the pores of pre-formed porous materials, leaching of enzymes from the pores is a critical issue [37]. The silanation process of blocking pores to prevent enzyme leaching also lead to denaturation of enzymes inside the pores [34]. It should be noted that the diffusion of enzyme molecules into these preformed nano pores or channels could be so sterically hindered that the loading level and uniformity of enzymes in the materials might be undesirable.
2.1.1.2. Immobilization via direct encapsulation of enzymes inside pores of sol-gel routed mesoporous silica

Immobilization of enzymes in silica xerogels has been reported, where, the enzyme was entrapped within the matrix of a forming sol-gel [28, 38]. In this method of immobilization, an enzyme solution was added to the precursor sol in the sol-gel reaction of metal alkoxides. The sol was then allowed to gel with the enzyme within the matrix [38]. The enzyme was thus retained within the pores that are formed during/after the gelation process. However, conventional sol-gel materials are microporous and the small pore diameter (< 15 Å) of these materials restricts the diffusion of substrate molecules to the enzyme inside the caged structure, which hinders the catalytic activity of the immobilized enzyme [26, 27].

Synthesis and development of nonsurfactant-templated mesoporous materials via sol-gel chemistry have been reported [39-41]. Our lab has developed the synthesis of mesoporous silica materials, where organic molecules are used as templates or pore forming agents. The various organic molecules used as templates included D-glucose, D-maltose, etc. After the synthesis, the extraction of the templates yielded mesoporous silica, which exhibited large pore volume, pore size and surface area [39, 40]. Since the process can be performed under biocompatible conditions, the direct (or in situ) immobilization of acid phosphatase (ACP) and alkaline phosphatase (ALP) enzymes in sol-gel mesoporous silica via the D-glucose templated route has been achieved by our group [1, 42].
The pore size of the sol-gel materials is a vital parameter in the direct encapsulation procedure. Since the enzyme is already within the pore channels after gelation, the pore channels needed to be wide enough just to allow the diffusion of substrate molecules to, and of product molecules away from, the enzyme caged within the matrix. The microporous materials with typical pore diameter < 1.5 nm usually severely restricted the diffusion of the substrate inside the pores, thus reducing the accessibility of the enzyme. But very large pore diameters, e.g., hydrogels, would lead to enzymes leaching out of the pore structures that defeat the purpose of immobilization. A pictorial representation of the phenomena is illustrated in Figure 2.1.

In this work, immobilization of cellobiase enzyme was done by the direct encapsulation of enzyme molecule in mesoporous silica via the sol-gel route, using D-fructose as a template or pore-forming agent. D-fructose was used as the non-surfactant template, as it is one of the most water-soluble sugars (compared to glucose and maltose), permitting easy template extraction. The enzyme, which was trapped inside the pore channels created by the template, could be accessed by substrates after template extraction. In the surfactant templated route to mesoporous materials, the synthesis often involves reagents and conditions that are detrimental to enzymes. In contrast, the D-fructose used here is very biocompatible with a lot of enzyme systems. Also, the removal of surfactant templates from mesoporous materials usually requires calcinations at high temperatures and other harsh solvent extractions, which are very lethal to enzyme systems. The D-fructose template used in this work is highly water soluble, and could be removed from the pores of the silica by simply washing in excess water or aqueous buffer systems.
A control over the pore size of the porous silica materials in the present work was achieved by adjusting the total template content during the synthesis. Variation in the concentration of pore forming agent or the template leads to silica materials with varying pore diameters. The pore volume and surface area of the silica materials could also be adjusted by varying the template content during the sol-gel synthesis.

2.1.2. Cellobiase enzyme

The enzyme immobilized in this work was cellobiase, which hydrolyzes various β-linked diglucosides and aryl β-glucosides. The immense importance of this enzyme lies in its role in the enzymatic hydrolysis of cellulose [43, 44]. Cellobiase (β-glucosidase), endo-1,4-β-gluconase and cellobiohydrolase comprise the enzyme system responsible for the breaking down of cellulose to glucose units [44]. Cellulose is a linear polymer consisting of D-glucose repeating units, which are linked via 1,4-β-D-glucosidic bonds. Cellobiase enzyme catalyzes the final step, involving the breaking down of cellobiose units to glucose units, during the hydrolysis of cellulose. Cellobiase acts in conjunction with cellulolytic enzymes, to improve the rate and extent of saccharification, and is the rate limiting factor for the enzymatic hydrolysis of cellulose [44-47]. The immobilization of cellobiase enzyme has drawn immense interest as it leads to reutilization of the enzyme and reduced product inhibition [43]. However, the substrate for cellobiase enzyme, i.e., cellobiose, is water-soluble. Hence, immobilization of the enzyme in water insoluble support materials is critical for its easy separation and reusability that would significantly reduce the cost of production of glucose and eventually ethanol [44].
In this chapter, immobilization of cellobiase enzyme in D-fructose templated sol-gel routed mesoporous silica host materials is presented. Tetraethyl orthosilicate (TEOS) was used as the silica precursor, and D-fructose as the nonsurfactant template. The activity of the immobilized enzyme was studied with varying pore size, surface area and pore volume of the mesoporous host materials.

2.2. Experimental section

2.2.1. Materials

Tetraethyl orthosilicate (TEOS, 98%), D-(−)-fructose (98%) and sodium acetate-trihydrate (99%) were obtained from Sigma Aldrich. The cellobiase enzyme (from Aspergillus niger) used was Novozym 188, a β-glucosidase obtained from Sigma Aldrich. The substrate cellobiose for the enzyme assay was also obtained from Sigma Aldrich. Spectrophotometric glucose assay reagents were obtained from Wako Autokit Glucose (cat # 439-90901) sold by Wako Pure Chemicals. All reagents were used as received without any further purification.

2.2.2. Synthesis of immobilized cellobiase sample

The immobilization of cellobiase was done via the acid catalyzed sol-gel reaction of TEOS with water (mol ratio of TEOS: H₂O: HCl = 1:2:0.005). D-fructose was used as the template in the synthesis. In a typical procedure for the preparation of 50 wt% fructose templated immobilized cellobiase sample, 9.0 g (0.5 mol) of distilled water was
mixed with 0.63 g 2M HCl in a two-necked 500-ml round-bottomed flask fitted with a thermometer and reflux apparatus. The mixture was stirred for 5 minutes at room temperature. To this mixture, 52 g of TEOS (0.25 mol) was added with slow stirring at room temperature.

After the addition, the reaction was allowed to stir at room temperature under nitrogen. The solution homogenized and cleared out in 10 minutes, with the reaction mixture temperature rising up to 35º C. The reaction mixture turned cloudy again for an instant, then finally became homogenous and clear, with the temperature increasing to 60º C. The reaction mixture was refluxed at 60º C under nitrogen for 1 hour. After the reflux, the reaction mixture was allowed to cool down to room temperature and high vacuum was applied to remove water and ethanol produced as a byproduct during the hydrolysis of TEOS. The reaction mixture was subject to high vacuum until it reached 50% of its original weight.

After the vacuum extraction, 30 g of 50% fructose solution (15 g of fructose dissolved in 15 g of distilled water) was added to the mixture with stirring. After a homogenous clear solution was obtained, the mixture was divided (by weight) equally into three 100 ml beakers. Each beaker contained 5 g of silica and 5 g of fructose. For each beaker, an enzyme solution of 625 µl of cellobiase in 5 ml 50 mM pH 5 sodium acetate buffers was prepared, and added to the sol-gel solutions under vigorous stirring at room temperature for 5 minutes.

After the enzyme solutions were added, the reaction mixtures were sealed with paraffin film and allowed to gel at 5º C. A light yellow colored transparent gel was
obtained. After gelling, 20-25 holes were made with a hypodermic syringe needle in the paraffin film and kept at 5º C for solvent evaporation. After 2 days, the samples were kept at 0º C under vacuum (using an oil vacuum pump), until no further weight loss was observed. Each reaction beaker contained 5 g silica, 5 g fructose and 625 µl of cellobiase enzyme. After the drying process, the transparent, light yellow colored somewhat hard, monolithic bio-glasses were grinded to 40-mesh size using a mortar and pestle. The fine white powder thus obtained was the enzyme containing mesoporous silica, which was stored at 5º C for further analysis. Three different samples were made with three different template concentrations, with identical enzyme content with respect to per gram silica in the sample. As control sample, cellobiase was immobilized under identical conditions without the addition of any fructose template.

2.2.3. Characterization

The template concentrations in the samples were determined from the weight loss using Thermogravimetric Analysis (TGA) on a TA Q50 Thermogravimetric Analyzer (TA Instruments Inc., New Castle, DE). The samples were preheated to 80º C and kept isothermal at 80º C for 45 minutes in N₂. During the actual thermal analysis, they were heated to 800º C at a heating rate of 10º C/min in air. Nitrogen adsorption-desorption measurements were done on a Quadrasorb SI Automated Surface Area and Pore Size Analyzer instrument at -196 ºC. The template and the enzyme were extracted from the samples by washing with large excess of distilled water. After extraction, the powder samples were dried at 40º C overnight, in a vacuum oven. The dried powders were
degassed at 100° C overnight, before analysis. Quantachrome QuadraWin software (Version 5.02) was used for calculating the pore size and surface area of the samples.

2.2.4. Procedures for assaying enzymatic activity

The enzymatic activity of immobilized cellobiase samples were determined by assaying with cellobiase substrate. A sodium acetate buffer solution was prepared according to Sigma Aldrich’s cellulase assay procedure. In this method, a 50 mM sodium acetate hexahydrate solution was prepared in distilled water, and the pH of the solution was adjusted to 5 by adding 1 M HCl solution. A 6.25 mg/ml solution of cellobiose in the sodium acetate buffer (50 mM, pH 5) was prepared and used as the substrate. Prior to doing the assays, the immobilized samples were washed in the pH 5 sodium acetate buffer, to extract the template. Appropriate amounts of the immobilized samples were taken in 15 ml falcon tubes, such that the amount of cellobiase enzyme present was 12.5 µl. In each tube, 5 ml of the sodium acetate buffer (50 mM, pH 5) was added; the tubes were sealed and agitated for 8 hours to extract the fructose template.

After washing, the supernatant liquid was poured out. To the tubes, different amounts of the substrate solution and buffer solutions were added, to have 0, 3.13, 6.25, 9.37, 12.5, 25.0, 50.0 and 62.5 mg/ml substrate concentrations. The tubes were sealed, incubated at 37° C and gently agitated for 2 hours. After 2 hours, the tubes were taken out of the water bath and kept over ice, and the solid particles were allowed to settle down at the bottom of the tube. The glucose content in the liquid phase was determined by the Wako Glucose Autokit method, which has been used by other researchers for the spectrophotometric determination of glucose yields after enzymatic hydrolysis of
cellulose [48-53], and expressed in terms of milligrams of glucose per milliliter of the solution (mg/ml). In this method, β-D-glucose is converted to hydrogen peroxide by β-D-glucose oxidase enzyme (GOD) enzyme. This peroxide is then treated with a chromogen dye (oxygen acceptor) in the presence of peroxidase, to generate a red pigment. This red quinoid pigment, which absorbs a 505 nm, is detected by using a spectrophotometer.

The ‘Autokit Glucose’ glucose determination kit from Wako included: Buffer solution (60 mmol/l phosphate buffer, pH 7.1); Color reagent (when reconstituted: 0.13 U/ml mutarotase, 9.0 U/ml GOD, 0.65 U/ml peroxidase and 0.5 mmol/l 4-amino antipyrene, 2.7 U/ml ascorbate oxidase); Standard solution I (200 mg glucose/dl) and Standard solution II (500 mg glucose/dl).

According to the procedure outlined in the kit, one bottle (150 ml) of the Buffer solution was added to one bottle of the Color reagent to prepare the Working solution. In a plastic (PMMA) semi-micro cuvette (path length of 1 cm), 1.5 ml of the Working solution was transferred, and its absorbance was recorded at 505 nm on a Perking-Elmer Lambda-35 UV/Visible Absorption Spectrometer. This was taken as the Blank absorbance ($A_{BL}$). To the cuvette, 10 µl of the supernatant from the hydrolyzed solution was added, and mixture was incubated at 37 °C for 5 minutes, during which, the color development occurred. The absorbance of this solution was measured at 505 nm. From this absorption, $A_{BL}$ was subtracted to get $A_{S}$ (absorption of sample). To quantify the glucose concentration, Standard solution I, and Standard solution II were used as standards. $A_{Std}$ (absorbance of the standard) was measured by subtracting the absorbance of the Working solution ($A_{BL}$) from the absorbance of the Working solution mixed with 10µl of the
Standard solution I/II and incubated at 37 °C. The glucose concentration of the sample was determined by the equation:

\[
\text{Glucose (g/l)} = A_s \times \frac{C_{\text{Std}}}{A_{\text{Std}}}
\]

where \( C_{\text{Std}} \) is the glucose concentration of the Standard solution I/II in g/l.

It must be noted here that all the activity assays were repeated 6–8 times, and the values reported are the mean values of the repetitions. The error bars in the activity diagrams represent the standard deviation from the mean value.

2.3. Results and discussion

2.3.1. Thermogravimetric analysis (TGA)

The fructose (template) content in the as-synthesized samples was determined by thermogravimetric analysis (TGA). The weight loss obtained after heating the samples in air at 10° C/ minute to 800° C are reported as TGA weight loss in Table 2.1. This weight loss observed is due mostly to the degradation of the fructose present in the samples. The TGA curves for all the as-synthesized samples are shown in Figure 2.2. Based on the residual mass, we can confirm that the original fructose contents in all the as-synthesized samples, calculated from the reaction stoichiometry, is in proportion to the TGA weight losses obtained. It is observed that the sample with 0% template (0%F) shows a weight loss of < 10%. This weight loss can be attributed to the presence of unreacted ethoxy groups in the silica [54], because of incomplete sol-gel reactions, as well as the weight loss due to the presence of water molecules tightly bound to the silica matrix [40].
weight loss of <10% for samples without template has also been reported elsewhere [1, 40].

2.3.2. Nitrogen adsorption-desorption

The Brunauer-Emmett-Teller (BET) surface area of the immobilized samples as well as their pore parameters as listed in Table 2.1, were determined from the nitrogen adsorption-desorption isotherms [55]. The nitrogen adsorption-desorption isotherms of the samples at -196 °C are shown in Figure 2.3. The control sample (0%F) with 0% fructose content, show a reversible type I isotherm [56], which are typically exhibited by xerogels having microporous structures [57]. In the 30%F sample, the adsorbed volume is much greater than that in 0%F sample. In the 50%F and 70%F samples, as the fructose content increases to 50 and 70 % by weight, respectively, the isotherms go to type IV isotherms with H2 hysteresis loops [56], which is a characteristic of mesoporous molecular sieves [41, 58, 59]. With increasing template content, the magnitude of the H2 hysteresis loop becomes bigger, shifting towards higher relative pressures (P/P₀). The pore size distributions and the pore volumes were calculated using the non-local density functional theory (NLDFT) method [60]. At 30% or higher fructose content, the surface area and the pore volume of the samples increase (Table 2.1), which suggests mesoporosity [40]. The pore size distribution is shown in Figure 2.4. The 0%F sample shows peak maxima at 15Å, which is microporous in nature. The pore size distribution peak maxima also indicates 30%F sample having pore diameter in 20-25 Å range, while 50%F and 70%F samples have pore diameters in 30-35 Å range, which are mesoporous
in nature. Increase in the template concentration from 0% to 70% also led to an increase in the surface area and pore volume (Table 1) of the immobilized samples. Clearly, increased template contents led to larger pore sizes, increased surface area and pore volumes. Hence, from the nitrogen adsorption-desorption, it is clear that the template (fructose) acted as a pore-forming agent.

2.3.3. Enzymatic activity of immobilized cellobiase

The enzymatic activity of the immobilized cellobiase samples with varying substrate concentrations are plotted in Figure 2.5, and their highest observed activities are listed in Table 2.1. The results demonstrate that the activities of the samples with higher template content (70%F and 50%F) are significantly higher than the one prepared without any template addition (0%F). The 30%F sample exhibits activity lower than 50%F and 70%F, but higher than the 0%F sample. When compared with the free enzyme, the enzymatic activity of 70%F and 50%F samples is about 80% of the free cellobiase enzyme, which is quite remarkably high for immobilized enzymes by other methods. The enzymatic activity of 30%F is ~ 22% and that of the 0F sample is ~ 8% of the free cellobiase enzyme.

The Michaelis-Menten plots for the four samples are shown in Figure 2.6 and the Michaelis-Menten kinetics was studied according to Eq. (1):

\[
\frac{1}{u_0} = \frac{K_m}{V_{max}} \cdot \frac{1}{[S]} + \frac{1}{V_{max}} \tag{1}
\]
where \([S]\) (mg/ml) is the substrate concentration, \(v_0\) (mg/ml.hr) is the initial rate of enzyme activity or the initial reaction rate at any given substrate concentration, \(V_{\text{max}}\) is the maximum reaction velocity or maximum rate of conversion and \(K_m\) is the substrate concentration at which the reaction velocity or the rate of conversion is half of \(V_{\text{max}}\) [61, 62]. The \(V_{\text{max}}\) and the \(K_m\) values of the immobilized samples and that of the free enzyme are listed in Table 2.1. The data clearly demonstrated that samples with higher template concentration have significantly higher \(V_{\text{max}}\) than the sample with no template. This observed activity and \(V_{\text{max}}\) enhancement could be attributed to the microstructure of silica matrix. The samples with high template concentration (70%F and 50%F) are mesoporous materials and have pore diameter in the range of 30-35 Å. The sample with 30% template (30%F) is also mesoporous, but the pore diameter is in the range of 20-25 Å. The 0%F sample (without any template) is microporous in nature, the pore diameter being ~ 15 Å.

The velocity of the enzymatic reaction and the enzymatic activity depend on the accessibility of the enzyme by the substrate. The rate of diffusion of the substrate to the enzyme inside the caged silica structure is the limiting factor in the accessibility of the enzyme. With increase in the template concentration, we can clearly see an increment in the surface area of the silica host. This is due to higher density of pores and channels (i.e., interconnected pores), which makes the materials more porous. With increased porosity and pore diameter, the rate of diffusion of the substrate is higher in the 70%F and 50%F samples, compared to 30%F and 0%F samples. In samples with small pore diameters, the substrate may not be able to even get inside the silica structure and access the enzyme inside. As a result, samples with higher template concentration exhibit significantly higher activities and \(V_{\text{max}}\).
Also, as listed in Table 2.1, the pore volume of the silica host materials increases with increase in the template content. As a result of this increased pore volume, the mobility of the substrate and also the accessibility of the enzyme to the substrate increase. The increased pore volumes, combined with increased porosity and pore diameters result in higher activities of samples with higher template content.

2.3.4. Leaching of enzyme from the silica matrix

The leaching of enzymes from the host materials is a critical issue in enzyme immobilization. For our immobilization method, we studied the enzyme leaching from the silica host materials by washing our immobilized samples in the sodium acetate buffer. It is assumed that any enzyme that leached out of the silica host material would be present in the washing liquid. The supernatant washing solution was assayed for detection of any leached enzyme. The wash solutions showed negligible enzyme activities. This suggested negligible enzyme leaching. This observation is in accordance with the fact that entrapped enzyme size (diameter of 51Å [63]) is much bigger than the pore diameters of the host silica matrix. Hence, the immobilized samples showed negligible enzyme leaching.

