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Potential Applications of Chitosan Nanoparticles as Novel Support in Enzyme Immobilization

[1]Hoda Jafarizadeh Malmiri, [1]Mohammad Ali Ghaz Jahanian and [2]Aydin Berenjian

[1]Department of Chemical Engineering, Sahand University of Technology, Tabriz, Iran
[2]School of Chemical and Biomolecular Engineering, The University of Sydney, Sydney, Australia

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ABSTRACT

Chitosan is an attractive natural biopolymer from renewable resources with the presence of reactive amino and hydroxyl functional groups in its structure. Due to the good biocompatibility of chitosan, it can be used in magnetic-field assisted drug delivery, enzyme or cell immobilization and many other industrial applications. In the past decade, nanotechnology has been a considerable research interest in the area of preparation of immobilized enzyme carriers. This study looks at characteristics and applications of chitosan and chitosan nanoparticles and their potentials as suitable supports for enzyme immobilization. Results indicated that activity of immobilized enzymes and performance of enzyme immobilization onto chitosan nanoparticles are higher than chitosan macro and microparticles. As compared to other biopolymers nanoparticles, application of chitosan nanoparticles to immobilize enzymes strongly increases stability of immobilized enzymes and their easy separability from the reaction mixture at the end of the biochemical process.

Keywords: Chitosan, Chitosan Nanoparticles, Immobilized Enzyme, Support, Enzyme Activity, Stability

1. INTRODUCTION

Enzymes are proteins that catalyze chemical reactions. Unlike more traditional organic and inorganic catalysts, enzymes are large and fragile molecules. Instability and sensitivity to process conditions are disadvantages of using soluble enzymes. Therefore, application of solid phase biocatalysts has become more and more important during the last decades (Biro *et al*., 2008). Immobilized enzymes have a great importance in industrial bioprocesses especially in food, nutritional and pharmaceutical technologies (Sheldon, 2007). There are several reasons for using an enzyme in an immobilized form. In addition to more convenient handling of the enzyme, it provides for its facile separation from the product. It helps to prevent the contamination of the substrate with enzyme/protein or other compounds, which decreases purification costs (Spahn and Minteer, 2008). Immobilization also facilitates the efficient recovery and reuse of costly enzymes, with longer half lives and less degradation, in successive batches, or the process can eventually be carried out in a continuously operating reactor (Shi *et al*., 2011b).

There are several methods used to immobilize enzymes and three of the most common methods are physical adsorption, entrapment (encapsulation) and cross linking or covalently binding to a support (Janssen *et al*., 2002; Sheldon, 2007). For practical purposes, carrier beads with size falling into millimeter range are mainly used. However, more and more results are reported on immobilization of enzymes onto microparticles possessing high specific surface area and numerous active sites available for the enzyme molecules to be fixed. Moreover, because of the smaller size of the support particles, the internal diffusion hindrance diminishes (Biro *et al*., 2009). Hindrance can be described as a function of solute size and geometric properties of the porous network. Diffusion of macromolecules in porous structures is hindered in comparison to diffusion in free solution (Schroder *et al*., 2006). Various supports have been used for enzymes immobilization such as synthetic organic polymers, biopolymers, hydrogels, smart polymers and inorganic

Corresponding Author: Hoda Jafarizadeh Malmiri, Department of Chemical Engineering, Sahand University of Technology, Tabriz, Iran

supports (Katchalski-Katzir and Kraemer, 2000; Lozinsky *et al*., 2003; Sheldon, 2007; Salemi, 2010). A variety of biopolymers, mainly water insoluble polysaccharides such as cellulose, starch, agarose and chitosan and proteins such as gelatin and albumin have been widely used as supports for immobilizing enzymes (Krajewska, 2004; Spahn and Minteer, 2008).

Chitosan with some primary amino groups is derived by deacetylation of chitin. Its pK_a is about 6.3 (Sorlier *et al*., 2001). At lower pH solutions ($<pK_a$), most of the amino groups are protonated, making chitosan a water soluble polyelectrolyte. When the pH is higher than pK_a, the amino groups are deprotonated and chitosan becomes insoluble. Because of its excellent film forming ability, biocompatibility, nontoxicity, high mechanical strength, cheapness and a susceptibility to chemical modifications, chitosan has been extensively used for immobilization of enzymes (Vaillant *et al*., 2000; Kilinc *et al*., 2002; Wei *et al*., 2002; Krajewska, 2004; Liang *et al*., 2005; Luo *et al*., 2005; Tang *et al*., 2006; Yang *et al*., 2010).