2.3.5. Recyclable use of immobilized cellobiase

As mentioned earlier, one of the most important targeted benefit and technological potential of enzyme immobilization is the easy separation of the enzyme
from the reaction mixture and reusability of the enzyme, leading to overall process cost reduction [1-4, 43, 64]. To demonstrate the reusability of our immobilized cellobiase system, the immobilized samples were run for enzymatic activity tests in multiple uses. After each run, the immobilized powdered samples were filtered out of the reaction mixture and were washed once with pH 4 sodium acetate buffer (50 mM, 5 ml) for 1 hour, and once with pH 5 sodium acetate buffer (50 mM, 5 ml) for 1 hour at room temperature, to remove any remaining substrate. New substrate and buffer (pH 5, 50 mM sodium acetate) was then added, and subsequently run for the next enzymatic activity assay.

Figure 2.7 shows the enzymatic activity of the immobilized samples in multiple uses. The results show that the enzymatic activities of the immobilized cellobiase samples are retained up to 9 uses with negligible loss in activity. Hence, it can be concluded that the enzyme is clearly immobilized inside the silica matrix, and not just adsorbed onto the silica surface, as it would have been washed away, if the enzyme were just adsorbed onto the surface of the silica host materials. It must be noted that the isoelectric point (pI) of cellobiase enzyme is 4.5 [65]. Since we have used pH 5 sodium acetate buffer to wash our samples before assay, any non-encapsulated, silica surface adsorbed enzyme should have been washed away with the washing solution. The retained activity of the immobilized samples also demonstrates the fact that there is very little or no leaching of the enzyme from inside the pores to the reaction mixture, which signifies very tight and efficient encapsulation of the cellobiase inside the silica host materials.
2.4. Conclusion

In this work, cellobiase enzyme was successfully immobilized in situ in silica materials via sol-gel processing with tetraethyl orthosilicate (TEOS) as the silica precursor. In this encapsulation method, D-fructose was used as the template or pore-forming agent during the sol-gel process to afford mesoporous silica host materials. By varying the template content in the silica host materials, different surface area, pore volume and pore diameters were obtained. These parameters defined the enzymatic activity of the immobilized cellobiase enzyme, because the immobilized enzyme activity is restricted by the rate of diffusion of the substrate to the enzyme inside the solid matrix. Hence, higher template content silica materials exhibited significantly higher enzymatic activity/ reaction rate of the immobilized cellobiase enzyme. Also, when used in multiple batches, the immobilized samples showed negligible loss of the enzyme activity, up to 9 recycles. This clearly demonstrated the fact that the cellobiase enzyme was definitely caged within the silica matrix, and not just adsorbed on the silica surface. The retained activity also demonstrates very efficient encapsulation, with negligible leaking of the enzyme from the silica matrix. The reusability of our immobilized enzyme system would significantly reduce the cost of the ethanol production from biomass hydrolysis.

2.5. Acknowledgments

A part of the enzymatic assay was done in collaboration with Dr. David Berke-Schlessel. We are indebted to Prof. Yury Gogotsi of the Department of Materials Science and Engineering, Drexel University, for letting us use his BET instrument. We also thank
Prof. Jun Xi and Prof. Heifeng (Frank) Ji of the Chemistry Department, Drexel University, for discussions and valuable suggestions.
Table 2.1. Template content, TGA weight loss, textural properties from nitrogen adsorption-desorption, enzymatic activity and Michaelis-Menten parameters of immobilized cellobiase samples.

<table>
<thead>
<tr>
<th>Sample name</th>
<th>Weight % template added&lt;sup&gt;a&lt;/sup&gt;</th>
<th>TGA weight loss (%)</th>
<th>BET Surface area (m²/g)</th>
<th>Pore diameter (Å)&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Pore volume (cm³/g)</th>
<th>Enzyme activity (mg/ml)</th>
<th>V&lt;sub&gt;max&lt;/sub&gt; (mg/ml/hr)</th>
<th>K&lt;sub&gt;m&lt;/sub&gt; (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Free Enzyme</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>12.96±0.95</td>
<td>9.88±0.42</td>
<td>13.75</td>
</tr>
<tr>
<td>0%F</td>
<td>0</td>
<td>&lt; 10</td>
<td>287</td>
<td>16</td>
<td>0.15</td>
<td>0.56±0.08</td>
<td>0.56±0.04</td>
<td>1.0</td>
</tr>
<tr>
<td>30%F</td>
<td>30</td>
<td>29</td>
<td>623</td>
<td>23</td>
<td>0.35</td>
<td>2.84±0.07</td>
<td>1.57±0.04</td>
<td>6.14</td>
</tr>
<tr>
<td>50%F</td>
<td>50</td>
<td>53</td>
<td>778</td>
<td>31</td>
<td>0.52</td>
<td>10.74±1.31</td>
<td>6.94±0.65</td>
<td>18.84</td>
</tr>
<tr>
<td>70%F</td>
<td>70</td>
<td>71</td>
<td>796</td>
<td>34</td>
<td>0.57</td>
<td>10.38±0.18</td>
<td>6.75±0.09</td>
<td>18.64</td>
</tr>
</tbody>
</table>

<sup>a</sup> Weight % template added was calculated by assuming complete conversion of TEOS to SiO₂.

<sup>b</sup> Obtained from maxima of pore size distribution plot.
Figure 2.1. Pictorial illustration of the effect of pore diameter on enzyme immobilization in sol-gel silica [66].
Figure 2.2. TGA curves for as synthesized immobilized cellobiase samples: 0%F containing 0 wt% fructose; 30%F containing 30 wt% fructose; 50%F containing 50 wt% fructose and 70%F containing 70 wt% fructose.
Figure 2.3. Nitrogen adsorption-desorption isotherms of the immobilized samples, after extraction of template.
Figure 2.4. Pore size distribution of the immobilized samples after template extraction.
Figure 2.5. Activity of free cellobiase and immobilized cellobiase samples after template extraction with distilled water. The substrate is cellobiose in 50mM pH 5 sodium acetate buffer.
Figure 2.6. Michaelis-Menten plots of the immobilized cellobiase samples. The x-axis represents the substrate concentration (mg/ml) and y-axis represents the initial reaction rate ($v_0$).
Figure 2.7. Activity of immobilized samples in recycled uses. All the tests were done in pH 5 sodium acetate buffer solution.
2.6. References


54. I. Mukherjee, A. Mylonakis, Y. Guo, S.P. Samuel, S. Li, R.Y. Wei, A. Kojtari, Y. Wei, *Effect of nonsurfactant template content on the particle size and surface


Chapter 3: Enzymatic hydrolysis of biomass with recyclable use of cellobiase enzyme immobilized in nonsurfactant templated sol-gel mesoporous silica.

3.1. Introduction

For the last 15 years or so, a great deal of research effort has been focused on the exploration of alternative energy sources [1, 2]. In the production of ethanol as an alternative fuel, cellulosic biomass has great potential as an abundant renewable energy source [3-5]. In the last two decades, extensive research has been conducted on the conversion of lignocellulosic materials to ethanol [1, 6-11], as it is the most abundant biomass available, which is capable of being converted to liquid fuel (ethanol) via enzymatic hydrolysis [12-14]. The method for converting lignocellulosic material to ethanol is mainly a two-step process [1, 13]. The first step involves the hydrolysis of the cellulose in the lignocellulosic materials to fermentable reducing sugars (e.g., glucose). The second step in the process ferments the sugar to ethanol.

The hydrolysis step is usually done by enzymatic saccharification/hydrolysis of cellulose, catalyzed by cellulase enzymes, while the fermentation of glucose to ethanol is usually carried out by yeasts or bacteria [1]. The enzymatic hydrolysis of cellulose in the biomass is affected by many factors, including the crystallinity of cellulose fibers and the lignin and hemicellulose contents [1, 15], which restrict the accessibility of the cellulose to the cellulase enzymes and lead to reduced efficiency of the process. The removal of lignin and hemicellulose as well as the reduction of cellulose crystallinity is achieved by pretreatment of the biomass [1, 5, 15, 16]. In this work, two different pre-treated
biomasses were investigated, i.e., pretreatment of biomass with 2.5% FeCl\textsubscript{3} solution and with 2.5% of 0.5M oxalic acid solution. These two pretreatments as studied in our laboratory have shown significantly higher hydrolytic efficiency with free cellobiase enzyme, when compared with non-pretreated biomass samples.

During the enzymatic hydrolysis, cellulose is broken down to monomeric or oligomeric sugars. This enzymatic hydrolysis is achieved by the application of highly specific cellulase enzymes [1]. The hydrolysis by the cellulase enzyme system primarily depends on three enzymes: β-1,4-endoglucanase, β-1,4-exoglucanase and cellobiase enzyme [17]. Cellulose is broken down to cellobiose units by the β-1,4-endoglucanase and β-1,4-exoglucanase, and then the cellobiase hydrolyzes the cellobiose units to produce glucose [1, 3, 5]. The hydrolysis of cellulose by cellulase enzymes is depicted in Scheme 1.4 [18].

However, the cost of enzymes is one of the major hurdles in the path of large-scale commercialization of the enzymatic hydrolysis of cellulose [2, 13, 19], and it accounts for as much as 60% of the total process cost [16, 20]. Recovery of enzymes by recyclization is one of the most important and effective ways of increasing the efficiency of the enzymatic hydrolysis process by lowering the enzyme cost [14, 19, 21-23]. Enzyme immobilization offers immense technological potential in this field as it extensively promotes enzyme reuse and overall process cost reduction [5, 14, 24-26]. Hence, considerable amount of research has been performed to optimize the enzymatic hydrolysis of biomass via immobilized cellulases [5, 27].
In the present work, the primary objective was the application of a recyclable cellobiase enzyme system on the enzymatic hydrolysis of pre-treated biomass. We developed the recyclable cellobiase enzyme through direct (i.e., in situ) encapsulation of the enzyme in mesoporous silica, via a non-surfactant templated sol-gel route. In the mesoporous silica host materials, pore sizes are bigger enough to allow easy diffusion of the substrate to the enzyme caged inside the solid support materials but small enough to prevent the enzyme molecules from leaking from the matrix. D-fructose was used as the template or pore-forming agent, because it is very economical, biocompatible, and after synthesis, it could be easily removed from the silica pores by simply washing the samples in excess water. Also, unlike surfactant templated mesoporous material synthesis, the D-fructose templated route does not involve harsh and stringent reaction conditions that are harmful to enzymes.

The porosity and the pore size of the silica materials could be controlled by varying the fructose content, which acted as a pore forming agent. Immobilized cellobiase samples having different pore sizes were prepared. The effect of the pore parameters on the activity of the cellobiase enzyme system during the enzymatic biomass hydrolysis was studied to see if there was any difference in the activity of the enzyme, as the pore size of the host materials restricts the diffusion of the substrate to the enzyme caged inside the host materials. The immobilized cellobiase system enabled the easy post-hydrolysis recovery of the enzyme from the reaction media, leading to efficient re-use of the enzyme in multiple batches.
3.2. Experimental procedures

3.2.1. Materials

Tetraethyl orthosilicate (TEOS, 98%), D-(-)-fructose and sodium acetate trihydrate (99%) was obtained from Sigma Aldrich. The cellobiase enzyme (from Aspergillus niger), liquid ≥ 250U/g used was Novozym 188, a β-glucosidase obtained from Sigma Aldrich (cat # C6105). The cellulase from Trichoderma reesei ATCC 26921, lyophilized powder, ≥ 1unit/mg solid was obtained from Sigma Aldrich (cat # C8546). The biomass used was poplar wood shavings obtained from Martin Millwork (Mercersberg, PA). The spectrophotometric assay reagents were included in a commercial glucose determination kit (Autokit Glucose, cat# 439-90901), obtained from Wako Pure Chemicals. All the reagents and materials were used as received without any further purification.

3.2.2. Synthesis of immobilized cellobiase samples

The immobilized cellobiase samples were prepared via acid catalyzed sol-gel reactions of TEOS with water. During the synthesis, D-fructose was used as the template or pore-forming agent. In a typical preparation of 52.19 g of 70% template content immobilized cellobiase sample, 9.0 g (0.5 moles) water and 0.63 g of 2M HCl was mixed in a 500 ml 2 necked round bottomed flask under mild stirring at room temperature. The round-bottomed flask was fitted with a thermometer and a reflux condenser. After 5 minutes of stirring, 52 g (0.25 moles) TEOS was slowly added with stirring at room temperature. After the TEOS addition, the mixture was stirred at high speed under N₂. The solution turned cloudy within 2 minutes of stirring, and then cleared out within
another 5 minutes of stirring, with the reaction temperature rising to 35 °C. With further stirring, the reaction mixture turned cloudy again, and then cleared out within a minute, with the temperature rising to 60°C. The homogenous reaction mixture was refluxed at 60 °C for 1 hour and then allowed to cool down to room temperature under constant N₂ purge.

After cooling down, the N₂ purge was removed, and the reaction mixture was connected to a high vacuum system (using an oil pump), to remove the ethanol produced as a byproduct in the sol-gel hydrolysis reaction. The mixture was subject to high vacuum until it showed 50% weight loss. To this mixture, 70 g of 50% (by weight) of D-fructose in water solution was added under vigorous stirring. The mixture was stirred and degassed simultaneously until a clear solution (solution A) was obtained. The solution A was then equally divided (by weight) into three 100 ml beakers. Each beaker contained 5.0 g of silica and 11.66 g of D-fructose. For each batch, an enzyme solution was prepared by adding 625 µl (732.1 mg) of cellobiase enzyme in 5.0 ml 50 mM sodium acetate buffer (pH 5.0). This enzyme solution was then added to the solution A under stirring at room temperature for 2 minutes. The reaction beaker was then sealed with paraffin and the reaction mixture was allowed to gel at 5 °C. After gelling, the 15-20 holes were made in the paraffin with a hypodermic syringe needle and the sample was stored at 5 °C for 48 hours for solvent evaporation. After the solvent evaporation, the samples were connected to a high vacuum system at 0 °C until no further weight loss was observed.
Each beaker contained 5.0 g of silica, 11.66 g D-fructose and 625 µl of cellobiase enzyme. After the drying process, the light yellow colored solid glassy samples were crushed to 40-mesh size powder and stored at 5 °C. Four different kinds of samples were made with template content of 0%, 30%, 50% and 70% with respect to the silica content by weight. In all the samples, the amount of enzyme per g of the sol-gel (silica + D-fructose) was kept constant.

3.2.3. Characterization of immobilized cellobiase samples

The template (D-fructose) content in the immobilized cellobiase samples were determined from weight loss in air using Thermogravimetric Analysis (TGA) on a TA Q50 Thermogravimetric Analyzer (TA Instruments Inc., New castle, DE). Prior to analysis, the samples were heated to 80 °C in N₂ and kept isothermal at 80 °C in N₂ for 45 minutes. For the weight loss analysis, the samples were heated at 10 °C/ minute to 800 °C in air. The pore size, pore volume and surface area of the cellobiase-encapsulated silica powders were determined by nitrogen adsorption-desorption on a Quadrasorb SI Automated Surface Area and Pore size Analyzer (Quantachrome Instruments). The pore size and surface area of the samples were calculated using the Quantachrome QuadraWin software (Version 5.02).

3.2.4. Procedure for enzymatic hydrolysis of biomass

For our biomass hydrolysis, we have used two enzymes: Cellulase from Trichoderma reesei, and cellobiase enzyme from Aspergillus niger. The cellulase from Trichoderma reesei was used to break the cellulose (in biomass) into cellobiose units. The cellobiose units were further broken down to glucose units by cellobiase enzyme [1,
Instead of free cellobiase enzyme, we have used immobilized cellobiase enzyme in our biomass hydrolysis, along with free cellulase (from *Trichoderma reesei*). The objective of using immobilized cellobiase was to enable reusability of the enzyme in multiple reaction batches. Since cellulose is a water insoluble substrate, free cellulase had to be used, so that the enzyme could travel to the cellulose substrate. However, the cellobiose units generated from the hydrolysis are water soluble, and hence can travel to the enzyme [2]. Hence, we have used immobilized cellobiase enzyme.

For the enzymatic hydrolysis of biomass, cellulase enzyme from *Trichoderma reesei* was first prepared. In this preparation, 5.0 mg of the lyophilized powder was taken in a 5 ml volumetric flask, and 5 ml distilled and de-ionized water was added to it. The mixture was shaken until the powder completely dissolved in the water. A sodium acetate buffer solution was prepared according to the Sigma Aldrich’s cellulase assay procedure. In this method, a 50 mM solution of sodium acetate trihydrate (99%) in distilled water was prepared. Then, the pH of the solution was adjusted to 5.0 using 1M hydrochloric acid (HCl). Prior to hydrolysis experiments, the immobilized cellobiase in the silica samples was washed in the sodium acetate buffer to remove the D-fructose that would make the enzyme molecules inside the pores accessible to substrates. For the washing process, appropriate amount of the immobilized samples was taken in 15 ml falcon tubes, such that the amount of cellobiase enzyme in each tube was 25 µl. To these tubes, 10 ml of the pH 5 sodium acetate buffer solutions (50mM) was added; the tubes were sealed and agitated on a rocker at room temperature for 15 hours. After washing, the liquid was decanted out.
To the washed cellobiase samples, fresh 2.0 ml sodium acetate buffer solution (50 mM, pH 5) and either 25, 50, 100, 200, 400 mg of untreated or pre-treated biomass was added. After this, 0.5 ml of the cellulase enzyme solution was added to each tube. The tubes were then sealed and shaken on a vortex mixer for 10 seconds and then agitated at 37 °C for 2 hours in a water bath.

After 2 hours of hydrolysis, the tubes were removed from the water bath and placed on ice until the contents were cooled. After cooling, the tubes were centrifuged at 500 rpm for 2 minutes. The supernatant was decanted into a sample vial and the glucose content in it was determined by the procedure as given in Wako Glucose Autokit, expressed as milligrams of glucose per milliliter of the solution (mg/ml). The method has been used by other researchers for the spectrophotometric determination of glucose yields, after enzymatic hydrolysis of cellulose [28-33]. The exact procedure has been detailed in chapter 2 (section 2.2.4) of this dissertation.

It must be noted here that all the activity assays were repeated 6 to 8 times for reproducibility. The reported values are average values, and the standard deviation from the mean values has been reported along with them.

3.3. Results and discussions

3.3.1. Thermogravimetric Analysis (TGA)

The template (i.e., fructose) contents in the as synthesized immobilized cellobiase samples were determined by the weight loss obtained in the thermogravimetric analysis (TGA). The samples were heated in air at 10 °C/ minute to 800 °C so that the template molecules were completely decomposed and removed from the samples. Figure 3.1
shows the TGA thermographs of the as synthesized samples. The weight losses obtained are listed as TGA weight loss in Table 3.1. The observed weight losses in the samples are due to the degradation of the fructose present in the samples. From the data listed in Table 3.1, we can confirm that the fructose content in the samples, as calculated from the reaction stoichiometry, is proportional to the TGA weight losses obtained.

The 0%F sample (i.e., 0% fructose content) exhibited a weight loss of <10%, which can be attributed to the degradation of the unreacted ethoxy groups on the silica, present due to incomplete sol-gel reactions [34]. This observed weight loss of <10% in samples without fructose is also partly due to the removal of water molecules that were tightly bound to the silica matrix [35], and has been reported elsewhere [35, 36].