In recent years, nanotechnology has showed a significant attraction to the preparation of immobilized enzymes. Nanomaterials due to their small size and large surface area to volume ratio have special characteristics, which make them favourable for enzyme immobilization (Tang *et al*., 2006; Li *et al*., 2008). Therefore the objectives of the present study are: (i) to evaluate characteristics of suitable immobilized enzyme supports such as chitosan, (ii) to study properties, production methods and applications of chitosan nanoparticles and (iii) to evaluate advantages of using chitosan nanoparticles as suitable support for enzyme immobilization.

1.1. Immobilized Enzyme Supports

Immobilization is often the key to optimizing the operational performance of an enzyme in industrial processes, particularly for use in non aqueous media. The immobilization of enzymes has been a growing field of research, because it has allowed enzymes to be easily reused multiple times for the same reaction with longer half lives and less degradation and has provided a straightforward method of controlling reaction rate as well as reaction start and stop time (Mateo *et al*., 2007; Spahn and Minteer, 2008; Shi *et al*., 2011b).

The properties of immobilized enzymes are governed by the properties of both the enzymes and the support materials (Sheldon, 2007). The interaction between the two lends an immobilized enzyme specific physico-chemical and kinetic properties that may be decisive for its practical application and thus, a support judiciously chosen can significantly enhance the operational performance of the immobilized system (Krajewska, 2004).

Suitable materials used as a carrier should have chemical, physical and biological stability during processing, as well as in the reaction conditions; sufficient mechanical strength, specially for its utilization in reactors and industries; should be nontoxic both for the immobilized enzyme/bioparticle, as well for the product; also should have adequate function groups for binding biocatalyst and high loading capacity (Gorecka and Jastrzebska, 2011). Other criteria, such as structural characteristics (porosity, swelling, compression, material and mean particle behavior), as well as possibility for microbial growth, biodegradability, high affinity to proteins, solubility, regenerability and ease of preparation in different geometrical configurations that provide the system with permeability and surface area are more application specific (Krajewska, 2004). Chemical structure of carrier material determines interaction with enzymes. If the support material is highly porous, pore size and pore size distribution will play an important role in determining the immobilized enzyme properties. A small pore size can cause diffusion limitation resulting in structural rearrangement of the enzymes and subsequent inactivity. However, for very large pore sizes, enzymes can cluster together and thus lose activity (Miletic *et al*., 2012).

Enzymes immobilization onto carriers has been extensively studied and applied in many fields, such as biocatalysts (Hou *et al*., 2007; Li *et al*., 2010) medical devices (Liang *et al*., 2000; Lao *et al*., 2008), drug delivery systems (Gan and Wang, 2007; Shi *et al*., 2011a) and biosensor (Ley *et al*., 2011; Samanta and Sarkar, 2011). Several carriers such as synthetic organic polymers (e.g., Eupergit C and polyurethane), biopolymers (e.g., alginate), hydrogels (e.g., Polyvinyl alcohol), smart polymers (e.g., poly-N-isopropylacrylamide) and inorganic supports (e.g., alumina, silica and zeolites) have been used in immobilization of enzymes (Katchalski-Katzir and Kraemer, 2000; Kirk and Christensen, 2002; Temino *et al*., 2005; Lutz *et al*., 2006; Awang *et al*., 2007; Sheldon, 2007). Biopolymers are one of the most applied supports for immobilizing enzymes, includes two types of mainly water insoluble proteins such as gelatin and albumin and polysaccharides such as starch, alginate and chitosan (Gorecka and Jastrzebska, 2011).

1.2. Enzyme Immobilization Techniques

Immobilization often stabilizes structure of the enzymes, thereby allowing their applications even under harsh environmental conditions of pH, temperature and organic solvents and thus enable their uses at high temperatures in non aqueous enzymology and in the fabrication of biosensor probes. Different methods for the immobilization of enzymes have been critically reviewed.