3.3.2. Determination of textural properties of the silica host material

The Brauner-Emmett-Teller (BET) surface area, pore size and the pore volume of the silica host materials were determined from the nitrogen adsorption-desorption isotherms and are listed in Table 3.1. Prior to analysis, the template and the enzyme were extracted from the samples by extensively washing the powder samples in a large excess of distilled water. The nitrogen adsorption-desorption analysis was done at -196 °C. Figure 3.2 shows the typical adsorption-desorption isotherms. The sample containing 0% fructose (0%F) exhibits a reversible type I isotherm, which are typical of xerogels having pores in the microporous range (i.e., pore size < 2 nm) [37, 38]. For the sample with 30% template (30%F), the porosity increases significantly though the type of isotherm remains similar. In the samples containing 50% (50%F) and 70% (70%F) template contents, the adsorption-desorption isotherms go to type IV, exhibiting H2 hysteresis loops, which are
characteristic of molecular sieves having pore structures in the mesoporous range (i.e., pore size >2 nm but <50 nm) [37, 39, 40].

Non-local density functional theory (NLDFT) method was used to calculate the pore size distribution, the pore volumes and the BET surface areas of the silica host materials [41]. From the pore size distribution (Figure 3.3) we can see that the 0%F samples has a peak maxima at < 2 nm (microporous), while the 30%F, 50%F and the 70%F samples show peak maxima in the 2-3.5 nm range, typical of mesoporous materials. The increase in the template content loading also led to an increase in the surface area and pore volume (Table 3.1) of the silica materials. This increase in the surface area of the samples with higher fructose loading indicates increased density of pore channels, and thereby making the materials more porous. Hence, from the textural properties of the silica host materials, we can see that the fructose addition clearly led to mesoporosity in the silica materials and acted as the pore-forming agent.

3.3.3. Enzymatic activity of immobilized cellobiase in the hydrolysis of biomass

The enzymatic hydrolysis of biomass with the immobilized cellobiase enzyme was performed according to the procedure described in Section 3.2.4. As discussed earlier, pre-treatment of the biomass is essential for enzymatic hydrolysis. In this work, two different pre-treated biomasses, 2.5% FeCl₃ treated biomass and 2.5% (w/V) of 0.5M oxalic acid treated biomass were used.

For the FeCl₃ pre-treated biomass and oxalic acid pre-treated biomass, the enzymatic activities of immobilized cellobiase samples with varying biomass concentrations, are shown in Figure 3.4 and Figure 3.5, respectively. The maximum
activities of the immobilized cellobiase samples as well as the native free cellobiase enzyme obtained are listed as Max activity (mg/ml) in Table 3.2. The efficiency of our immobilized system compared to the free enzyme is listed as hydrolysis efficiency (%) in Table 3.2, and was calculated according to equation (1):

\[
\text{Hydrolysis Efficiency (%) = } \frac{\text{Max activity of sample}}{\text{Max activity of free cellobiase}} \times 100 \quad (\text{Equation 1})
\]

From the enzymatic activities listed in Table 3.2, it is demonstrated that the immobilized cellobiase system attained up to 74% (FeCl₃ pre-treated biomass with 70%F sample) and 81% (oxalic acid pre-treated biomass with 70%F sample) hydrolysis efficiency, in the biomass hydrolysis. The hydrolysis efficiency of 0%F samples are lower, at 54% for FeCl₃ pre-treated biomass and 51% for oxalic acid pre-treated biomass. This observed difference in max activity and hydrolysis efficiency could be attributed to the unique micro-/nano-structure of the silica host materials. As listed in Table 3.1, the samples with higher fructose contents (70%F and 50%F) are mesoporous and have bigger pore diameters (3.4 nm and 3.1 nm, respectively) compared to 0%F samples that is microporous in nature (pore diameter < 2 nm). The bigger pore diameters allowed easier diffusion of the substrate (i.e., cellobiose) into the pores, thus increasing the accessibility of the enzyme. In the 0%F sample, the smaller pore diameter hindered the diffusion of the substrate, thereby restricting the accessibility of the enzyme. As evident from Table 1, the surface area and the pore volume of the 70%F and 50%F samples also are higher compared to the 0%F sample. The high surface area signifies greater concentration of pores in the host material. High template (fructose) content opened up more
interconnected pores/channels in the silica, thereby increasing the porosity of the host silica. Hence, the bigger pores and higher porosity in 70%F and 50%F samples led to higher enzymatic activities, compared to the 0%F sample.

It must be noted that the cellobiase enzyme molecules are trapped within the silica matrix, via direct encapsulation by gelation of the silica precursor sol around the protein molecules. The template actually increased the diameter and concentration of the interconnected open pore channels, leading to the caged enzyme inside the silica matrix. In 70%F and 50%F (and to some extent in 30%F) samples, there is template-induced porosity, which makes the pore sizes bigger, thus promoting easy diffusion of substrate through the pore channels, to the enzyme caged within the host matrix. In 0%F sample, the pore diameters are smaller (due to absence of template-induced porosity), thus reducing the accessibility of the enzyme caged within the silica microstructure, by the substrate.

The Michaelis-Menten kinetics for the immobilized cellobiase samples in the hydrolysis of biomass was studied according to Equation (2) [42]:

\[
\frac{1}{v_0} = \frac{K_m}{V_{max}} \frac{1}{[S]} + \frac{1}{V_{max}} \quad (2)
\]

Where \(v_0\) is the initial rate of enzyme activity or the initial reaction rate of hydrolysis at any given substrate (i.e., biomass in this work) concentration, \([S]\) is the substrate concentration, \(V_{max}\) is the maximum rate of conversion or the maximum rate of hydrolysis and \(K_m\) is the substrate concentration at which the rate of reaction or rate of hydrolysis is half of \(V_{max}\). The Michaelis-Menten plots for the immobilized cellobiase samples are shown in Figure 3.6 and Figure 3.7, and the \(V_{max}\) values obtained for the
immobilized cellobiase samples as well as the free cellobiase samples for the two pre-treated biomass hydrolysis are listed in Table 3.2. The data listed in Table 3.2 demonstrated that our immobilized cellobiase samples yielded $V_{\text{max}}$ values ~ 65% (70%F with FeCl$_3$ pre-treated biomass) and 83% (50%F with oxalic acid pre-treated biomass) as compared to biomass hydrolysis with free cellobiase enzyme. The 0%F showed a relatively lower $V_{\text{max}}$ value. This observation is in accordance with the max activity values. The smaller pore size and lower porosity greatly restricted the accessibility of the enzyme inside the caged structure. This led to lower rates of reaction for the 0%F sample, as evident from the low $V_{\text{max}}$ value obtained.

Hence, from the hydrolysis efficiency results reported, it is evident that our immobilization method produced highly efficient immobilized enzyme systems, retaining up to a remarkably high (i.e., 81%) hydrolysis efficiency after the immobilization process.

3.3.4. Reusability of the immobilized cellobiase in biomass hydrolysis

As discussed earlier, the most important aspect and the primary goal of this work was to generate an immobilized enzyme system, that could be easily separated from the biomass reaction mixture, and readily be recycled for multiple uses in cellulose hydrolysis. To enable multiple use of our immobilized enzyme system in biomass hydrolysis, we designed a two-step hydrolysis process, based on the understanding that during the enzymatic hydrolysis, the cellulase enzyme first breaks down the cellulose into cellobiase units, and thereafter, the cellobiose units are broken into glucose by the cellobiase enzyme [1, 3]. The biomasses used for the recyclable application were FeCl$_3$
pre-treated biomass and oxalic acid pre-treated biomass. The 50%F and 70%F immobilized cellobiase samples were used at the biomass concentration of 400 mg/ml. The aforementioned samples and concentration were chosen because they showed the highest enzymatic activity as discussed in Section 3.3.3. In the first step of our experimental design, the biomass (cellulose) was broken down to cellobiose by cellulase enzyme. For this, 400 mg of FeCl$_3$ pre-treated biomass was taken in a 15 ml falcon tube. To this tube, 0.5 ml of cellulase enzyme solution and 2 ml sodium acetate buffer were added; the tube was sealed and mixed in a vortex mixer for 10 seconds. Then, the tube was agitated at 37°C for two hours. After the hydrolysis of cellulose to cellobiose, the second step was conducted. Thus, the liquid phase of the reaction mixture containing the cellobiase was taken out and added to another 15 ml falcon tube which contained appropriate amount of either the 50%F or the 70%F immobilized cellobiase samples (enzyme content was 25 µl in each sample). It was then mixed in a vortex for 10 seconds, and then agitated at 37°C for 2 hours. After the hydrolysis of the cellobiose, the glucose content in the liquid phase was determined by the Wako Glucose Autokit method, as described in section 3.2.4. The activity of the immobilized cellobiase was expressed in terms of milligrams of glucose per milliliter of solution. After the run, the immobilized samples were filtered out and washed twice with 5 ml sodium acetate buffer (50mM, pH 5) at room temperature, and the whole process was repeated for the next subsequent run. In this way, the immobilized cellobiase samples were reused in 10 multiple batches.

Figure 3.8 shows the activity of the immobilized cellobiase with FeCl$_3$ pre-treated biomass and oxalic acid pre-treated biomass hydrolysis in multiple uses. From the results, it is clearly demonstrated that with up to 10 reuses, our immobilized cellobiase samples
maintained their enzymatic activities during the biomass hydrolysis. This retained activity also confirms that the enzyme is well encapsulated within the silica matrix with negligible leaking. Cellobiase is a globular protein with a diameter of 51 Å [2], and hence bigger than the pore channels. The smaller diameter of the pore channels in the silica host materials thus prevented the enzyme from coming out of the caged structure. The elution of the enzyme from the host materials was also checked by washing the immobilized samples in sodium acetate buffer (50 mM, pH 5) and assaying the washing solutions. All the washing solutions showed negligible enzymatic activity. Hence, the results clearly established that with our experimental design, recyclable application of the immobilized cellobiase system is attainable in the enzymatic hydrolysis of biomass with high hydrolysis efficiency.

3.4. Conclusion

In this work, an immobilized cellobiase enzyme system was used in the enzymatic hydrolysis of biomass. The cellobiase enzyme was directly immobilized in silica host materials via the sol-gel reactions of TEOS with water in the presence of D-fructose as a nonsurfactant template or pore-forming agent. By varying the fructose content, the silica materials could have controlled the porosity, pore size and the pore volume. These pore parameters, in turn, affected the activity of the immobilized enzyme during hydrolysis, as they regulate the diffusion of the substrate to the enzyme inside the silica matrix. When immobilized cellobiase systems were applied in the hydrolysis of pre-treated biomasses, hydrolysis efficiency up to 74% and 95% were obtained as compared to hydrolysis with free cellobiase enzyme. A new experimental design was set up to allow easy post-hydrolysis separation of the immobilized enzyme from the reaction mixture. The recycled
enzyme, when used repeatedly in multiple batches, showed negligible loss of activity in the biomass hydrolysis. Thus, we have demonstrated that such a novel immobilized cellobiase system, when used in the enzymatic hydrolysis of biomass, enabled the recyclable use of the enzyme. This recyclability ensures the re-usability of the enzyme while maintaining the high efficiency in biomass hydrolysis.

Our immobilized cellobiase enzyme thus opens up great potential in the production of ethanol as biofuel from the hydrolysis of renewable sources such as biomass. The recycling and reusability of the immobilized enzyme system would actively bring down the enzyme cost, leading to the overall decrease in the cost of ethanol production in the biomass hydrolysis process.

3.5. Acknowledgment

A part of the biomass pretreatment and a part of the activity measurement were carried out by Dr. David Berke-Schlessel. The nitrogen adsorption-desorption experiments were conducted in the Department of Materials Science and Engineering, Drexel University. We are indebted to Prof. Yury Gogotsi and John McDonough of the Department of Material Science and Engineering, Drexel University for helping us with the BET measurements. We also thank Prof. Hai-Feng Ji of the Chemistry Department, Drexel University, for valuable suggestions and discussions.
Table 3.1. Template content, TGA weight loss and textural properties of the immobilized cellobiase samples.

<table>
<thead>
<tr>
<th>Sample code &amp; spec. vol.</th>
<th>Weight % template added (^a)</th>
<th>TGA weight loss (%)</th>
<th>BET Surface area (m(^2)/g)</th>
<th>Pore diameter (nm)(^b)</th>
<th>Pore volume (m(^3)/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0(%F)</td>
<td>0</td>
<td>&lt; 10</td>
<td>287</td>
<td>1.6</td>
<td>0.15</td>
</tr>
<tr>
<td>30(%F)</td>
<td>30</td>
<td>29</td>
<td>623</td>
<td>2.3</td>
<td>0.35</td>
</tr>
<tr>
<td>50(%F)</td>
<td>50</td>
<td>53</td>
<td>778</td>
<td>3.1</td>
<td>0.52</td>
</tr>
<tr>
<td>70(%F)</td>
<td>70</td>
<td>71</td>
<td>796</td>
<td>3.4</td>
<td>0.57</td>
</tr>
</tbody>
</table>

\(^a\) Weight % template added was calculated from the reaction stoichiometry, assuming complete conversion of TEOS to silica.

\(^b\) Obtained from the peak maxima of the pore size distribution plot.
Table 3.2. Maximum activity, Hydrolysis Efficiency and $V_{\text{max}}$ values of immobilized cellobiase and free cellobiase enzyme in biomass hydrolysis. 2.5% FeCl$_3$ pre-treated biomass and 2.5% 0.5M oxalic acid pre-treated biomasses were used for the hydrolysis.

<table>
<thead>
<tr>
<th>Sample</th>
<th>FeCl$_3$ pre-treated biomass</th>
<th></th>
<th>Oxalic acid pre-treated biomass</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Max activity (mg/ml)</td>
<td>Hydrolysis Efficiency (%)</td>
<td>Vmax (mg/ml.hr)</td>
<td>Max activity (mg/ml)</td>
</tr>
<tr>
<td>0%F</td>
<td>0.58±0.06</td>
<td>54</td>
<td>0.31±0.03</td>
<td>0.59±0.12</td>
</tr>
<tr>
<td>30%F</td>
<td>0.53±0.05</td>
<td>50</td>
<td>0.29±0.03</td>
<td>0.75±0.06</td>
</tr>
<tr>
<td>50%F</td>
<td>0.79±0.03</td>
<td>73</td>
<td>0.42±0.02</td>
<td>0.84±0.06</td>
</tr>
<tr>
<td>70%F</td>
<td>0.80±0.12</td>
<td>74</td>
<td>0.48±0.09</td>
<td>0.95±0.16</td>
</tr>
<tr>
<td>Free Cellobiase</td>
<td>1.08±0.14</td>
<td>100</td>
<td>0.74±0.07</td>
<td>1.16±0.14</td>
</tr>
</tbody>
</table>
Figure 3.1 TGA thermographs of the as-synthesized cellobiase encapsulated silica samples for verifying the added template (fructose) content. The nomenclatures of the samples are described in Table 3.1.
Figure 3.2 Nitrogen adsorption-desorption isotherms of the silica host materials of the immobilized samples. The template and enzyme was extracted out of the samples by washing with excess water. This figure has been reproduced from Figure 2.3, as we have used the same samples.
Figure 3.3 Pore size distribution of the host silica materials of the immobilized samples. This figure has been reproduced from Figure 2.4, as we have used the same samples.
Figure 3.4 Activity of immobilized cellobiase samples with varying substrate (biomass) concentration. The biomass used was pre-treated with 2.5% FeCl₃ solution. The hydrolysis reactions were done in pH 5 sodium acetate buffer at 37°C.
Figure 3.5 Activity of immobilized cellobiase samples with varying substrate (biomass) concentration. The biomass used was pre-treated with 2.5% 0.5M oxalic acid solution. The hydrolysis reactions were done in pH 5 sodium acetate buffer at 37°C.
Figure 3.6 Michaelis-Menten plots for the immobilized samples with FeCl₃ pre-treated biomass. The x-axis represents the biomass concentration and y-axis represents initial reaction rate ($v_0$).
Figure 3.7 Michaelis-Menten plots for the immobilized samples with oxalic acid pre-treated biomass. The x-axis represents the biomass concentration and y-axis represents initial reaction rate ($v_0$).
Figure 3.8 Activity of 70%F and 50%F immobilized cellobiase samples, recycled and used in multiple batches. The biomass used was pre-treated with 2.5% FeCl₃ solution. All the runs were done in pH 5 sodium acetate buffer at 37°C. 70%F and 50%F samples were used in recycled use, as they exhibited the highest max activities as mentioned in section 3.3.3.
3.6. References


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Chapter 4: Encapsulation of Candida rugosa lipase enzyme in nonsurfactant templated sol-gel silica materials

4.1. Introduction

In Chapter 2, the technology of immobilization of enzymes via direct encapsulation in non-surfactant templated sol-gel silica materials has been discussed. The effect of template content on the activity of the immobilized enzymes has been discussed in detail. In this chapter, immobilization of lipase enzyme from Candida rugosa in sugar templated sol-gel silica is described.

Lipases are an important class of enzymes that catalyze a number of reactions including esterification, interesterification and hydrolysis [1]. Owing to their high selectivity, lipases function as valuable biocatalysts in the synthesis of chiral drug intermediates and neutraceutical lipids[2, 3]. Extracellular lipases, which are produced by microorganisms, have been studied extensively due to their wide biotechnological applications [4, 5]. A huge amount of research is being done to screen various lipase-producing organisms and to utilize them in biotechnological applications for human welfare [6, 7]. This worldwide effort led to the introduction of Candida rugosa lipases [8, 9]. Secreted by the Candida rugosa yeast, the lipase from Candida rugosa is currently the most extensively studied lipase [10].

Lipase from Candida rugosa has been widely used in aqueous and water-restricted catalytic reactions. It has been applied as catalyst in stereo-specific as well as non-specific hydrolysis, esterification, trans-esterification and inter-esterification reactions [6,
7, 9]. For further expansion of its synthetic utility, the reusability of Candida rugosa lipase is very important to compensate for its increasing price. To meet this need, efficient immobilization of lipase in solid supports is very necessary, as it enables enzyme reuse and leads to process cost reduction [11-13].

A variety of methods have been reported for the immobilization of lipase enzyme [14, 15]. These include adsorption onto solid supports, covalent bonding and encapsulation of the enzyme within polymeric matrix or sol-gel silica gels [16]. It has been suggested by many authors that physical entrapment within hydrophobic materials, preferably polymer matrix or sol-gel silica materials, are the most suitable lipase immobilization methods [1, 17]. The tight gel-network obtained through sol-gel process allows stabilization of tertiary structure of the encapsulated enzyme molecules [18, 19].

Entrapment of lipases in sol-gel silica from various silica precursors like tetraethoxysilane (TEOS), methyltrimethoxysilane (MTMS) and polydimethoxysilane (PDMS) in the presence of poly(vinyl alcohol) (PVA) and poly(ethylene glycol) (PEG) has been reported [1, 20, 21]. Entrapment of lipase from Candida rugosa in silica, obtained via sol-gel reactions of TEOS in the presence of PEG as additive was reported to yield high hydrolytic activity [1, 22]. Lipases hydrolyze ester bonds of triacylglycerols. These substrates are insoluble in aqueous media, and hence, during hydrolysis with lipase, they are emulsified with gum arabic solution to form large emulsion droplets [22]. Diffusional resistance in the transport of the substrate from bulk solution to the catalytic sites largely hinders the activity of the entrapped enzyme. Therefore, pore size of the silica matrix is a critical issue in the immobilization of lipase.
In this work, lipase from Candida rugosa was immobilized via direct encapsulation of the enzyme in silica host materials via the nonsurfactant templated sol-gel processing. The silica matrix was generated through the acid catalyzed hydrolysis and polycondensation of tetraethyl orthosilicate (TEOS). D-fructose was used as a template or pore-forming agent to regulate the porosity of the sol-gel silica. The catalytic activity of the encapsulated lipases was evaluated as a function of pore parameters of the silica matrix. The re-usability of the immobilized lipase samples was also demonstrated in this work.