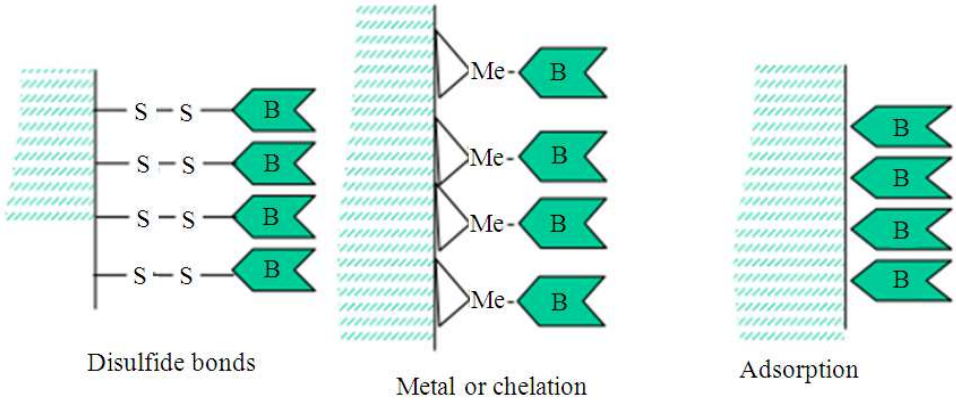

Fig. 1. Reversible methods of enzyme immobilization. B: Biocatalyst (Enzyme) **Source:** Gorecka and Jastrzebska (2011)

These methods are divided into two main categories namely; reversible and irreversible immobilization (Sheldon, 2007). Adsorption, disulphide bonding and chelation or metal binding are the reversible methods for enzyme immobilization (Gorecka and Jastrzebska, 2011).

Adsorption is the elementary and probably the simplest method of immobilization. This method is based on weak forces (e.g., van der Waals), however still enabling an efficient binding process. A wide range of both organic and inorganic materials can be used as a support in this method (Cetinus *et al*., 2007). Disulphide binding may be seen as a variation of covalent bonding, as there are stable covalent bonds formed between activated support and free thiol group (e.g., on cysteine) in the biocatalyst (Gulla *et al*., 2004). Chelation is based on the ability of the side chains of certain amino acids (e.g., histidine, tryptophan, tyrosine, cysteine and phenylalanine) to substitute weakly bonded ligands in the metal ions that have been immobilized by chelating group covalently bound to a solid support (Gorecka and Jastrzebska, 2011). Schematics of reversible enzyme immobilization methods are shown in **Fig. 1**.

Covalent bonding, entrapment, encapsulation and cross linking, fall into the irreversible enzyme immobilization methods. Covalent bonding is one of the most widely used methods for enzymes immobilization. Popularity of this approach is mainly connected with the stability of the bonds formed between the enzyme and the support, which prevents enzyme release into the environment (Sheldon, 2007). Entrapment is based on incorporating enzymes into the lattices of a semi permeable gel or enclosing the enzymes in a semi permeable polymer membrane (Lei *et al*., 2003). Encapsulation is similar to entrapment. In this process, enzymes are restricted by the membrane walls (usually in a form of a capsule) (Ma *et al*., 2009; Briones and Sato, 2010). Cross linking method is widely used to immobilize enzymes and is based on intermolecular cross linking of enzymes by bi-functional or multi functional reagents (Seo *et al*., 2012). In fact, this method does not require a support and this is the advantage of this method. Immobilization of an enzyme on a carrier often leads to the loss of more than 50% native activity, specially at high enzyme loadings. The design of carrier bound immobilized enzymes also relies largely on laborious and time consuming trial and error experiments, because of the lack of guidelines that link the nature of a selected carrier to the performance expected for a given application (Sheldon, 2007; Spahn and Minteer, 2008). Schematics of irreversible enzyme immobilization techniques are shown in **Fig. 2**.

1.3. Chitosan

Chitin is a copolymer of N-acetyl-D-glucosamine and D-glucosamine units linked with ß-(1- 4) glycosidic bond, where N-acetyl-d-glucosamine units are predominant in the polymeric chain (Agnihotri *et al*., 2004). The deacetylated form of chitin refers to chitosan (Lee *et al*., 2009). Basically, the process consists of deproteinization of the raw shell material with a dilute NaOH solution and decalcification with a dilute HCl solution. To result in chitosan, the obtained chitin is subjected to N-deacetylation by treatment with a 40-45% NaOH solution, followed by purification procedures (Krajewska, 2004). Thus, production and utilization of chitosan constitutes an economically attractive means of crustacean shell wastes disposal sought worldwide.