4.2. Experimental

4.2.1. Materials

Tetraethyl orthosilicate (TEOS), 98% (cat # 131903); D- (-) –fructose, 99% (cat # F0127); Olive oil (highly refined, low acidity, cat # O1514-500ML); sodium phosphate monobasic (cat # S-0751); sodium phosphate dibasic (cat # S9390); gum Arabic (cat # 51198) and potassium hydroxide, reagent grade (90%, cat # 484016) were purchased from Sigma Aldrich. The enzyme, lipase from Candida rugosa (product # L1754), was obtained from Sigma Aldrich. All the reagents were used as received without any further purification.

4.2.2. Synthesis of immobilized lipase samples

Lipase from Candida rugosa was encapsulated in porous silica matrix via acid catalyzed sol-gel reactions of TEOS, with D- (-) fructose as the template or pore-forming agent. In a typical preparation of 30% (by weight) D-fructose containing immobilized lipase sample, 9 g (0.5 mol) of distilled water and 0.63 g of 2M HCl were
mixed in a 500 ml 2-necked round bottomed flask fitted with a thermometer and reflux apparatus. The mixture was stirred at room temperature for 5 minutes. After stirring, 52 g of TEOS (0.25 mol) was added to the mixture drop-wise, and the mixture was allowed to stir under nitrogen.

The mixture turned cloudy and then cleared out in about 10 minutes, with the temperature rising to 37 °C. The mixture turned cloudy again, and finally cleared out and became homogenous with the temperature rising to ~ 55°C. The reaction mixture was then refluxed at 60 °C for 1 hour under nitrogen. After reflux, the reaction was allowed to cool down to room temperature and high vacuum (by oil pump) was applied to evacuate water and ethanol that was produced as a byproduct. The high vacuum was applied until the reaction mixture showed 50% weight reduction. To this, 12.8 g of 50% D- (–) -fructose solution (6.4 g fructose + 6.4 g distilled water) were then added drop-wise with vigorous stirring, until a clear homogenous solution was obtained. This mixture was then divided into 3 equal parts (by weight) in 3 separate beakers. Each beaker contained 5 g of silica and 5 g of fructose.

A 100 mM sodium phosphate buffer (pH 7.0) was prepared. For each beaker, an enzyme solution of 11.66 mg of lipase in 7.0 ml of the 100 mM sodium phosphate buffer (pH 7.0) was prepared. The enzyme solutions were then added to the respective sol-gel mixtures under vigorous stirring at 0 °C. After enzyme addition, the mixtures were stirred until the mixtures gelled. A transparent gel was obtained. Each beaker was sealed with paraffin film, and 20-25 holes were drilled using a hypodermic needle in the film. The beakers were then stored at 5 °C for solvent evaporation. After 2 days, the beakers were kept under high vacuum at 0 °C for further drying until no further weight loss was
observed. Each sample beaker now contained ca. 5 g silica, 2.13 g fructose and 11.66 mg lipase enzyme. After drying, the transparent and hard, enzyme containing monolithic bioglasses was grinded to 40-mesh size, and stored at 5 °C for further analysis. Samples with 0%, 10% and 30% fructose contents were prepared similarly with identical enzyme loading per gram of silica.

4.2.3. Characterization of immobilized lipase samples

The surface area, pore size and pore volumes of the lipase encapsulated silica samples were determined by nitrogen adsorption-desorption experiments on a Quadrasorb SI Automated Surface Area and Pore Size Analyzer instrument at -196 °C. Prior to adsorption-desorption, the template (i.e., fructose) was extracted out of the samples by washing in large excess of water, and the samples were dried at 40 °C overnight in a vacuum oven. Before analysis, the powders were degassed at 100 °C overnight. Quantachrome QuadraWin software was used to analyze the data. The template contents in the as-synthesized immobilized lipase samples were determined from the weight loss obtained in thermogravimetric analysis (TGA), performed on TA Q50 Thermogravimetric Analyzer (TA Instruments Inc., New castle, DE). Prior to TGA measurements, the samples were heated to 80 °C and kept isothermal at 80 °C for 45 minutes. During the weight loss experiment, the pre-heated samples were heated from 25 °C to 800 °C at a heating rate of 10 °C/minute in air. TA Universal Analysis software was used to analyze the data obtained.
4.2.4. Enzymatic activity of lipase enzyme

The enzymatic activities of the free and immobilized lipase enzymes were determined by the olive oil emulsion method [23]. In this method, olive oil was used as the substrate, which was hydrolyzed by lipase enzyme to liberate fatty acids. For the preparation of the substrate, a 7% (W/V) solution of gum Arabic was made in distilled water. 50 ml of the gum Arabic solution was mixed with 50 ml of olive oil, and the mixture was stirred for 5 hours to make an emulsion. Prior to doing the assays, the immobilized lipase samples were washed with phosphate buffer to remove the template. Thus, appropriate amounts of the immobilized samples were taken in beakers, such that the amount of lipase enzyme present was 0.59 mg. To the beaker, 10 ml of the phosphate buffer (100 mM, pH 7) was added and the mixture was stirred for 1 hour to extract the template. To the washed lipase samples, a fresh 10 ml portion of the phosphate buffer was added. To this, 5.0 ml of the olive oil emulsion substrate was added, and the mixture was stirred at 37 °C for 15 minutes. After hydrolyzing for 5 minutes, the liquid phase was decanted out from the reaction mixture, leaving the immobilized lipase sample in the beaker. The liberated fatty acids, present in the emulsion, were titrated with 25 mM potassium hydroxide solution, using phenolphthalein indicator. One unit of activity (U) was obtained in terms of μmol of fatty acid liberated per minute (U = μmol minute$^{-1}$). As a control experiment (i.e., using free lipase), 0.59 mg of the lipase enzyme powder was dissolved in 10 ml of phosphate buffer. This enzyme solution was then assayed under identical conditions as was done for the immobilized samples. All assays were repeated 3-4 times for reproducibility. All the activities of the immobilized samples in this work
has been expressed in terms of % residual activity, based on 100% activity of free lipase enzyme in 100 mM, pH 7.0 phosphate buffer, according to the following equation:

\[
\% \text{ Residual Activity} = \left( \frac{U_S (\text{µmol minute}^{-1})}{U_L (\text{µmol minute}^{-1})} \right) \times 100
\]

where \( U_S \) is the activity of the immobilized sample and \( U_L \) is the activity of free lipase.

**4.3. Results and discussions:**

**4.3.1. Thermogravimetric analysis (TGA)**

Thermogravimetric analysis technique was used to verify the template contents in the immobilized lipase samples. The samples were heated from room temperature to 800 °C at a heating rate of 10 °C/minute. The weight loss observed can be attributed to the degradation of fructose present in the samples. The weight losses observed in the as-synthesized samples are reported as TGA weight loss in Table 4.1 and the TGA thermographs are shown in Figure 4.1. From the data listed in Table 4.1, it can be seen that the fructose content in the samples, as calculated from the reaction stoichiometry, closely matches the TGA weight loss obtained. The weight loss of < 10%, as observed in the 0% sample (without any fructose) is due to degradation of un-reacted ethoxy groups from the TEOS, which are present in the silica, because of incomplete sol-gel reactions [24]. A part of this weight loss is also due to the removal of water molecules, which were tightly bound to the silica matrix [25].

**4.3.2. Nitrogen adsorption-desorption**

Nitrogen adsorption-desorption experiments were done to determine the Brauner- Emmett-Teller (BET) surface area, pore diameter and pore volume of the silica matrix.
Prior to the nitrogen adsorption-desorption experiments of the immobilized lipase samples, the template and enzyme were extracted by extensive washing with distilled water. The adsorption-desorption isotherms, measured at -196 °C, for the different samples, are shown in Figure 4.2. The adsorption-desorption isotherm exhibited by 0% and 10% samples are type I reversible isotherms, which are characteristic of xerogels with microporous structures [26]. Non-linear local density functional theory (NLDFT) method was used to determine the pore size distribution, surface area and the pore volumes of the samples [27]. The pore diameters of the samples were determined from the maxima of the pore size distribution plot [28], shown in Figure 4.3. The 30% sample exhibits a peak at ~ 2.3 – 2.5 nm range. The 10% and 0% samples exhibit maxima at <2 nm, which are microporous in nature. Hence, from the nitrogen adsorption-desorption, it is evident that the different fructose contents in the samples led to different template induced porosity in the host silica materials. A more detailed discussion of the template-induced porosity is given in the following section.

4.3.3. Enzymatic activity of immobilized lipase

The enzymatic activities of 0%, 10% and 30% immobilized lipase samples are shown in Figure 4.4. The activity of free lipase was taken as 100% and all the activities of the immobilized lipase samples has been expressed as percentage with respect to the free lipase activity. The activities up to 3 successive uses are shown in Figure 4.4, and listed in Table 4.2. Figure 4.5 shows the activities of the washing solutions, after the samples were washed in phosphate buffer, as described in section 4.2.4. The activities of the washing solutions were studied to monitor the leaching of enzymes from the silica matrix during washing to remove the template.
From the activity profiles of the immobilized lipase samples in Figure 4.4, we can see that the 30% immobilized lipase sample exhibited higher activity compared to 0% and 10% samples. This observation can be explained through the porosity of the host silica matrix. From Table 4.1, we can see that with increasing template (fructose) concentration, the surface area of the host silica matrix increases. This increase in surface area can be attributed to the formation of more pores in the silica materials. The fructose acted as a pore-forming agent, and with increased fructose content, more interconnected pores/channels are formed. As evident from Table 4.1, higher fructose content (30% sample) also led to larger pore volumes and pore diameters, compared to samples with lower fructose content (0% and 10%). Thus, it is evident that higher fructose content led to higher porosity of the silica host materials. The enzymatic activity of the immobilized lipase depends on the rate of diffusion of the substrate to the enzyme caged within the silica host [22]. Since the substrate is insoluble in aqueous media, the difficulty in transport of the substrate from the bulk solution to the catalytic sites via diffusion largely hinders the reaction rate [22]. This substrate diffusion rate depends on the porosity of the host materials. With increased concentration of pore channels, substrate diffusion is easier. Hence, with increased porosity and bigger pore sizes, the rate of diffusion of the olive oil substrate to the enzyme inside the silica structure is higher and easier in the 30% immobilized lipase sample. This substrate diffusion is hindered in 0% and 10% samples, owing to their lower porosity. Hence, the activity of the immobilized lipase in 30% sample is significantly higher than 0% and 10% samples. It should be noted that we also prepared immobilized lipase samples with 50% fructose content. However, the 50%
samples showed high enzyme leaching during the washing process (owing to their bigger pore diameters), and the washed solid samples showed little enzymatic activity.

4.3.4. Multiple use of the immobilized lipase and enzyme leaching from the silica matrix

As discussed in section 4.2.4, the immobilized samples were washed in phosphate buffer solution to remove the template (fructose) from the pore channels and allow accessibility of the enzyme to the substrate. After the washing, the washing solution was assayed to study the leaching of enzymes from within the pores to the washing solution. Figure 4.5 shows the enzymatic activity of the washing solutions for the immobilized lipase samples. From the figure and data listed in Table 2, it can be seen that the 0%, 10% and 30% immobilized samples showed comparatively negligible ($\leq 5\%$) residual activity. The lipase enzyme has been reported to have an ellipsoid shape with dimensions of $5.3 \times 5.0 \times 4.1$ nm [29]. The pore sizes of the host silica matrices, being smaller, prevent enzyme leaching.

As mentioned earlier, one of the most important objectives of enzyme immobilization is reusability of the enzyme catalyst. We studied the activity of our immobilized lipase samples in multiple batches. For this, the solid samples were separated from the reaction mixture as described in section 4.2.4. The separated samples were washed with pH 7 phosphate buffer to remove any residual substrate. Then, fresh substrate was added to the catalyst and the whole assay procedure was repeated for the run. The activity profiles of our immobilized lipase samples with multiple uses are shown in Figure 4.4, and the activities are listed in Table 4.2.
As can be seen from Figure 4.4, when used in multiple batches, the 30%, 10% and 0% immobilized samples retained their catalytic activities. This retained hydrolytic activity of the immobilized enzyme also further suggests minimal enzyme leaching from the silica host materials.

4.3.5. Stability of immobilized lipase in organic solvent

Lipases are one of the most robust enzymes. However, their industrial applications in synthetic organic processes are still short of significant level [22]. This has been attributed to their limited long-term stability and difficulties in recycled use of the enzyme. We studied the stability of our immobilized lipase sample in ethanol as the organic solvent. The 30% sample (sample weight corresponding to 0.59 mg of enzyme) was taken in a beaker, and 10 ml of ethanol was added to it. The immobilized sample was stored in ethanol for 1 hour and 2 hours at room temperature. After the desired times, the ethanol was decanted out and the solid samples were assayed for their activities using the procedure as described in section 4.2.4. Figure 4.6 shows the residual activities of the sample. It can be seen that our 30% sample retained ~20% and 12% residual activities after 1 and 2 hours, respectively. The free lipase enzyme showed complete deactivation in 1 hour, when stored in ethanol at room temperature.

4.4. Conclusions

Lipase enzyme from Candida rugosa was in situ encapsulated in porous silica host materials via sol-gel reaction of TEOS in the presence of nonsurfactant template. During the synthesis, D-fructose was used as the template or pore-forming agent. The higher template content generally tends to result in greater porosity. The porosity of the host
silica matrix played a very important role in the enzymatic activity of the immobilized lipase enzyme, as it regulates the diffusion of the substrate molecules to the enzyme inside the pores of the silica matrix. Samples with 30% fructose content exhibited higher enzymatic activities, compared to samples with lower fructose content (0% and 10%). The immobilized lipase samples were also used in multiple batches successfully and established the reusability of the immobilized lipase enzyme. The samples retained their activities in reusability study. Our immobilized lipase samples also showed stability in organic solvent, compared to free lipase enzyme. It should be noted that the substrates for lipase are mostly hydrophobic molecules while the silica matrix is hydrophilic. Therefore, in the future studies, we plan to prepare organic-inorganic hybrid silica with controlled porosity and to carry out post-synthesis modification of the silica host materials with hydrophobic functionalities.

### 4.5. Acknowledgement

This work was a collaborative effort with Mr. Tom Hughes and Mr. Collin Murray from Reacta Corporation. I am indebted to my former colleagues, Mr. Somang Kim, Dr. Indraneil Mukherjee and Dr. Andreas Mylonakis for valuable suggestions and very fruitful discussions. I also thank Dr. Hai-Feng Ji for his very helpful suggestions and discussions.
Table 4.1. Sample nomenclature, TGA weight % template and textural properties of immobilized lipase samples.

<table>
<thead>
<tr>
<th>Sample Name</th>
<th>Weight % template added</th>
<th>TGA Weight loss (%)</th>
<th>BET Surface Area (m$^2$/g)</th>
<th>Pore diameter$^a$ (nm)</th>
<th>Pore volume (cm$^3$/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0%</td>
<td>0</td>
<td>&lt; 10</td>
<td>416</td>
<td>&lt; 2</td>
<td>0.26</td>
</tr>
<tr>
<td>10%</td>
<td>10</td>
<td>16</td>
<td>427</td>
<td>&lt; 2</td>
<td>0.21</td>
</tr>
<tr>
<td>30%</td>
<td>30</td>
<td>29</td>
<td>687</td>
<td>2.3</td>
<td>0.34</td>
</tr>
</tbody>
</table>

$^a$ From maxima of pore size distribution plot (Figure 4.3).
Table 4.2. Enzymatic activity of the immobilized lipase samples and the washing solutions.

<table>
<thead>
<tr>
<th>Sample</th>
<th>% Residual Activity&lt;sup&gt;b&lt;/sup&gt;</th>
<th>1&lt;sup&gt;st&lt;/sup&gt; use</th>
<th>2&lt;sup&gt;nd&lt;/sup&gt; use</th>
<th>3&lt;sup&gt;rd&lt;/sup&gt; use</th>
<th>Washing solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>0%</td>
<td></td>
<td>10±0.65</td>
<td>10±0.92</td>
<td>7±0.03</td>
<td>4±0.43</td>
</tr>
<tr>
<td>10%</td>
<td></td>
<td>9±0.58</td>
<td>8±0.94</td>
<td>11±0.86</td>
<td>3±0.86</td>
</tr>
<tr>
<td>30%</td>
<td></td>
<td>20±0.63</td>
<td>23±1.02</td>
<td>18±1.23</td>
<td>5±0.65</td>
</tr>
</tbody>
</table>

Activities of all samples were calculated based on 100% activity of free lipase in pH 7, 100 mM sodium phosphate buffer.
Figure 4.1. TGA thermographs of the as synthesized immobilized lipase samples. The 0%, 10% and 30% samples contained 0, 10 and 30 weight % template (D-fructose) respectively.
Figure 4.2. Nitrogen adsorption-desorption isotherms of the template and enzyme extracted silica samples. The 0%, 10% and 30% samples contained 0, 10 and 30 weight % template (D-fructose) respectively.
Figure 4.3. Pore size distribution of the template and enzyme extracted silica samples.
Figure 4.4. Enzymatic activity of the immobilized lipase samples. The 0%, 10% and 30% samples contained 0, 10 and 30 weight % template respectively.
Figure 4.5. Enzymatic activity of the washing solutions for the immobilized lipase samples. The 0%, 10% and 30% samples contained 0, 10 and 30 weight % template respectively.
Figure 4.6. Residual activity of 30% immobilized lipase sample in ethanol at room temperature. Free lipase enzyme showed complete deactivation after 1 hour under identical conditions.
4.6. References


Chapter 5: Synthesis and characterization of novel bio-applicable inorganic-organic silica-curcumin hybrid materials

5.1. Introduction

During the last decade, inorganic-organic hybrid materials have attracted large research attention because of their immense potential applications [1]. The combination of organic polymers and inorganic materials in the molecular level has been of immense interest since the late 80’s [2] and a number of review articles on inorganic-organic hybrids have been published [3-6]. The classical approach of combining the properties of different materials introduced the concept of combining two different phases with complementary physical properties, to generate composite materials or blends [7]. Hybrid materials involve the combination of chemical groupings of two different phases on a nanoscopic or molecular level [7, 8].

5.1.1. Inorganic-organic hybrid materials via sol-gel approach

Because of its mild reaction conditions, the sol-gel process could efficiently combine an inorganic oxide with an organic phase, and is a very widely used method for the preparation of inorganic-organic hybrid materials [1, 3, 7-10]. Basically, the sol-gel process is an inorganic polymerization, which leads to a highly cross-linked structure through hydrolytic polycondensation reactions. In the first step of the sol-gel process, a monomeric, metal or semimetal alkoxide precursor, represented as [M(OR)$_4$], where M is a network-forming element (typically Si, Ti, Zr, Al, B) and R is an alkyl group, is hydrolyzed to form M-OH groups. In the second step, these M-OH groups undergo polycondensation to form metal oxide network (M-O-M). The hydrolysis step is either
acid catalyzed or base catalyzed. In general, after the initiation of the hydrolysis step, the hydrolysis and condensation of the precursor occurs simultaneously, and both the processes occur by nucleophilic substitution mechanisms [8]. The sol-gel reactions of non-silicate alkoxides occur without any catalyst, owing to their high reactivity. A base or acid catalysis is typically required for silicon based metal alkoxides.

The main advantage of sol-gel method over other inorganic network forming methods is that it uses very mild reaction conditions and is compatible with a broad range of solvents [11]. The low temperature (room temperature) processing conditions of sol-gel makes it an ideal method to incorporate polymeric/low-molecular weight organic moieties into the inorganic networks, under temperatures in which organic molecules can survive [8]. Because of the compatibility with a lot of solvents, the method is flexible enough to incorporate a wide range of organic reagents and polymers, which has reactive groups that can react during the hydrolysis-condensation process [1]. The synthesis of a number of novel polymeric-inorganic hybrid materials with good performance has been reported using sol-gel process [12].

5.1.2. Bonding of the organic phase with the inorganic phase in hybrids

The nature of interaction at the interface of the inorganic and organic phases is an important criterion in the synthesis of hybrid materials. Inorganic-organic hybrid materials are generally prepared by either the covalent bonding of the inorganic and organic components, or by specific intermolecular interactions between the inorganic and the organic moieties, which includes van der Waals forces, hydrogen bonding or electrostatic forces [3, 7, 8].
The incorporation of various organic polymers into silica networks has attracted a lot of interest [12]. Organoalkoxysilanes has been used as a precursor in sol-gel reaction to incorporate organic groups in inorganic silica networks [13]. Our group has pioneered the synthesis of vinyl polymer-silica hybrid materials, in which vinyl polymers such as poly(methyl methacrylate), polystyrene, etc., were homogeneously distributed in and covalently bonded to silica or other metal oxide networks [14-17]. Sol-gel process has also been used to incorporate polymers like poly(N-vinylpyrrolidone), poly(N,N-dimethylacrylamide) and poly(ε-caprolactone) into silica networks [1, 12, 18-21]. In this work, an inorganic-organic hybrid system was developed by covalently incorporating an organic phase with potential biomedical functions in an inorganic silica network via sol-gel reactions of a silicon alkoxide.

5.1.3. Curcumin organic phase in inorganic-organic hybrid materials

Curcumin \(1,7\)-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione) [22, 23] is a natural polyphenol and it is the main constituent of the Indian cooking spice turmeric [22-24]. Curcumin is also known as diferoyl methane, and it contains a β-diketone unit linked to phenolic units on either side via C=C bonds (Scheme 5.1). It has been reported from the X-ray crystal structure analysis that in the solid state, curcumin and its bisacetoxy derivative exist as keto-enol tautomers (Scheme 5.2) [25]. Curcumin is a fluorescent molecule [22], giving the curry sauces their characteristic yellow color. As a widely edible compound, curcumin is highly biocompatible and biodegradable, as it gets metabolized very easily [26]. Various other pharmacological activities including anti-inflammatory, anti-carcinogenic and anti-oxidant activities of curcumin have also been reported [24, 27-30]. It has also shown some effectiveness against cystic fibrosis [31],
Alzheimers disease [32], malaria [33] and rheumatoid arthritis [34], in addition to its anti-HIV activities. The anti-inflammatory function of curcumin is regarded as one of its most important properties and numerous review articles have been published on this activity of curcumin [35].

Scheme 5.1. Chemical structure of curcumin.

Scheme 5.2. Keto-enol tautomerism of curcumin.

In this chapter, the synthesis and characterization of a new class of silica-curcumin organic-inorganic hybrid materials are presented. The organic curcumin phase was introduced in the silica during the polycondensation of the hydrolyzed silicate. The
two phenolic hydroxyl groups in curcumin are capable of covalently linking to the silanol groups during the sol-gel polycondensation process. This new class of inorganic-organic silica hybrids may find wide applications in implant and scaffold materials, with the curcumin phase rendering the hybrid materials with reduced inflammation properties [23]. It is assumed that curcumin, being highly biocompatible and biodegradable, will also impart higher biocompatibility and biodegradability to the silica materials.

5.2. General methodology

Sol-gel chemistry has been utilized in this work to prepare the silica-curcumin hybrid materials. In the sol-gel process, a liquid phase, which is mostly colloidal in nature, transforms to a gel phase, which leads to solid phase end products. In the first step of this process, a liquid metal alkoxide precursor (TEOS in our synthesis) is hydrolyzed to yield silanol species (Scheme 5.3), which then undergo polycondensation to form soluble oligomeric intermediates of silicates called sol.

![Scheme 5.3. Acid-catalyzed hydrolysis reaction of TEOS with water.](attachment:scheme53.png)

The polycondensation process can proceed through condensation reactions with other hydrolyzed TEOS molecules (Scheme 5.4) or with non-hydrolyzed TEOS molecules (Scheme 5.5).
Scheme 5.4. Self-condensation reaction of hydrolyzed TEOS molecules.

Scheme 5.5. Condensation reaction of hydrolyzed TEOS with non-hydrolyzed TEOS molecule.

Finally, these intermediates link together in a three dimensional network to form a gel. During the gelling process, polymerization occurs through condensation reactions to produce water or ethanol as byproducts (Scheme 5.4 and Scheme 5.5). In our hybrid material synthesis, these condensation reactions of the hydrolyzed silica sol were carried out in the presence of curcumin. A curcumin molecule contains two hydroxyl groups, which can undergo covalent bonding with the silanol (Si-OH) groups through condensation reactions. The condensation of silanol groups with the hydroxyl groups of 2-hydroxyethyl methacrylate (HEMA) and glycidyl methacrylate (GMA) in sol-gel reactions has been reported [9, 36].
In our reaction system, the sol-gel reaction of TEOS was allowed to proceed in the presence of curcumin, until the inorganic-organic phase separation disappeared, leading to a homogenous reaction mixture. The hydrolysis of TEOS led to the generation of silanol groups, which then reacted with other silanol groups as well as the hydroxyl groups of curcumin (Scheme 5.6). This allowed the covalent incorporation of curcumin in the silica network.

Scheme 5.6. Condensation reaction of hydrolyzed TEOS with curcumin. The phenolic OH groups of curcumin react with the free silanol groups of the silica.

5.3. Experimental section

5.3.1. Materials
Tetraethyl orthosilicate (TEOS, 99%) was obtained from Sigma Aldrich. Curcumin (98+ %, cat. # 218580100) and tetrahydrofuran, 99.9%, anhydrous (THF cat. # 181500010) were obtained from Acros Organics. Hydrochloric acid (HCl) was obtained from Fisher Scientific. Tetrahydrofuran (THF) was purified by distillation and stored with molecular sieves. All the other reagents were used as received without further purification.

5.3.2. Synthesis of silica-curcumin hybrid materials

Silica-curcumin hybrid materials were prepared by the acid catalyzed sol-gel reactions of tetraethyl orthosilicate (TEOS) in the presence of curcumin. In a typical procedure for preparing silica-curcumin hybrid materials with 35% curcumin content, 0.80 g of curcumin (0.0020 moles) was added to 8.0 ml anhydrous THF. The mixture was stirred under nitrogen until the curcumin dissolved completely to give a clear yellow solution. In a three-necked round-bottomed flask, 0.9 g H₂O and 0.06 g of 2M HCl was added. This mixture was stirred for 5 minutes, and then, the curcumin solution was added to it dropwise. The color of the curcumin solution changed from yellow to clear dark yellow. The solution was stirred for 5 minutes to homogenize, and then 5.68 g of TEOS (0.025 moles) was added to the solution dropwise, at room temperature. The TEOS to curcumin molar ratio in this mixture was 12.5:1. The solution turned turbid after the TEOS addition, and the temperature of the solution was 30 °C. The solution was allowed to stir under nitrogen. After 10 minutes of stirring, the solution cleared out, with the temperature rising to 33 °C. The reaction mixture turned cloudy again for an instant, then finally became homogenous and cleared out, with the temperature rising to 36 °C. The solution color became very dark red. This reaction mixture was refluxed at 60 °C under
nitrogen for 4 hours. After refluxing, the clear dark red solution was allowed to cool to room temperature. Upon cooling, the solution was poured into a 100 ml beaker, and allowed to gel at room temperature. The solution gelled in 8 hours. After gelling, the sample was further cured in an oven at 70 °C overnight (8 hours). Then, the glassy sample was grinded using a mortar and pestle to a fine powder, and stored for further analysis. The total curcumin content (theoretical) in this sample was 35% with respect to the silica content. This theoretical curcumin content was calculated assuming complete conversion of the TEOS to SiO$_2$ in the sol-gel reactions.

Samples with 12%, 20%, 35% and 50% curcumin contents were prepared using the above procedure. Sample with 0% curcumin content was also synthesized as a control. The nomenclature of the samples, along with the molar ratio of curcumin to TEOS and the curcumin content are listed in Table 5. 1.

5.4. Characterization

FTIR spectra were recorded using a Perkin-Elmer Spectrum One FTIR spectrometer (Perkin-Elmer Co., Norwalk, CT) in the attenuated total reflectance (ATR) mode. Thermogravimetric analysis (TGA) was performed on a TA Q50 instrument (TA Instruments Inc., New castle, DE). The samples were run in air at a flow rate of 40 ml/minute. Before the TGA run, samples were grinded to fine powder and dried in a vacuum oven at 80 °C for 24 hours. Differential scanning calorimetry (DSC) measurements were performed on a TA Q100 instrument (TA Instruments Inc., New castle, DE). The samples were run in nitrogen at a flow rate of 50 ml/minute. Before the run, all samples were grinded into fine powder and dried in a vacuum oven at 80 °C for
24 hours. Typical TGA and DSC samples weighed ~ 5-10 mg. TA Universal Analysis 2000 software was used to analyze all TGA and DSC data. UV-Vis spectra were obtained on a Perkin-Elmer Lambda 35 UV-Vis spectrometer. The absorption was recorded between 300 nm and 900 nm at a scan speed of 480 nm/minute. The slit width of the detector was 1 nm. Fluorescence spectra were obtained on a Hitachi F-7000 FL Spectrophotometer. A wavelength scan was performed at a scanning speed of 1200 nm/minute, with an excitation wavelength of 425 nm. The emission was recorded between 445 nm and 700 nm. The excitation and emission slit width were 5 nm.

5.5. Results and discussion

5.5.1. Fourier Transform Infra Red (FTIR) analysis

Figure 5.1 shows the FTIR spectra of washed silica-curcumin hybrid sample (35%) along with pure curcumin and pure silica samples. For the silica-curcumin hybrid materials (Si curcumin), the broad peak observed at 3560 cm\(^{-1}\) is representative of the phenolic – OH group. The peak at 2800 cm\(^{-1}\) corresponds to the C–H methyl stretch of the curcumin molecule. The peak at 1607 cm\(^{-1}\) corresponds to the C=C aliphatic stretch and the peak at 1508 cm\(^{-1}\) corresponds to the C=C aromatic stretch of the curcumin molecule. These representative peaks in the silica-curcumin hybrid confirm the presence of curcumin in the hybrids.

5.5.2. Thermogravimetric analysis of as-synthesized silica-curcumin hybrids

The curcumin content in the as-synthesized hybrid samples were verified by the weight loss observed in the thermogravimetric analysis (TGA). The TGA thermographs of the as-synthesized samples are shown in Figure 5.2. The samples were heated at 10°C/
min to 1000°C, and the weight losses obtained are reported as Weight % Curcumin TGA (as synthesized) in Table 5.1. The TGA thermograph of pure curcumin is showed in Figure 5.3. From the figure, we can see that curcumin shows complete weight loss in the temperature range of 300 °C – 600 °C. Since only silica remained at 1000 °C, it can be concluded that the weight losses observed in the hybrid samples in the 300°C - 600°C temperature range are due to the degradation of the curcumin present in the samples. The weight loss observed < 300°C can be attributed to the vaporization of volatile compounds like ethanol (by-product during synthesis) and water. From the data listed in Table 5.1, we can see that the curcumin content in our as-synthesized samples approximates to the amount of curcumin added during the synthesis of the hybrid materials. It is noted that the 0% sample (control sample without any curcumin) shows a weight loss of <10%. This observed weight loss in the 0% sample could be partially attributed to the degradation of un-reacted ethoxy groups present due to incomplete sol-gel reactions [37], and partially to the vaporization of water molecules present in the sample that were tightly bound to the silica matrix [38].

5.5.3. Extraction of un-reacted curcumin

The extent of curcumin incorporation into the silica matrix was studied by washing the as-synthesized silica-curcumin hybrid materials in acetone to wash away any un-reacted curcumin. The samples were washed exhaustively with acetone for 9 days, until no further weight loss was observed. After washing, the samples were dried in a vacuum oven at 50 °C for two days to remove the acetone from the samples.
5.5.4. Thermogravimetric analysis of the washed silica-curcumin hybrid materials

The curcumin content in the washed hybrid materials were determined from the weight loss obtained in TGA. Figure 5.4 shows the TGA thermographs of the washed hybrid samples. In the TGA run, the samples were heated at 10 °C/minute to 1000 °C, and the weight losses obtained are listed as Weight % Curcumin TGA (washed) in Table 5.1. As discussed in the earlier section, the weight loss observed in the 200 °C – 600 °C temperature range corresponds to the degradation of curcumin present in the samples. The TGA thermographs clearly exhibit that after extensive washing with acetone, curcumin in retained in our hybrid samples. From the data listed in Table 5.1, it is demonstrated that the amount of curcumin incorporated into the silica matrix depended on the curcumin content added during the sol-gel synthesis of the hybrid materials. In the samples with lower curcumin contents, i.e. 12% and 20% samples, the amounts of curcumin incorporated, as listed in Table 5.1 were 11% and 17%, respectively. However, in the sample with high, 50% curcumin content, the amount of curcumin incorporated, as observed in the TGA weight loss (Table 5.1) was only ~15%. The 35% sample (curcumin content of 35%) shows 27% incorporated curcumin.

Hence, from the thermogravimetric analysis, it can be concluded that curcumin has been incorporated into the silica matrix during the sol-gel synthesis of the hybrid materials. The retained curcumin in the hybrids even after extensive washing with acetone definitely suggests covalent linkage of curcumin with silica. The amount of curcumin incorporated into the silica matrix depended on the amount of curcumin added during the synthesis of the hybrid materials. Lower curcumin content samples (12% and
(20%) showed higher degree of curcumin incorporation, compared to high curcumin content samples.

5.5.5. Differential scanning calorimetry of silica-curcumin hybrid materials

Differential scanning calorimetry (DSC) measurements were performed to study the covalent incorporation of the curcumin in the hybrids. The samples were heated at 15 °C/minute to 300 °C in nitrogen and the DSC thermographs of the hybrids obtained are shown in Figure 5.5. The DSC thermograph of pure curcumin is shown in Figure 5.6. It shows a sharp endothermic peak at ~180°C. This endothermic peak corresponds to the melting point of curcumin [39, 40]. However, in the DSC thermographs of the hybrid materials (Figure 5.5), this sharp endothermic peak is absent. There is a very broad endothermic peak at ~150°C, which is also present in the DSC of pure silica (0% sample). This can be attributed to the vaporization of water, ethanol and un-reacted ethoxy groups present due to incomplete sol-gel reactions [1]. The DSC thermograph of a physical mixture of silica (sol-gel synthesized from TEOS) and curcumin is shown in Figure 5.6. The sample clearly exhibits the sharp endothermic melting peak of curcumin at ~180 °C, in addition to the broad endothermic peak as observed in the pure silica sample. The absence of this clear calorimetric melting peak in the hybrid materials implies the formation of homogeneous dispersion of curcumin in silica matrix with possible silicate–curcumin covalent bonding [40].

5.5.6. Fluorescence analysis of the silica-curcumin hybrid materials

Curcumin has intrinsic fluorescence characteristics and fluorescence spectroscopy was used to study the binding of curcumin to the silica matrix. Figure 5.7 shows the
fluorescence spectra of pure curcumin and silica-curcumin hybrid materials in DMSO. The silica-curcumin hybrids were sparingly soluble in DMSO, while pure curcumin was highly soluble in DMSO. Both samples were excited at a wavelength of 425 nm. The pure curcumin showed a fluorescence peak at 520 nm. The hybrid material showed a red shifted peak at 540 nm.

A shift in the fluorescence peak suggests binding of the curcumin molecule with other substrates [24, 41]. A blue shift in the fluorescence peak has also been reported, when curcumin is bound to hydrophobic surfaces [22, 24, 41]. Hence, the curcumin, in our system, could be covalently bonded to silicate networks, which might result in the observed red shift of the fluorescence peak of curcumin from 520 nm to 540 nm. The fluorescence study thus suggests the covalent bonding of the organic curcumin phase to silicate in the hybrid materials.

5.6. Conclusions

In this work, a new class of silica-curcumin inorganic-organic hybrid materials was prepared via sol-gel reactions of tetraethyl orthosilicate as the silica precursor. Curcumin was inducted into the inorganic silica matrix by reacting the phenolic hydroxy groups of curcumin with the silanol groups during the sol-gel synthesis. The retention of curcumin in the hybrid materials as verified by TGA analysis, after washing them in acetone, as well as the representative IR peaks of curcumin in the acetone washed hybrid samples confirm the incorporation of curcumin in the silica matrix. DSC analysis and fluorescence studies of the hybrid system also suggest bonding of the curcumin (organic phase) to the inorganic silicate.
The inorganic-organic hybrid materials presented in this work represent a new class of bio-based hybrids, where the organic phase is highly biocompatible and biodegradable, and is obtained from a renewable plant source. These bio-based silica hybrids may find potential applications in implant and scaffold materials for tissue engineering with anti-oxidant and reduced inflammatory properties.

5.7. Acknowledgment

I am indebted to Prof. Hai-Feng Ji of the Chemistry Department, Drexel University, for letting me use his fluorescence instrument, and also for his valuable suggestions related to the project. I am also indebted to Dr. Hong Wang of the Chemistry Department, Drexel University, for helping me with fluorescence measurements. I also thank my former and current lab-mates Dr. Indraneil Mukherjee and Dr. David Berke-Schlessel for very fruitful discussions and valuable suggestions.
Table 5.1. Mole ratio of TEOS:Curcumin, theoretical weight % and the TGA weight % of curcumin in as-synthesized and washed hybrid materials.

<table>
<thead>
<tr>
<th>Sample</th>
<th>TEOS : Curcumin (Mole ratio)</th>
<th>Weight % Curcumin Theoretical(^a)</th>
<th>Weight % Curcumin TGA (as synthesized)</th>
<th>Weight % Curcumin TGA (Washed)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0%</td>
<td>1:0</td>
<td>0</td>
<td>&lt;10</td>
<td>&lt;10</td>
</tr>
<tr>
<td>12%</td>
<td>50:1</td>
<td>12</td>
<td>14</td>
<td>12.5</td>
</tr>
<tr>
<td>20%</td>
<td>25:1</td>
<td>20</td>
<td>23</td>
<td>16.0</td>
</tr>
<tr>
<td>35%</td>
<td>12.5:1</td>
<td>35</td>
<td>34</td>
<td>22.0</td>
</tr>
<tr>
<td>50%</td>
<td>6:1</td>
<td>50</td>
<td>49</td>
<td>16.0</td>
</tr>
</tbody>
</table>

\(^a\) Theoretical Weight % of curcumin in the samples were calculated assuming complete conversion of TEOS to SiO\(_2\) via sol-gel reactions.
Figure 5.1. FTIR spectrum of washed 35% silica-curcumin hybrid sample (denoted as Si Curcumin). The spectra of pure curcumin (denoted as Curcumin) and pure silica (denoted as Pure Si) are also shown.
Figure 5.2. TGA thermographs of the as-synthesized hybrid samples. The TGA samples were run at 10°C/minute and heated to 1000°C in air. A typical TGA sample weighed ~5-10 mg. The sample codes has been described in Table 5.1.
Figure 5.3. TGA thermograph of pure curcumin. The sample was heated to 1000 °C at a rate of 10 °C/minute in air.
Figure 5.4. TGA thermographs of washed hybrid samples. The samples were extensively washed in acetone to remove un-reacted curcumin, until no further weight loss was observed. The washed samples were dried in a vacuum oven at 50 °C for 2 days prior to TGA analysis. The sample codes has been described in Table 5.1.
Figure 5.5. DSC curves of washed hybrid samples. The samples were heated from room temperature to 300 °C in nitrogen, at a heating rate of 15 °C/minute. The washed samples were dried in a vacuum oven at 50 °C for 48 hours, prior to the DSC analysis. Typical DSC samples weighed ~ 5-10 mg. The sample codes has been described in Table 5.1.
Figure 5.6. DSC curves of pure curcumin and a physical mixture of silica and curcumin (denoted as Silica Curcumin mixture).
Figure 5.7. Fluorescence spectra of pure curcumin and silica-curcumin hybrid materials (denoted as Si-cur hybrid) in DMSO. The excitation wavelength was 425 nm, and the emission was monitored from 445 nm to 700 nm.
5.8. References


Chapter 6: Encapsulation of savinase enzyme in mesoporous sol-gel silica for protection against high pH aqueous media

6.1. Introduction

Reactions that are catalyzed by enzymes often proceed under relatively mild conditions of temperature and pressure, compared to chemical catalysis [1]. Most enzymes are eco-friendly and non-toxic. Hence, enzyme catalyzed reactions are becoming increasingly attractive, as they are environmentally beneficial and offer lower energy costs. Owing to the mild reaction conditions, enzymes have been in laundry detergents for over four decades. The application of enzymes in commercial laundry detergents enables specific and safe stain removal, without applying harsh stain treatment conditions [2]. The enzymes eliminate the need of some harmful chemicals in laundry detergents and also bypass high temperature washing, thus creating much safer and efficient washing conditions for fabrics.