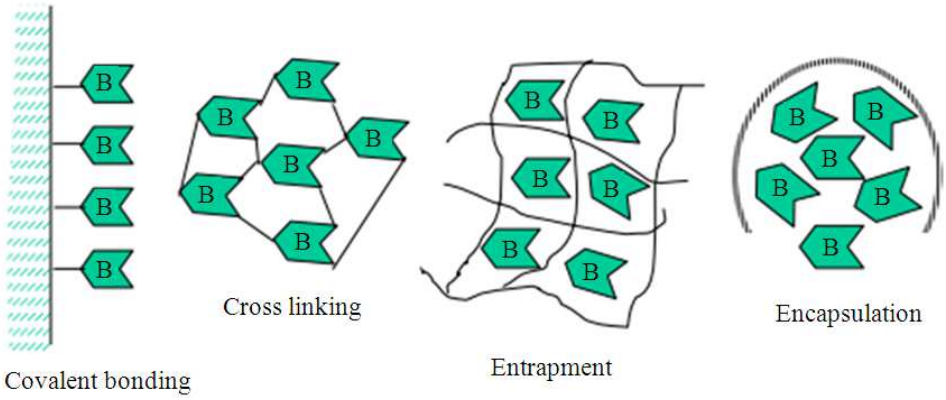

Fig. 2. Irreversible methods of enzyme immobilization. B: Biocatalyst (Enzyme) **Source:** Gorecka and Jastrzebska (2011)

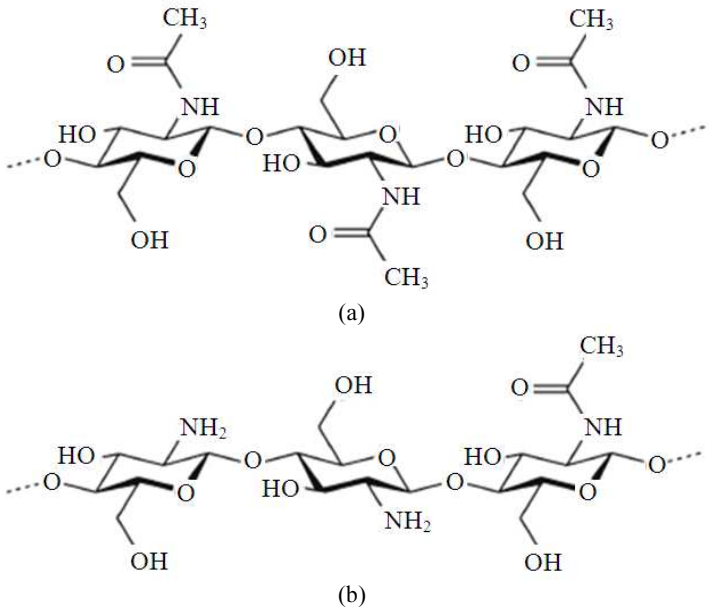

Fig. 3. Chemical structure of chitin (a) chitosan (b) **Source:** Krajewska (2004)

These renewable resources are found in many naturally occurring organisms (e.g., fungi and yeast) and is the principal component in the exoskeleton of sea crustaceans such as crabs, shrimps, lobsters and krills (Shi *et al*., 2011b). Chitosan represents long chain polymers having molecular mass up to several million Daltons. Chitosan is relatively reactive natural biopolymer due to presence of reactive amino and hydroxyl functional groups in its structure and can be produced in various forms such as powder, gels and films, sponges, intragastric floating tablets and especially spherical micro and nanoparticles (Agnihotri *et al*., 2004; Racovita *et al*., 2008). If degree of deacetylation and molecular weight of chitosan can be controlled, then it would be a material of choice for developing micro and nanoparticles. Chitosan has ability to control the release of active agents and avoid the use of hazardous organic solvents while fabricating particles since it is soluble in aqueous acidic solution. It is a linear polyamine containing a number of free amine groups that are readily available for cross linking and its cationic nature allows for ionic cross linking with multivalent anions. Chitosan has mucoadhesive character, which increases residual time at the site of absorption (Agnihotri *et al*.,

2004; Grenha *et al*., 2005; Kim *et al*., 2008; Racovita *et al*., 2008). Chemical structures of chitin and chitosan are shown in **Fig. 3**.