In the global market for enzymes, detergent applications hold a considerable 28% of the total market share, second only to food processing applications [3]. However, for an enzyme to be used in detergent application, there are certain criteria to be fulfilled by the enzymes [4]. These include: (a) the maximum activity of the enzyme should be at an alkaline pH; (b) the enzyme must be stable at temperatures as high as 60 °C; (c) the enzyme should be able to remove stains and remain stable in an environment which also has surfactants, bleaches and other detergent ingredients present in it. In the U.S. market, the enzymes mostly used in detergent applications include amylases, proteases and
lipases [4]. Amylases (α-amylase) are used to catalyze the hydrolysis of α-1,4-glucosidic bonds of starch chains, thereby removing starch based stains. Lipases are used for the removal of triglycerides containing soil stains. Proteases catalyze the hydrolysis of peptide bonds in amino acids, thereby removing blood, egg, sweat and other protein containing stains. Among the above mentioned types of enzymes, proteases are used most extensively in detergent formulations [4, 5]. Savinase®, Esperase®, and Ovozyme® are very widely used proteases in laundry detergent applications [5].

For application of enzymes in laundry detergents, a major concern is the storage stability of the enzymes in the detergent [3]. In the high pH environment of detergents, the non-covalent bonds in the enzyme breaks down, leading to unfolding (denaturing) of the polypeptide chains. Other ingredients present in the detergent, namely, cationic surfactants and bleach, in addition to the high pH, lead to deactivation of the enzyme in the detergent [6-8]. Application of immobilized enzyme is potentially an effective way of protecting the enzyme against the harsh detergent environment. This work focuses on the protection of savinase enzyme from the high pH media of detergents, via encapsulation of the enzyme in porous silica host materials.

Savinase® is a protease enzyme secreted by alkalophilic bacterium Bacillus lentus. The enzyme is stable in the pH range of 7-10 and shows activity in the pH range of 8-12 [9, 10]. The enzyme exhibits high thermal stability owing to a large number of weak interactions, which are present due to a large number of internal salt bridges [9, 11].
6.1.1. Considerations for savinase encapsulation in detergent application

For the application of immobilized enzyme in detergents, there are certain criteria, which need to be addressed. Since the immobilized enzyme is stored in the detergent, the encapsulation should provide stability to the enzyme under the high pH condition of the detergent. The encapsulation should also be resistant to surfactants and other detergent ingredients, which are detrimental to the enzyme. Apart from the above-mentioned criteria, the most important aspect of the encapsulation is the release of the enzyme in wash. In detergent application, under wash conditions, the enzyme should be able to come out of the pores of the host materials, and interact with the substrate (stain).

During encapsulation in solid matrix, the enzyme is confined within pores of the host materials. The limited space within pores restricts the unfolding of the peptide chains under high pH conditions. Hence, stability under high pH of detergents is achievable in encapsulated enzymes. During wash, in order for the enzyme to come out of the pores and interact with the substrate, the pore size of the host material needs to be controlled. Pore sizes bigger than the enzyme would facilitate the release of enzymes easily. However, during storage, the enzyme needs to be confined within the pores. Only during washing, it is desired that the enzyme be released. Hence, this trigger mechanism necessitates blocking of the pores, which would hold the enzyme inside pores during storage, and come off only during washing, which would release the enzyme.

6.1.2. Our approach to stabilize savinase in detergents

In this work, an encapsulated savinase enzyme system was developed by direct encapsulation of savinase inside nonsurfactant templated mesoporous sol-gel silica.
Samples with different degrees of porosity were prepared, by varying the template content during synthesis. D-fructose was used as the nonsurfactant template. It also acted as a pore-blocking agent to protect the enzyme inside the pores by preventing the detergent from entering the pores during storage. However, since D-fructose is highly water soluble, during wash, water could easily dissolve the D-fructose, thus removing the pore blockade. This allowed the encapsulated enzyme to come out of the pores during wash, and interact with the substrate.

However, during storage stability test, pore blocking by D-fructose alone did not show the desired degree of stability in a commercial liquid detergent. Over time, the detergent was able to penetrate through the D-fructose layer and deactivate the enzyme inside. Furthermore, silica materials are known to be vulnerable to strong basic media. To lessen or solve these problems, a “double encapsulation” approach was developed. In this method, the enzyme and D-fructose containing encapsulate was further coated with an acrylic polymer layer, which was only sparingly soluble in water. The large dilution only during washing could remove the coating and allow enzyme release in wash water.

6.2. Experimental

6.2.1. Materials

Tetraethyl orthosilicate (TEOS, 98%, cat.# 131903), D-(-)-fructose (99%, cat.# F0127), trishydroxymethyl aminomethane (Trizma base, 99%, cat.# T1378), triethyleneglycol dimethacrylate (TEGDMA, 95%, cat.# 261548), poly(ethylene glycol) (PEG, Mₙ = 4600, cat.# 373001), camphorquinone (CQ, 97%, cat.# 124893) and N-phenylglycine (NPG, 97%, cat.# 330469) were purchased from Sigma Aldrich. The
substrate for enzymatic assay, azocaesin (cat.# A2765), trichloroacetic acid (TCA, 99%, cat.# T6399) and urea (99.5%, cat.# U1250) were also purchased from Sigma Aldrich. The enzyme used was savinase® concentrate (lot # PPA-26291), and was provided to us by Novozymes North America Inc. The liquid detergents used for storage stability were Purex™ Free and Clear provided by Novozymes North America Inc. and Purex™ Original fresh Aroma Fresco with Pure Clean Technology (UPC # 2420004820) purchased from a local grocery store. A pH 12 calcium hydroxide buffer solution (cat.# S11M008) was purchased from Radiometer Analytical. All the reagents were used as received, without any further purification.

6.2.2. Encapsulation of savinase enzyme in mesoporous silica

The encapsulation of savinase enzyme in mesoporous silica was achieved by D-fructose templated acid catalyzed sol-gel reactions of TEOS. In a typical procedure for making 50% fructose templated immobilized savinase sample, 52 g of TEOS (0.25 mol), 9 g distilled water (0.5 mol) and 0.63 g 2M HCl were used with the [TEOS]:[H₂O]:[HCl] molar ratio of 1:2:0.005. In a 250 ml round bottomed flask, 9 g of distilled water and 0.63 g of 2M HCl were charged and magnetically stirred for 5 minutes at room temperature. The round-bottomed flask was fitted with a reflux condenser and a thermometer. After 5 minutes of stirring, 52 g of TEOS was added slowly to the flask under moderate stirring at room temperature. Immediately after TEOS addition, the reaction was maintained under a nitrogen gas (N₂) purge. The reaction mixture initially turned cloudy, and cleared out in 10 minutes, with the temperature increasing to 35 °C. After the initial clearing, the reaction mixture turned cloudy again for a moment and then finally cleared out, with the temperature rising to 60 °C. The reaction mixture was then heated at 60 °C for 1 hour.
under N₂. Assuming complete hydrolysis of the ethoxy groups of TEOS, 15 g of SiO₂ should be generated from 52 g TEOS. After an hour of refluxing at 60 °C, the reaction mixture was allowed to cool down to room temperature. In the hydrolysis of TEOS, ethanol was produced as a byproduct. This ethanol was extracted out of the reaction mixture by applying high vacuum. The high vacuum was applied until the reaction mixture showed 50% weight loss. To the ethanol-extracted mixture, 30 g of 50% D-fructose solution (prepared by dissolving 15 g fructose in 15 g distilled H₂O) were added, and stirred until a clear solution was obtained. This reaction mixture now contained 15 g SiO₂ and 15 g fructose (50% fructose content with respect to silica) in addition to water and trace amounts of ethanol. The reaction mixture was then equally divided (by weight) in three 100 ml beakers. Each beaker should yield sample containing 5 g SiO₂ and 5 g fructose.

For each beaker, 5.4 g of 50% savinase solution in 0.2 M Trizma buffer (pH 8.5) was prepared. These enzyme solutions were added to the respective sol-gel mixtures, and stirred for 5 minutes. After stirring, the beakers were sealed with paraffin film, 20-25 holes were drilled with hypodermic syringe needle in the film and kept at 5 °C. All the solutions gelled within 30 minutes. The transparent yellow colored gels were then dried at 0 °C under high vacuum for 3 weeks, until complete evaporation of volatiles (i.e., water and residual ethanol). The samples were then grinded to 40-mesh size, and kept at 5 °C for further analysis. Encapsulated savinase samples with 70%, 50%, 30% and 0% fructose contents were prepared. The nomenclatures of the samples are described in Table 6.1.
6.2.2. Double encapsulation for encapsulated savinase samples

The savinase enzyme samples, encapsulated in D-fructose templated mesoporous silica, were further coated with another layer of acrylic polymer using photo polymerization. For this double encapsulation (coating the savinase encapsulated silica particles with acrylic polymer layer), 0.5 wt% camphorquinone (CQ) and 0.3 wt% N-phenylglycine (NPG) photo initiators were blended into triethyleneglycol dimethacrylate (TEGDMA) in dark, at room temperature. A yellow colored transparent solution was obtained. Nine parts (by weight) of this solution was then mixed with one part of poly(ethylene glycol) (PEG, Mₙ = 4600). The PEG was completely dissolved in the solution, and then this TEGDMA-PEG solution was mixed with the powders of encapsulated savinase samples in 1:1 (by weight) ratio. This mixture was then smeared as a thin layer onto a glass slide, and photo polymerized under a LED Curedome light curing unit. Curing was done for 4 minutes, with intermediate crushing of the samples after every minute. A light yellow colored coarse powder was obtained, which was further crushed to 30-mesh size. These double encapsulated samples were stored at 5 ºC for further analysis. The outer acrylic polymer coating thus contained 10% (by weight) of PEG. The nomenclatures of the double encapsulated samples are as follows:

50%F-TP: 50% fructose containing savinase encapsulated silica sample coated with acrylic polymer having 10% water-soluble PEG.

70%F-TP: 70% fructose containing savinase encapsulated silica sample coated with acrylic polymer having 10% water-soluble PEG.
6.2.3. Characterization of encapsulated savinase samples

Thermogravimetric analysis (TGA), on a TA Q50 Thermogravimetric Analyzer (TA Instruments Inc., New Castle, DE), was used to determine the contents of template and polymer coating in the single and double encapsulated savinase samples. During analysis, the samples were heated to 800 °C in air at a heating rate of 10 °C/minute. The pore size, surface area and the pore volume of the host silica materials were determined from nitrogen adsorption-desorption measurements on an ASAP 2010 Surface area and pore analyzer instrument (Micrometrics Inc., Norcross, GA). Prior to analysis, the template was extracted out of the samples by extensive washing in excess water and dried under vacuum. The analysis was performed at -196 °C.

6.2.4. Procedure for assaying enzymatic activities of the encapsulated samples

The encapsulated savinase samples were assayed with azocaesin substrate according to the Novozymes 2004-110706-01 method [12]. For a typical procedure, in a 100 ml preparation of azocaesin solution, 0.6 g of azocaesin was dissolved in 10 ml of 50% (w/v) urea solution in water. 10 ml of 2M trizma buffer (pH 8.5) was added, and the volume was adjusted to 100 ml, along with pH adjustment to 8.5 using 1.0 M H₂SO₄. This was the azocaesin substrate solution.

Required amount of the encapsulated powder sample was added into a 100 ml Erlenmeyer flask, such that the amount of savinase in the sample was 30 mg. To this, 50 ml distilled water was added, and the mixture was sonicated for 20 minutes in a Branson 2510 sonicator at room temperature. During the sonication, the enzyme is assumed to come out of the pores, due to washing away of the D-fructose layer, as well as the double
encapsulation layer. After sonication, the solid particles were filtered out and the liquid, which contained the enzyme, was filtered into another 100 ml flask. 1.0 ml of this enzyme solution was taken into a 16 × 150 mm test tube, and incubated at 40 °C in a water bath for 1 minute. 5.0 ml of the azocaesin solution was then added to the enzyme solution, the test tube was shaken in a vortex mixer, and incubated at 40 °C for 30 minutes. The reaction was stopped after 30 minutes, by adding 5.0 ml of 10% aqueous trichloroacetic acid (TCA) solution, and the reaction mixture was allowed to stand. In 15 minutes, all the azocaesin precipitated out, and the solution was filtered into a glass vial. The absorbance of this solution at 390 nm was measured using a Perkin Elmer Lamda2 UV-Vis instrument using distilled water as the reference. This absorbance was reported as A_{390}. All the activity assays has been done 4 to 6 times for reproducibility. The values reported are mean values, and the standard deviation from the mean values are reported in the activity tables.

The assay for pure free savinase was done by the same procedure, starting with 30 mg of free enzyme, instead of the encapsulated powder sample. The A_{390} value of 1.77 was obtained for free savinase. This was taken as 100% enzyme activity.

6.3. Results and discussion

6.3.1. Thermogravimetric analysis (TGA)

The TGA thermographs for the as-synthesized single encapsulated samples are shown in Figure 6.1. The weight losses obtained are reported in Table 6.1. From the data listed in Figure 6.1, we can see that 70%F, 50%F and 30%F samples exhibited weight losses that are approximately in agreement with the stoichiometric amounts of the
template added during synthesis. The sample with 0% fructose content (0%F) exhibited 20% weight loss. This observation is in accordance with weight loss obtained for immobilized cellobiase enzyme with 0% template (Chapter 2, section 6.3.1). It is explained by the presence of un-reacted ethoxy groups and tightly bound water molecules in the silica matrix [13, 14]. This observation is consistently made in all our results. The reason why the sol-gel reactions of TEOS in the absence of fructose always yield more unreacted groups while less in the presence of fructose could be that sugars, such as fructose, somehow facilitate the sol-gel reactions. This postulation will need further research to be firmly validated.

6.3.2. Nitrogen adsorption-desorption

The surface area and other pore parameters of the sample silica matrices were determined from the nitrogen adsorption-desorption experiments and are listed in Table 6.1. The adsorption-desorption isotherms for the samples are shown in Figure 6.2. Before analysis, the template and enzyme were extracted out of the samples by washing extensively in excess water. Figure 6.2 shows that all the samples exhibit type IV isotherm with H2 hysteresis loops, which are characteristic of mesoporous molecular sieves [15-17]. However, compared to 70%F, 50%F and 30%F, the hysteresis loop in 0%F is much less prominent, and has some type II character. Possibly, the enzyme, water and ethanol (sol-gel reaction byproduct) which are present within the matrix during synthesis, rendered partial mesoporosity to the F0 sample. The BJH pore size distribution plots for the samples are shown in Figure 6.3. Though the peak maxima for all the samples lie in the 3-3.5 range, the peak maxima for 0%F is less prominent, compared to other 3 samples. The surface area of the samples showed an increase with increasing
fructose content. This increased surface area can be attributed to the increased density of pore channels, formed by increased fructose content. So, addition of higher fructose content led to higher porosity in the silica matrix.

Hence, from the nitrogen-adsorption results, it is evident that fructose acted as a pore-forming agent during synthesis.

6.3.3. Verification of double encapsulation

As discussed in section 6.2.2, the savinase encapsulated silica samples were further coated with another acrylic polymer layer. The amount of this coating (% content) was verified by weight loss obtained by the double encapsulated samples in TGA. As mentioned earlier in section 6.2.2, the acrylic polymer content in the double encapsulated samples was targeted to be 50%. The TGA thermographs for the double encapsulated samples are shown in Figure 6.1. For determining the acrylic polymer content, the residual SiO$_2$ content of the double encapsulated sample was compared to the residual SiO$_2$ content of the corresponding single encapsulated sample in Figure 6.1. The 70%F-TP sample showed 13% residual SiO$_2$ content. The 70%F sample (single encapsulated) showed 28% residual SiO$_2$ content. Comparing the two residual SiO$_2$ contents, it can be concluded that in 70%F-TP, the targeted 50% acrylic polymer content was achieved.

6.3.4. Enzymatic activity of encapsulated savinase samples

The enzymatic activities of the as-synthesized single and double encapsulated savinase samples are listed in Table 6.2. The absorbance for free savinase at 390 nm ($A_{390}$) was taken as 100% activity, and the activities of all encapsulated samples was reported as percentage with respect to free savinase. As discussed in section 6.2.4, during
the enzymatic activity evaluation, the encapsulated samples were dispersed in water and sonicated, to trigger the release of the enzyme in the water. This enzyme solution (in water) was assayed for enzymatic activity. Hence, our enzymatic activity solely represents the activity of the enzyme that was released from the silica pores into the water. Figure 6.4 shows the enzymatic activity of the encapsulated savinase samples with increasing fructose content. From the results, it is evident that the activity of the samples increased with increasing template (fructose) content. The 70%F showed 75% activity compared to free savinase. The 0%F sample exhibited a significantly lower activity of 16%. This observation can be attributed to the host silica microstructure. As discussed in section 6.3.2, increased fructose content led to an increased number of pore channels in the silica matrix. This template-induced porosity with increased concentration of interconnected pore channels led to easier release of the enzyme. This enzyme release is highly restricted in the F0 sample, due to absence of the template-induced interconnected pores. The restricted enzyme release in 0%F led to its lower enzymatic activity. Another important factor is that the enzyme assay medium with pH of 8.5 could at least partially dissolve the sol-gel silica matrix to release the enzyme. The higher silica porosity should lead to faster dissolution of the silica framework, which in turn resulted in higher enzymatic activities as observed.

6.3.5. Storage stability of encapsulated savinase samples in commercial detergent

As mentioned earlier in the introduction section, the main objective behind the encapsulation work was the protection and stabilization of the savinase enzyme in a commercial laundry detergent. For the stability test of our encapsulated samples, Purex™
*Original fresh Aroma Fresco* with *Pure Clean Technology* detergent was used. The detergent had a pH of 10.8.

For the enzymatic activity of immobilized savinase in the detergent, appropriate amount of immobilized samples was taken in a 15 ml Falcon tube, such that the amount of savinase was 30 mg. To this, 5 g of the detergent was added; the tube was sealed and placed on a Blood Rocker (Lab-Line Maxi Rotor) for the desired duration. After the completion of intended time period, the contents of the falcon tube (immobilized sample + Purex detergent) were poured into a 100 ml Erlenmeyer flask. The Falcon tube was rinsed with 50 ml distilled water (in 10ml portions) to transfer all contents of the tube to the flask. After the transfer, the contents in the flask (immobilized savinase + detergent +distilled water) were sonicated for 20 minutes. After sonication, the liquid phase (released enzyme + distilled water + detergent) of the mixture was filtered out. This filtrate was then assayed for enzymatic activity as described in section 6.2.4.

For our storage stability measurements, the immobilized savinase samples and also free savinase was stored in the Purex detergent for 1, 2 and 4 weeks. The residual activity of the samples after the intended time periods is listed in Table 6.3. A plot of the residual activity with time is shown in Figure 6.5. As can be seen from Figure 6.5, the residual activity of free savinase drops to ~20% in 1 week. The 0%F and 30%F samples had comparatively lower initial activities to begin with. This was because of lower porosity of the silica matrix, which restricted the enzyme release in 0%F and 30%F samples. The residual activities of 70%F and 50%F samples (which had higher initial activity) also dropped down to ~22% and 10% respectively in 4 weeks. Hence, from the above results, it is evident that the single encapsulation did not provide stability to the
savinase enzyme during detergent storage. Out of the 4 different single encapsulated samples, 70%F and 50%F showed somewhat better results, but still they all were not stable enough for 4 weeks of storage. Hence, from the storage stability of single encapsulated samples, it is evident that over an extended period of time, the detergent was able to penetrate through the fructose layer (which acted as a template and pore-blocking agent), and deactivate the enzyme. The high water content in the detergent probably led to the dissolution of the fructose during storage, which exposed the enzyme to the detergent. However, to counter this problem, decreasing the fructose content in the samples was not a feasible option. Decreased fructose content would lead to decreased porosity, which was a major factor in enzyme release, as discussed in section 6.3.4.

To enhance the stability of our immobilized savinase, the 70%F and 50%F single encapsulated samples were double encapsulated. 70%F and 50%F samples were used as they exhibited the better activities in section 6.3.4. These double encapsulated samples, when used in the study of storage stability, exhibited enhanced residual activities. The residual activity of 50%F-TP dropped down to ~32%, but the 70%F-TP sample maintained high residual activity and showed a residual activity of ~ 61% after 4 weeks in the harsh liquid detergent medium of pH 10.8. Hence, it is evident that the acrylic polymer layer provided enhanced stability over the single encapsulated samples in commercial detergent, with the 70%F-TP formulation being the best.

6.3.6. Mechanism of enzyme protection in double encapsulation

As discussed earlier, the primary objective of this work was to stabilize the savinase enzyme in a commercial laundry detergent, via encapsulation. In the double
encapsulation process, the savinase loaded fructose templated silica matrix was further coated with an acrylic polymer layer. This outer layer consisted of a crosslinkable acrylic polymer and poly(ethylene glycol) (PEG). A pictorial illustration of the double encapsulation is provided in Figure 6.6. An acrylic polymer was chosen as the outer layer, as they have high alkali resistance ability. In the acrylic polymers, the C–O bond, which is susceptible to alkali, is on the main chain and is sterically protected. This gives the polymers increased resistance against high pH media [18].

The polymer used in our double encapsulation was a crosslinkable acrylate polymer, which was polymerized from a difunctional acrylic monomer TEGDMA. The structure of TEGDMA is shown in Scheme 6.1.

![Scheme 6.1: Structure of triethyleneglycol dimethacrylate (TEGDMA).](image)

The crosslinking ability of the polymer was an important issue. Since detergent formulations contain large proportion of water, the outer coating layer was desired to be water insoluble. This would keep the coating intact during storage in detergent. In our formulation of the outer layer, TEGDMA was mixed with PEG, and the mixture was photopolymerized on the savinase containing silica particles. The PEG ($M_n = 4600$) used was water soluble, and acted as a binder in the acrylic polymer layer during storage in detergent. This acrylic polymer-PEG layer restricted the exposure of fructose to the
detergent during storage. However, during the wash, large dilution and extensive agitation resulted in the dissolution of the PEG. This created voids or breaks in the acrylic polymer layer (Figure 6.6), resulting in its complete collapse due to extensive agitation in water, thus exposing the fructose (which blocked the pores of the silica matrix) to the aqueous media. The fructose was readily dissolved, making the pores de-blocked for the enzyme to come out.

The double encapsulation approach was thus successful in retaining high residual activity of the encapsulated savinase in commercial detergent. This double coating layer was designed for added protection to the single encapsulated samples during storage in high pH aqueous media. It cannot increase the activity of a single encapsulated sample. From the data listed in Table 6.2, we can see that the activity of 50%F-TP and 70%F-TP are marginally higher than 50%F and 70%F, respectively. This was possibly due to sampling issues, arising because of irregular distribution of the enzyme in the silica matrix. It should be noted that the stability in storage and the fast release of enzymes in wash are mutually contradictory. The system we have developed demonstrates the feasibility of achieving a balance of these 2 adversary factors. Further research might lead not only new detergent products, but also new general methodology for enzyme encapsulation.

6.4. Conclusion

In this work, savinase enzyme was encapsulated in mesoporous silica host materials via acid catalyzed non-surfactant templated sol-gel route. D-fructose was used both as template and as pore-blocking agent. The encapsulated of savinase enzyme was
designed for use in commercial laundry detergent. The encapsulation formulation was designed to trigger enzyme release from the silica pores under large dilution during washing. From the nitrogen adsorption-desorption results, it is evident that the savinase was encapsulated in mesoporous silica matrix. The enzyme release from the pores of the silica host materials was largely affected by the fructose content in the formulation. With increased fructose content, the porosity of the silica matrix increased, as evidenced from nitrogen adsorption-desorption measurements. The increased porosity led to easier enzyme release. With 70% fructose content (70%F sample), enzymatic activity of 75% (with respect to free savinase) was obtained.

The encapsulated savinase samples were tested for storage stability in a commercial liquid laundry detergent with very high pH values of 10.8. The activities of the encapsulated samples showed a sharp decrease with time, during storage in detergent because of denaturation of the enzyme molecules. In an effort to maintain the activity of the encapsulated samples in storage, the samples were further coated with TEGDMA/PEG polymer layer. This double encapsulation provided enhanced stability to the encapsulated samples during storage in detergent. A residual activity of 61% was obtained for the polymer coated sample (70%F-TP) after 4 weeks of storage in the detergent. Further exploration of such a double-encapsulation technique might lead to new enzyme encapsulation and stabilization methods.

6.5. Acknowledgment

This work was a collaborative effort with Mr. Tom Hughes and Mr. Collin Murray from Reacta Corporation, Dr. Solomon Praveen of Einstein Medical College, and
Dr. Indraneil Mukherjee from Kraft Foods Inc. Dr. Mukherjee, who was my lab
colleague during this work, was largely responsible for the double encapsulation
approach. I am also indebted to Dr. Andreas Mylonakis and Dr. Alpa Patel for very
resourceful discussions. I am also thankful to Dr. Ole Simonsen and Dr. Vic Casella from
Novozymes North America Inc. for their valuable suggestions. Partial financial support
for this project from Novozymes is gratefully acknowledged.
Table 6.1. TGA weight loss, pore diameter, surface area and pore volume of the single encapsulated savinase samples with different fructose content.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Weight % fructose added</th>
<th>TGA weight loss (%)</th>
<th>BJH desorption pore diameter (nm)</th>
<th>BET Surface area (m²/g)</th>
<th>Pore volume (cm³/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0%F</td>
<td>0</td>
<td>20</td>
<td>3.2</td>
<td>473</td>
<td>0.30</td>
</tr>
<tr>
<td>30%F</td>
<td>30</td>
<td>41</td>
<td>3.3</td>
<td>538</td>
<td>0.39</td>
</tr>
<tr>
<td>50%F</td>
<td>50</td>
<td>55</td>
<td>3.3</td>
<td>588</td>
<td>0.48</td>
</tr>
<tr>
<td>70%F</td>
<td>70</td>
<td>72</td>
<td>3.5</td>
<td>651</td>
<td>0.48</td>
</tr>
</tbody>
</table>
Table 6.2. Activity of single and double encapsulated savinase samples with different fructose content.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Enzymatic activity&lt;sup&gt;a&lt;/sup&gt; (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0%F</td>
<td>16 ± 29</td>
</tr>
<tr>
<td>30%F</td>
<td>40 ± 11</td>
</tr>
<tr>
<td>50%F</td>
<td>66 ± 10</td>
</tr>
<tr>
<td>70%F</td>
<td>75 ± 7</td>
</tr>
<tr>
<td>50%F-TP</td>
<td>75 ± 13</td>
</tr>
<tr>
<td>70%F-TP</td>
<td>79 ± 3</td>
</tr>
</tbody>
</table>

<sup>a</sup> Enzymatic activity (%) was calculated using the activity of free savinase (as described in section 6.2.4) as 100% activity.
Table 6.3. Storage stability of single and double encapsulated savinase samples in commercial detergent.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Residual activity a (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 week</td>
</tr>
<tr>
<td>Free savinase</td>
<td>23±3</td>
</tr>
<tr>
<td>0%F</td>
<td>7±2</td>
</tr>
<tr>
<td>30%F</td>
<td>31±8</td>
</tr>
<tr>
<td>50%F</td>
<td>29±8</td>
</tr>
<tr>
<td>70%F</td>
<td>46±8</td>
</tr>
<tr>
<td>50%F-TP</td>
<td>75±9</td>
</tr>
<tr>
<td>70%F-TP</td>
<td>77±1</td>
</tr>
</tbody>
</table>

a All activities were calculated based on activity of free enzyme (as described in section 6.2.4) as 100%.
Figure 6.1. TGA weight loss of as-synthesized encapsulated savinase samples.
Figure 6.2. Nitrogen adsorption-desorption isotherms of the encapsulated savinase samples. Before analysis, the template (fructose) was extracted out by extensive washing in excess distilled water.
Figure 6.3. BJH desorption pore size distribution plot for the savinase encapsulated silica matrix. Before analysis, fructose was extracted out of the silica matrix by extensive washing of the samples in a large excess of distilled water.
Figure 6.4. Variation of enzymatic activity of encapsulated savinase samples with increasing template (fructose) content in the samples.
Figure 6.5. Activity profile of single and double encapsulated savinase samples with time in Purex™ Original fresh Aroma Fresco with Pure Clean Technology.
Figure 6.6. A pictorial illustration of the double encapsulation approach and its mechanism for enzyme release during washing [19].
References


Chapter 7: Overall Summary and Future Work

This research work has primarily focused on the development of immobilized enzyme systems and silica hybrid materials for various catalytic and bio-based applications. In the second chapter, cellobiase enzyme was successfully immobilized in situ in silica materials via sol-gel processing with tetraethyl orthosilicate (TEOS) as the silica precursor. D-fructose was used as the template or pore-forming agent, and different pore sizes, surface areas and pore volumes were obtained by varying the template content. These parameters defined the activity of the immobilized enzyme, as they restricted the diffusion of the substrate to the enzyme caged within the host silica microstructure. The results indicated the generation of a highly efficient cellobiase catalyst system. The easy separation of the developed enzyme catalysts from the reaction mixture, and their reusability has been successfully demonstrated in this work.

The third chapter of this dissertation focused on the enzymatic hydrolysis of biomass with the use of the newly developed immobilized cellobiase enzyme catalyst system. The efficiency of the immobilized enzyme system, in hydrolysis of pretreated biomass, compared to free cellobiase enzyme has been studied. The results indicated high efficiency of the immobilized catalyst system. A biomass hydrolysis experimental set-up was designed to enable easy separation of the catalyst, to enable multiple use of the enzyme catalyst. The high efficiency of the immobilized enzyme systems, coupled with their reusability opens up great potential in process cost reduction of enzymatic
hydrolysis of renewable sources (i.e. biomass) to generate cellulosic ethanol for biofuel production.

The fourth chapter focused on the immobilization of lipase enzyme in nonsurfactant templated sol-gel silica materials. The results indicated reusability of the developed lipase enzyme catalyst. The sol-gel silica materials generated in this work are hydrophilic. Since most lipase enzyme substrates are hydrophobic in nature, further optimization of the process, to encapsulate the lipase enzyme organic-inorganic hybrid silica, rather than pure inorganic sol-gel silica materials might result in higher enzymatic activities.

The fifth chapter of this dissertation focused on the synthesis of silica-curcumin inorganic-organic hybrid materials, via the sol-gel route. The organic curcumin phase in the newly developed hybrid materials is highly biocompatible and biodegradable, and is obtained from a renewable plant source. The developed materials were characterized by thermal analysis and spectroscopic methods and the degree of incorporation of the curcumin phase in the hybrid materials has been studied. These bio-based organic-inorganic silica hybrids may find wide potential applications in implant and scaffold materials for tissue engineering with reduced inflammatory properties. Further optimization and incorporation of templates during the synthesis of the hybrid materials may lead to mesoporous silica-curcumin hybrid materials with enhanced catalytic, drug-delivery and bio-based applications.

In the sixth chapter, stabilization of savinase enzyme via in situ encapsulation in mesoporous silica, in high pH commercial laundry detergents has been studied. A double
encapsulation (acrylic polymer coating of the enzyme encapsulated silica particles) technology has been developed, to stabilize the enzyme during storage in laundry detergents. The encapsulation formulation was specifically designed to protect the enzyme from the detergent media during storage, and trigger the release of the enzyme from the silica host materials during the wash cycle, which involves large dilution of the detergent. This work demonstrates the feasibility of achieving a balance between the storage stabilization and fast release of the enzyme in detergents. Further optimization of the encapsulation process might lead to new detergent formulations and enzyme immobilization methodologies.
Appendix A: An Independent Research Proposal, which was part of requirements for Ph.D. candidacy, and was defended successfully on November 5th, 2008.

**Flux and fouling resistance improvement in thin film composite (TFC) polyamide reverse osmosis membranes**

*Abstract:*

In reverse osmosis, the main goal of current research is to increase the water permeability of the membranes for energy savings and to have membranes with more fouling-resistant surfaces to maintain permeability over an extended period of time [1]. This work proposes the development of a hybrid reverse osmosis membrane, designed to address the above-mentioned aspects of flux improvement and microbial biofouling reduction at the same time. The water permeability is improved by the incorporation of hydrophilic surface-modified multiwalled carbon nanotubes (MWNT) in the nonporous polyamide thin film layer, which acts as a coating on the porous polysulfone supports. The surface of the nanocomposite thin film is then further modified and grafted with hydrophilic polyethylene glycol (PEG) chains to improve the fouling resistance of the thin film surface.

A.1. Introduction

Developing an effective and efficient water purification technology for desalination of water and separation of numerous organic compounds is a major challenge to science and engineering. The USEPA studies have identified more than 160 organic compounds in water systems throughout the US [2]. In recent years, reverse osmosis (RO) has become a major water purification technology for cleaning water from non-traditional water
sources [1]. In normal osmosis process, solvent flows through a membrane, from a region of low solute concentration to a region of high solute concentration, under no external pressure. Reverse osmosis (RO) is a separation process in which a solution is allowed to flow under an external pressure through a semi permeable membrane, which retains the solute on one side and allows the solvent to pass to the other side. Hence, RO allows solvent to go from a high solute concentration to a region of low solute concentration, because of the applied external pressure, which is in excess of the osmotic pressure. Across semi permeable membranes, the diffusion of solutes present in a feed stream will be much slower (or not at all) than the water, leading to solute-free permeate stream.

A.1.1. **Solute rejection in reverse osmosis:**

The rejection of various organic compounds and salts in reverse osmosis follows complex mechanisms and is not fully understood [3]. It is generally believed that in RO, rejection of dissolved solutes in a solution occurs through mainly two mechanisms [1,2,3,4,5,6]:

- Restricted solute diffusion through RO membrane due to electrostatic interactions between solute and membrane surface;
- Restricted solute transport due to steric hindrance.

For charged solutes or ions (salt solutions), the rejection takes place due to electrostatic interaction between the membrane and the charged solute particles. The Donnan exclusion method [6] explains this type of rejection. In this method, when a membrane with fixed charge on its surface is placed in a salt solution, equilibrium is reached between the membrane and the solution. The concentration of the counter ions with
respect to the membrane charge is higher in the membrane–solution interface than in the bulk solution phase. The co–ion concentration with respect to the membrane charge is lower in the interface than the bulk solution phase. To counteract the transport of counterions to the bulk phase and co–ions to the membrane interface, a potential difference at the membrane solution interface, called the Donnan potential is created. When a pressure gradient is applied across the membrane, water flows through the membrane. The co–ions are repelled from the membrane–solution interface by the Donnan potential. The counter–ions are also rejected due to electroneutrality in the solution, leading to salt retention.

For filtration of uncharged solutes, the solute particle transport occurs by convection due to pressure gradient and by diffusion due to a concentration gradient across the membrane. The retention of solute particles by the membrane occurs due to a sieving mechanism. This is explained by the preferential sorption–capillary flow mechanism [2]. In this method, the solute is adsorbed onto the membrane surface. Then, solute transport takes place through the pores of the membrane by diffusion or convection. The factor that influences the migration of solute through the membrane the most is the steric interaction. The membrane pore size and the molecular geometric size of the solute give rise to rejection of solute by the membrane.

A.1.2. Membranes for reverse osmosis:

The membranes used for RO can be classified into two different types: composite membranes and asymmetric membranes. A composite membrane typically consists of a thick porous polymeric layer. This porous, non–selective layer is coated with a thin skin layer, which is of different chemical composition. Figure A.1 gives a schematic diagram
of a typical commercial thin film composite (TFC) membrane. The reinforcing base layer is a woven or non-woven fabric, which is coated with an anisotropic microporous polymer. This microporous layer, usually polysulfone [7], is coated with an ultrathin polymeric skin layer. This thin skin layer is the active layer that provides controlling properties as to semi permeability.

Figure A.1: Schematic diagram of a Thin Film Composite (TFC) membrane [7].

Asymmetric membranes consist of a dense skin on a porous sub-layer. The skin and the sub-layer underneath are of the same chemical composition. They are usually formed from a polymer–containing dope, which is cast into a homogenous film by a single step-phase inversion method [7]. It should be mentioned here that for the practical application of RO in water purification, the surface layer of the RO membrane should be as thin as possible to minimize the resistance to water flow to the porous layer underneath.
A.1.3. **Advantages of composite membranes over asymmetric membranes:**

Relative to the asymmetric membrane, the composite membrane approach has some key advantages.

- In the composite membrane approach, the ultra thin barrier skin layer and the porous layer can be optimized independently for maximum performance capability.
- A vast kind of chemical compositions can be formed into extremely thin skin barrier layers [1,7].
- The skin layer can be chosen to have a chemical composition that promotes a combination of high solvent flux and high solute rejection.
- The porous support layer can be chosen to give high strength and compression resistance along with low resistance to permeate (solvent) flow.
- Both linear and crosslinked polymers can be used as the thin skin layer. However, in asymmetric membrane approach, the skin and the sub layer have the same chemical composition. Only a few polymers have the right combination of flux and solute rejection, quite limited to linear, soluble polymers [7], mainly cellulose acetate and linear polyamides.

Various polymers have been tested for their suitability for RO membranes. Currently, thin film composite (TFC) polyamide membranes have become the most important and widely used membrane for RO [8] and are accepted widely as the optimal system for RO membranes [9]. In TFC polyamide membrane, the ultra thin skin barrier layer is mostly
an aromatic polyamide. It is generally prepared by interfacial polymerization of an aromatic polyamine, m–phenylene–diamine (MPD) with aromatic polyacyl halide, like trimesoyl chloride (TMC). The microporous polymeric layer underneath the skin layer is usually polysulfone.