1.4. Applications of Chitosan

Chitosan due to its unique physico-chemical and biological properties is an attractive material for use in various applications (Sorlier *et al*., 2001; Yang *et al*., 2010). These properties include: biodegradability, lack of toxicity, antibacterial and antifungal effects, wound healing acceleration and immune system stimulation (Shi *et al*., 2011a). Because of excellent biological and physico-chemical properties of chitosan, it has the ability to bind to particular materials including cholesterols, fats, proteins, metal ions and even tumor cells (Grenha *et al*., 2005). This allows chitosan to be used as a chelating agent in various applications.

Over the last decade, a number of potential products based on chitosan have been increasingly produced and applied in several areas such as: waste water treatment, due to its high adhesive and insolubility properties (e.g., removal of heavy metal ions and membrane purification processes); food industries (e.g., anticholesterol and fat binding, preservative, packaging material and animal feed additive); agriculture (e.g., seed and fertilizer coating, controlled agrochemical release); pulp and paper industries, because of its wet strength to paper (e.g., surface treatment, photographic paper) and cosmetics and toiletries due to its fungicidal and fungistatic properties (e.g., moisturizer, body creams, bath lotion) (Krajewska, 2004; Nakorn, 2008; Azeredo, 2009). Fibers made of chitosan are useful as absorbable sutures and wound dressing materials (Nakajima *et al*., 1984). Chitosan has also important applications in photography due to its resistance to abrasion, optical characteristics and film forming ability (Nuzzarelli *et al*., 1997). Due to its unique molecular structure, chitosan has an extremely high affinity for many classes of dyes, including disperse, direct, reactive, acid, vat, sulfur and naphthol dyes (Krajewska, 2005).

Chitosan ability to development of drug delivery systems is one of the most well known of its characteristics. Different types of chitosan made drug carriers have been conceived for various administration routes, such as oral, nasal, transdermal, parenteral, vaginal, cervical and rectal due to its biocompatibility, biodegradability and ecological safety (Racovita *et al*., 2008). Tissue engineering is an important therapeutic strategy for present and future medicine. Recently, functional biomaterial researches have been directed towards the development of improved scaffolds for regenerative medicine. Chitosan and its derivatives are promising candidates as a supporting material for tissue engineering applications owing to their porous structure, gel forming properties, ease of chemical modification, high affinity to in vivo macromolecules and so on (Kim *et al*., 2008). Moreover, it is one of the most promising immobilization matrices with excellent characteristics as a support matrix for enzyme immobilization (Nakorn, 2008).

1.5. Immobolized Enzymes onto Chitosan

Chitosan has shown favorable biocompatibility characteristics, as well as the ability to increase membrane permeability (Nakorn, 2008). Moreover, it is one of the most promising immobilization matrices due to an excellent membrane forming ability, good adhesion, low cost, low immunogenicity and nontoxicity, high mechanical strength and hydrophilicity as well as the improvement of stability (Dutta *et al*., 1997; Monteiro and Airoldi, 1999; Kumar *et al*., 1999; Vazquez-Duhalt *et al*., 2001; Colonna *et al*., 2008). These properties have prompted extensive applications of chitosan as a matrix for enzyme immobilization. In fact, polycationic nature of chitosan and presence of large primary amine groups in its chemical structure (**Fig. 3b**) make it potentially useful in immobilization of various enzymes (Fang *et al*., 2009). Results of several studies have been shown that after selection of suitable enzyme immobilization method, chitosan reactive amino and hydroxyl groups offer a good enzyme coupling efficiency (Luo *et al*., 2005; Biro *et al*., 2009; Yang *et al*., 2010).

Chitosan has been extensively used in immobilization of various enzymes groups because of its many characteristics like improved resistance to chemical degradation, avoiding disturbance of metal ions to enzyme and antibacterial property (Tang *et al*., 2006). Simsek-Ege *et al*., 2002; Vaillant *et al*., 2000) used chitosan to immobilize carbonic anhydrase and pectin lyase, respectively, which both of them are classified in lyase enzymes group. Applications of chitosan in immobilization of oxidoreductase, hydrolase and transferase enzymes are summarized in **Table 1-3**, respectively.

1.6. Chitosan Nanoparticle

In recent decades, nanostructured materials have attracted much attention because of their unique properties and interesting applications. Nanotechnology is the ability to work on a scale of about 1-100 nm in order to understand, create, characterize and use material structures, devices and systems with new properties derived from their nanostructures (Azeredo, 2009).

Table 1. Some of immobilized oxidoreductases onto chitosan

Enzeme	Support	Type of immobilization	Reference
Alanine dehydrogenase	Chitosan beads	Covalent binding with G	Kiba *et al*. (1993)
Alcohol dehydrogenase	Chitosan beads	Covalent binding with G	Soni *et al*. (2001)
Alcohol oxidase	Chitosan beads	Covalent binding with G	Taniai *et al*. (2001)
Catalase	F3GA attached-chitosan beads	Adsorption on support	Cetinus *et al*. (2007)
Galactose oxidase	Chitosan membrane	Covalent binding with G	Wang *et al*. (2003)
Glucose oxidase	Chitosan capsules	Micro encapsulation	Briones and Sato (2010)
Glutamate dehydrogenase	Chitosan membrane	Covalent binding with G	Petach and Driscoll (1994)
Glutamate oxidase	Chitosan membrane	Covalent binding with G	Wang *et al*. (2003)
Horseradish peroxidase	Chitosan membrane	Covalent binding with G	Lei *et al*. (2003); Monier *et al*. (2010)
Laccase	Magnetic chitosan microspheres	Adsorption followed by cross linking with G	Jiang *et al*. (2005)
Lactate oxidase	Chitosan-enzyme beads	Gel inclusion	Wei *et al*. (2003)
Leucine dehydrogenase	Chitosan capsules	Micro encapsulation	Ma *et al*. (2009)
Octopine dehydrogenase	Chitosan beads	Covalent binding with G	Shin *et al*. (1998)
Oxalate oxidase	Chitosan powder	Adsorption followed by cross linking with G	Ramakrishnan *et al*. (1997)
Putrescine oxidase	Chitosan beads	Covalent binding with G	Okuma *et al*. (1992)
Sulfite oxidase	Chitosan-PHEMA membrane	Adsorption on support	Ng *et al*. (1998)
Uricase	Chitosan membrane	Covalent binding with agents other than G	Yao *et al*. (2003)
Xanthine oxidase	Chitosan beads	Covalent binding with G	Park and Kim (1999)

G: Glutaraldehyde

Table 2. Some of immobilized hydrolases onto chitosan

Enzyme	Support	Type of immobilization	Reference
Acid phosphatase	Chitosan beads	Adsorption on support	Kurita (2001)
Alkaline phosphatase	Chitosan beads	Covalent binding with G	Agarwal and Gupta (1995)
Alkaline protease	Chitosan powder	Covalent binding with G, adsorption on support	Abdel-Naby *et al*. (1998)
Aminoacylase	Chitosan-coated alginate beads	Gel inclusion	Lee *et al*. (1992)
α-Amylase	Chitosan beads	Covalent binding with G	Tripathi *et al*. (2007)
β-Amylase	Chitosan beads	Adsorption on support	Noda *et al*. (2001)
α-l-Arabinofuranosidase	Chitosan powder	Adsorption, cross linking and covalent binding with G	Martino *et al*. (2000)
Bromelain	Chitosan beads	Covalent binding with agents other than G	Seo *et al*. (1998)
Candida ntarctica lipase	chitosan-based hydrogels	Covalent binding with G	Silva *et al*. (2012)
Cellulase	Chitosan beads	Adsorption	Darias and Villalong (2001)
Chitinases	chitosan beads	Adsorption followed by cross linking with G	Seo *et al*. (2012)
α-Chymotrypsin	Chitosan beads	Adsorption followed by cross linking with G	Ge and Zhang (1996)
Creatininase	chitosan-g-polyaniline with iron oxide	Covalent binding with G, adsorption on support	Yadav *et al*. (2012)
Creatinine deaminase	Chitosan membrane	Adsorption on support	Magalhaes and Machado (2002)
Endo-1,4-β-Xylanase	Chitosan powder	Adsorption on support	Abdel-Naby (1993)
Ficin	Chitosan beads	Covalent binding with agents other than G	Hayashi and Ikada (1991)
β-Galactosidase	Chitosan beads	Covalent binding with G	Dwevedi and Kayastha (2009)
α-Glucosidase	Chitosan beads	Covalent binding with G	Sheu *et al*. (1998)
β-Glucosidase	Chitosan powder	Covalent binding with G	Martino *et al*. (2000)
β-Glycosidase and precipitate	Chitosan powder	Adsorption followed by cross linking with G	D'Auria *et al*. (1996)
Invertase	Chitosan powder and solution	Adsorption on support	Hsieh *et al*. (2000)
Lipase	Chitosan flakes and beads	Adsorption on support	Pereira *et al*. (2001)
Neutral proteinase	Chitosan precipitate	Adsorption followed by cross linking with G	Guo *et al*. (1996)
5-Nucleotidase	Chitosan beads	Adsorption followed by cross linking with G	Park and Kim (1999)
Papain	Chitosan beads	Adsorption followed by cross linking with G	Lei *et al*. (2003)
Pectinase	Chitosan beads	Adsorption on support	Lei and Bi (2007)
Pepsin	Chitosan beads	Adsorption followed by cross linking with G	Altun and Cetinus (2007)
Phospholipase A2	Chitosan beads	Covalent binding with agents other than G	Chen and Chen (1998)
Proteases	Chitosan beads	Covalent binding with G	Li *et al*. (2011)
Urease	Chitosan-triphosphate beads	Covalent binding with G	Kayastha and Sirvastava (2001)
β-Xylolidase	Chitosan powder and beads	Adsorption on support, covalent binding with G	Abdel-Naby (1993)

G: Glutaraldehyde

Table 3. Some of immobilized transferases onto chitosan

Enzyme	Support	Type of immobilization	Reference
Cyclodextrin glycosyltransferase	Chitosan powder	Adsorption on support	Sobral *et al*. (2002)
Limonoid glucosyltransferase	Chitosan powder	Covalent binding with G	Karim and Hashinaga (2002)
Nucleoside phosphorylase	Chitosan beads	Covalent binding with G	Park and Kim (1999)
Transglutaminase	Chitosan beads	Covalent binding with G	Nonaka *et al*. (1996)
W-transaminase	Chitosan beads	Covalent binding with G	Yi *et al*. (2007)

G: Glutaraldehyde

Reducing the particle size of materials is an effective method for improving their properties. Nanoparticles have proportionally larger surface area and consequently more surface atoms than their microscale counterpart, which in turn affects their physico-chemical, optical, catalytic and other reactive properties (Rai *et al*., 2009). In polymer composites conjugated with nanoparticles, a uniform dispersion of nanoparticles leads to a very large matrix/filler interfacial area, which changes the molecular mobility, the relaxation behavior and the consequent thermal and mechanical properties of the materials (Azeredo, 2009). In contrast to other biopolymers, chitosan is a hydrophilic polymer with positive charge that comes from weak basic groups, which give it special characteristics from the technological point of view (Lopez-Leon *et al*., 2005). Recently, chitosan nanoparticles have attracted great attention in several fields due to their unique physico-chemical and biological properties (Azeredo, 2009; Racovita *et al*., 2008; Shi *et al*., 2011a).

1.7. Chitosan Nanoparticles Preparation Methods

Several methods have been used to prepare chitosan nanoparticulate. The selection of any of these methods depends on shape and particle size requirements (Agnihotri *et al*., 2004). Emulsion cross linking, emulsion-droplet coalescence, coacervation/precipitation, ionotropic gelation, reverse micelles, template polymerization and molecular self assembly are the main preparation methods of chitosan nanoparticles (Agnihotri *et al*., 2004; Lopez-Leon *et al*., 2005; Tang *et al*., 2006; Biro *et al*., 2008; Shi *et al*., 2011a).

In emulsion cross linking method, the reactive functional amine group of chitosan crosses link with aldehyde groups of the cross linking agent. In fact, chitosan solution is emulsified in oil phase (water-in-oil emulsion) and the aqueous droplets are stabilized using a suitable surfactant. The stable emulsion is then reacted with an appropriate cross linking agent such as glutaraldehyde to stabilize the chitosan droplets. The nanoparticles are then washed and dried (Agnihotri *et al*., 2004).

Ionotropic gelation method is commonly used to prepare chitosan nanoparticles. This method is based on the electrostatic interactions between the chitosan amine group and a polyanion such as tripolyphosphate. In this method chitosan is dissolved in