![Chemical structure of polysulfone membrane.](image)

Figure A.2: Chemical structure of polysulfone membrane.

A.1.4. **Interfacial polymerization:**

Interfacial polymerization is a condensation polymerization, in which the polymerization occurs at the interface of two liquid phases, each containing one reactant. The temperature range usually employed for this process is between 0°C to 50°C. The two liquid phases are immiscible, one is an aqueous phase and the other is the organic phase. The monomers dissolved in the aqueous phase and the organic phase diffuse to the interface of the two liquids, and polymerization occurs at the interface. In polyamidation, the diamine is dissolved in water, and the acid chloride is in the organic phase. When the two solutions are brought in contact, polymerization occurs at the interface, forming polyamide, which precipitates. Generally, the reaction takes place at the organic side of
the interface [10]. This is due to the better solubility of the diamine in the organic phase and the negligible solubility of the acid chloride in the aqueous phase.

In polyamidation, due to the very high reaction rate between the acid chloride and the diamine, monomers diffusing to the interface react with the growing polymer chain ends before they can penetrate the polymer film and start a new chain. Hence with this technique, an ultra thin film (skin layer) formation takes place at the interface, with film thickness under half micron [10]. Another feature of interfacial polymerization is that it does not require bulk stoichiometry. At the interface, reaction stoichiometry exists automatically.

Figure A.3: The chemical structure of a TFC polyamide membrane skin layer [7].
Over the last 30 years, the performance of TFC polyamide RO membranes has continually improved [1]. However, RO is an energy-intensive process. The pressing needs for improved membranes for desalination and purification of water from various organic/biological contaminants require the RO process to have improved water flux through the membranes and improved resistance to membrane fouling.

This proposal aims to address the above-mentioned aspects of improving the water flux and fouling resistance of TFC polyamide RO membrane at the same time. The development of a TFC polyamide membrane is discussed, in which, an attempt to improve water permeability is made by incorporating hydrophilic carbon nanotubes within the polyamide matrix. The surface of the polyamide is further modified to improve hydrophilicity of the membrane surface, which leads to better resistance to organic fouling.

A.2. Flux improvement in TFC membranes:

Reverse osmosis is an energy-intensive process. To minimize energy expenditure, a lot of current research in TFC RO membranes is focused on improving water flux through the membrane, while maintaining or increasing selectivity/ solute rejection [11]. The water permeability/ flux rate of TFC membranes mainly depend on the thickness of coating layer and its hydrophilicity [12]. Various methods have been reported to increase the hydrophilicity of the coating layer [1,11,12]. Work has been done to study the relationship between surface morphology of the coating layer and water permeability. Madaeni [13] has reported that increasing the surface roughness of TFC polyamide RO
membranes results in lower flux. However, Kwak and Ihm [14] have studied the performance of commercially available polyamide TFC membranes. They observed that the roughest membrane showed highest flux, although a linear relationship between the surface roughness and water flux could not be established. Yao et al. [15] have reported that the surface roughness of TFC polyamide RO membranes affects the membrane flux, but more work is underway to establish a clear relation. Flux enhancement has also been done by modification of processing conditions during the fabrication of TFC polyamide membranes [11].

Research has been done on introducing appropriate hydrophilic pore channels into the coating layer during synthesis. Recent studies on use of organic–inorganic polymer composite membranes in molecular separations have inspired the above method. By using sol–gel method, ZrO$_2$ has been incorporated into the Pebax copolymer coating (Zoppi et al [16]) to prepare hybrid water filtration membranes. However, no marked improvement in water flux was observed. Merkel and co–workers [17] incorporated nonporous, nanoscale fumed silica particles in high–free volume glassy polymers. Enhancement in gas permeability and gas separation was reported. It was suggested that disruption in the polymer chain packaging was achieved because of the incorporated nanoparticles, which yielded more polymer/ particle interfacial area. This disruption in the chain packing affected the molecular transport.

Fabrication of high flux membranes from carbon nanotubes [12,18] and zeolite films [1] has been reported. Chu et al [12] have introduced surface-oxidized multiwalled carbon nanotubes (MWNT) into the coating layer of membranes made from electrospun polyvinyl alcohol (PVA) as the substrate layer. The coating layers used were PVA
hydrogel and Pebax (poly (ethylene oxide)-block-polyamide 12 copolymer) containing surface oxidized MWNT. The MWNT was dispersed in PVA and Pebax coating layer solution. This coating layer solution was slightly crosslinked to form gel of PVA with incorporated MWNT. The composite membranes were prepared by cast coating the polymer/MWNT solution on the electrospun PVA substrate and allowing slow solvent evaporation. In table 1, the water permeability for composite membranes with varying concentration of MWNT is shown. The membrane is a composite membrane of electrospun PVA substrate coated with Pebax/MWNT layer.

<table>
<thead>
<tr>
<th>Pebax/MWNT</th>
<th>flux (L/m²-h)</th>
<th>rejection (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pure Pebax</td>
<td>58</td>
<td>99.9</td>
</tr>
<tr>
<td>6 wt % MWNT/Pebax</td>
<td>105</td>
<td>99.8</td>
</tr>
<tr>
<td>8 wt % MWNT/Pebax</td>
<td>161</td>
<td>99.8</td>
</tr>
<tr>
<td>12 wt % MWNT/Pebax</td>
<td>310</td>
<td>98.3</td>
</tr>
</tbody>
</table>

Table A.1: Water flux variation with MWNT concentration in Pebax/MWNT coating layer.

<table>
<thead>
<tr>
<th>PVA hydrogel/MWNT</th>
<th>flux (L/m²-h)</th>
<th>rejection (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pure PVA</td>
<td>67</td>
<td>99.8</td>
</tr>
<tr>
<td>5 wt % MWNT/PVA</td>
<td>143</td>
<td>99.8</td>
</tr>
<tr>
<td>10 wt % MWNT/PVA</td>
<td>330</td>
<td>99.8</td>
</tr>
<tr>
<td>15 wt % MWNT/PVA</td>
<td>445</td>
<td>98.8</td>
</tr>
</tbody>
</table>

Table A.2: Water flux with MWNT concentration in PVA/MWNT coating layer.
The water flux is given by: Flux (J) = Q / [A(Δt)]

Q is the permeation volume of the testing solution, A is the area of the membrane surface and Δt is the time required for the permeation.

Rejection is given by: Rejection (%) = [(C_f - C_p) / C_f] 100

C_f is the organic concentration of the feed solution and C_p is the organic concentration of the permeate solution.

From the above data, we see that with the inclusion of MWNT in the PVA and Pebax matrix (coating layer on electrospun PVA substrate layer), the water flux shows an increment, without altering the rejection abilities of the pure PVA or pure Pebax layers. In this proposal, a high flux TFC polyamide membrane development is proposed by incorporating surface oxidized MWNT into the polyamide coating by interfacial polymerization of MWNT/polyamide nanocomposite as the skin layer on microporous polysulfone substrate.

A.3. Fouling of membranes:

One of the major drawbacks of TFC membranes for industrial application is the flux decline due to fouling of the membrane surface [1, 7, 8, 9, 18-25]. Fouling can be defined as the irreversible deposition of materials on the membrane surface, which leads to flux decline [8]. For application of membranes in water purification processes, membrane replacement due to fouling is regarded as the single largest operating cost [22]. Since RO
is a highly energy-dependent process, the gradual decline in the water flux would make it uneconomical because of the high costs of energy to maintain the flux [18]. Irreversible adhesive fouling is caused by the macrosolute adsorption onto the membrane surface [23]. Foulants, which include colloids, microbes, oils, proteins and undissolved hydrocarbons (usually high molecular weight organic compounds), adhere to the membrane surface due to hydrophobic interactions, hydrogen bonding and extracellular macromolecular interactions [2,22, 25].

The main drawback of TFC polyamide membrane is its proneness to organic fouling. This fouling primarily arises due to the hydrophobicity of the PA active layer [8]. Previous studies have shown that significant fouling of RO membranes is caused by the natural organic materials in surface water [26]. Many natural organic compounds easily adsorb onto the surface, which leads to the eventual decrease in the flux.

A.3.1. Improving membrane fouling resistance:

The nature of the membrane surface is a very important factor for the adhesion of molecular layers or precipitates onto the membrane. Surface modification of membranes with suitable hydrophilic and charged functional groups is a widely used method for controlling membrane fouling [27]. The hydrophilic and charged groups are generally expected to inhibit adsorption of microorganisms and hydrophobic solutes. In case of microfiltration and ultrafiltration membranes, it has been shown that making the surface more hydrophilic and/or negatively charged leads to less fouling [24]. Hence, hydrophilization of the PA active layer surface is a potential way to solve the fouling problem. Increasing the hydrophilicity of the surface leads to inhibition of non–specific
binding between membrane surface and retained molecules [28]. So, hydrophilization of the membrane surface decreases the adherence of the organic matter, leading to improved fouling resistance.

A.3.2. Hydrophilization of TFC polyamide membranes:

Several reported surface modification methods, to decrease fouling by changing the surface chemistry of the membranes include: a) physically coating the membrane surface with water soluble polymers or charged surfactants, b) coating the membrane with hydrophillic polymers by heat curing and c) covalent grafting of hydrophilic polymers onto the membrane surface [23]. Considering the durability of these methods, the covalent grafting method is desirable. The covalent grafting methods include grafting by chemical coupling [22], redox – initiated grafting [9,8,24], UV–induced or plasma–initiated grafting. Various hydrophillic polymers, grafted to increase the hydrophilicity of TFC membranes include polyacrylic acid [8], polymethacrylic acid [9] and polyethylene glycol (PEG) [9,22]. Among the hydrophillic polymers, PEG has been used widely because its high hydrophilicity prevents hydrophobic or large molecule adsorption onto the membrane surface [22]. Immobilization of PEG molecules on membrane surface to improve anti fouling properties of ultrafiltration membranes have been investigated in recent years [22]. Cao et al [22] have modified TFC polyamide membrane surface by grafting PEG molecules on the surface. Aminopolyethylene glycol monomethylether (MPEG–NH₂) was used as grafting monomer. However, this process required the synthesis of MPEG-NH₂ from monomethoxy – polyethylene glycol (MPEG) through a series of reactions.
A.4. Proposed Research:

In this work, the proposed idea is to synthesize a TFC polyamide reverse osmosis membrane, having improved water flux and better resistance to fouling. The flux improvement is attempted by making the polyamide skin layer more hydrophilic. To make the membrane more fouling resistant, the surface of the membrane is made more hydrophilic, which leads to better fouling resistance. With this aim in mind, the polyamide layer is impregnated with hydrophilic surface oxidized multi walled carbon nanotubes (MWNT). Oxidation of MWNT generates –COOH, –C=O and –OH functionalities on its surface. This improves its compatibility with the polymer and also makes it hydrophilic. The hydrophilic MWNT’s are introduced into the polyamide matrix by interfacial polymerization of MPD and MWNT dispersed solution of TMC as monomers on a polysulfone support layer. The negatively charged, hydrophilic MWNT’s are expected to provide preferential flow paths for water permeation, maintaining the solute rejection through Donnan exclusion and steric hindrance to solute molecules. The surface of the synthesized membrane is further modified to generate –COOH and –NH₂.
groups by partial hydrolysis of the polyamide layer with hydrofluoric acid (HF) solution. The \( -\text{NH}_2 \) groups generated are used for grafting PEG chains onto the surface of the membrane by reacting \( -\text{NH}_2 \) groups with carboxyl group of methoxypolyethylene glycol acetic acid. The hydrophilic PEG chains on the surface of the membrane inhibit adsorption of various organic compounds and hydrophobic solute onto the membrane surface, reducing fouling. The \( -\text{COOH} \) groups generated on the surface during the partial hydrolysis will increase the surface negative charge on the membrane, thus improving solute rejection through Donnan exclusion. Figure A.5 shows a schematic of the proposed idea.
Figure A.5: Schematic of proposed idea.
A.4.1. **Development of the hybrid reverse osmosis membrane:**

To prepare hydrophilic composite membrane for water purification, pre-formed polysulfone ultra filtration membranes are used as non-woven porous support. It is then coated with the MWNT – polyamide nanocomposite film by interfacial polymerization of the composite layer on the polysulfone support. The polyamide film is synthesized from m – phenylenediamine (MPD) and trimesoyl chloride (TMC). The MWNT are surface oxidized by treating them with a concentrated H$_2$SO$_4$/ HNO$_3$ (1:3) solution. This treatment generates surface acidic groups including –COOH, –C=O and –OH functionalities [11].

The surface oxidized MWNT’s are then dispersed in a solution of TMC. To do this, a given amount of the modified MWNT is added to a 0.1% (w/v) TMC–hexane solution and sonicated. Complete dispersion is achieved by ultrasonication for 2 hours at room temparature.

The TMC (with MWNT’s dispersed) is then interfacially polymerized with MPD on the polysulfone layer. To do this, the polysulfone membrane is immersed in a 2% (w/v) aqueous solution of MPD for 5 minutes. After this, the MPD-soaked membrane is placed on a rubber sheet and rolled with a rubber roller to remove excess MPD. Now, the MPD-soaked polysulfone membrane (excess MPD removed) is immersed in the MWNT-dispersed TMC solution. In this step, interfacial polymerization occurs between the TMC and MPD to generate a MWNT-dispersed thin polyamide film on the polysulfone membrane surface. This interfacial polymerization is allowed to occur for 1 minute at room temperatur. After 1 minute of polymerization, the membrane is removed from the
TMC solution and rinsed thoroughly with deionized water to remove excess TMC. At this point, we have a thin film of polyamide-MWNT composite skin layer on the polysulfone ultra filtration membrane.

Now, we hydrophilize the membrane surface by grafting hydrophilic PEG chains onto the polyamide surface, to make it more fouling resistant. To graft PEG chains, methoxypolyethylene glycol acetic acid (MPEG-AA) is used as the grafting monomer. The structure of MPEG-AA is shown in Figure A.6. The acidic –OH group of the MPEG-AA is used for grafting it to the membrane surface, by reacting the –OH group with –NH₂ groups generated on the membrane surface.

![Figure A.6: Chemical structure of MPEG-AA.](image)

Surface modification of the polyamide membrane is done to generate the –NH₂ groups. For this, the polyamide coated TFC membrane is immersed in a 15% aqueous solution of hydrofluoric acid (HF) and kept for 24 hours to allow the partial hydrolysis of the polyamide skin. This partial hydrolysis of the polyamide generates –NH₂ and –COOH groups on the membrane surface. The reaction scheme for this step is shown in Figure A.7.

After the membrane is taken out of the HF solution, it is thoroughly washed with deionized water. Now, the thoroughly washed surface modified membrane is immersed
in a 5% aqueous solution of MPEG-AA to react the surface –NH₂ groups of the membrane with the –COOH functionalities of MPEG-AA. The reaction is allowed for 5 minutes. Then the membrane is taken out of the MPEG-AA solution and thoroughly washed with deionized water.

In Figure A.7, the chemical modification of the polyamide skin layer is depicted. It is an acid catalyzed hydrolysis of the amide linkages in of the polyamide layer. In this reaction, the carbonyl oxygen of the amide is protonated initially by the H⁺ from HF solution. In the next step, the O from H₂O acts as a nucleophile and reacts with the C=O of the carbonyl group. In the next step, deprotonation of the O atom (from H₂O) occurs, followed by the protonation of the of the amide bond. This is followed by the breaking of the amide bond to generate the amine functionality. In the final step, deprotonation of the carbonyl C=O occurs to generate the –COOH functionality.

This modification leads to no loss in the rejection properties of the membrane [29]. Moreover, the –COOH groups generated add to the surface charge of the membrane, which helps in ion rejection and fouling resistance of the membrane. The grafting of the hydrophilic PEG chains onto the surface modified membrane skin is shown in Figure A.8. After the grafting, the skin surface still has the –COOH functional groups, which helps in increasing the overall negative charge on the surface. As discussed earlier, this helps in the overall rejection of ions in the water.
Figure A.7: Hydrolysis of the polyamide layer.
It should be mentioned here that the ability to repel proteins by the surface grafted membrane depend on the density of the grafting and also on the molecular weight of the grafting polymer. Among these, the grafting density is the major factor [30]. By varying
the degree of hydrolysis of the polyamide skin, the number of available –NH₂ groups for PEG grafting on the membrane surface can be varied. This will lead to variation of the graft density.

A.5. Characterization of the synthesized membrane:

The characterization of the modified TFC membranes is done by transmission electron microscopy (TEM) and attenuated total internal reflection Fourier transform infrared spectroscopy (ATR-FTIR).

A.5.1. Characterization of MWNT in the polyamide layer:

The MWNT nanoparticles within the polyamide skin layer can be seen in the TEM spectroscopy. The TEM micrograph of an unmodified TFC polyamide membrane is shown in Figure A.9.

Figure A.9: TEM of TFC polyamide membrane [1].
In case of the composite membrane with nanoparticles, the incorporated MWNT’s should appear as dark spots within the polyamide matrix. The dispersion of the nanoparticles within the polyamide layer is also viewed through TEM spectroscopy and the numbers of dispersed nanoparticles depend on the concentration of the MWNT in the TMC-hexane solution.

A.5.2. Characterization of grafted PEG chains:

ATR-FTIR is used to characterize the polymer chains grafted onto the membrane surface. We compare IR spectra of the ungrafted and the grafted membranes. In the PEG-grafted spectra, we would see C–C and C–O stretch of the PEG at 1080 cm$^{-1}$, CH$_2$ rock and C–C stretch of PEG at 943 cm$^{-1}$ [21]. These would not be present in the ungrafted spectra.

A.6. Summary:

In this proposal, the synthesis of a high flux-high fouling resistant TFC polyamide reverse osmosis membrane has been discussed. The water flux is improved by forming a MWNT-polyamide nanocomposite thin film on the polysulfone substrate layer by interfacial polymerization. The surface oxidized MWNT would provide preferential flow paths for water permeation, while maintaining the solute rejection of pure polyamide membranes. The resistance to fouling by macrosolute adsorption on the membrane surface is improved by increasing the hydrophilicity of the surface skin layer. Grafting of hydrophilic PEG chains on the membrane surface provided better hydrophilicity of the
membrane surface. It should be mentioned here that for the grafting of the PEG chains, the MWNT-polyamide nanocomposite film is partially hydrolyzed with aqueous HF solution. This partial hydrolysis step is very critical, as high degrees of hydrolysis may lead to lowering of solute rejection. The repulsion of proteins and other organic molecules by the membrane surface depends primarily on the PEG grafting density. This density depends on the number of available –NH$_2$ groups generated during the hydrolysis of the polyamide layer. Hence, various concentrations of the acid solution need to be tried as well as the reaction time of hydrolysis, for obtaining optimum result.
Reference:


VITA

Sudipto Das was born in Kharagpur, India and attended the Hijli High School. He received his Integrated Master of Science degree in Industrial Chemistry from the Indian Institute of Technology (IIT), Kharagpur in 2003. He received the award for best senior research project at IIT Kharagpur in 2003. He joined Drexel University, Philadelphia in 2005 to pursue a Ph.D. degree in chemistry. He has been working under the supervision of Dr. Yen Wei at the Center of Advanced Polymers and Materials Chemistry. His research interests include sol-gel chemistry, immobilization of enzymes and biological molecules in sol-gel silica materials, thermal analysis of polymeric materials, development of bio-based organic-inorganic hybrid materials, and processes for enzymatic hydrolysis of biomass for renewable energy sources. He received an award for his poster at the Thermal Analysis Forum of Delaware Valley in 2008.

Selected publications:
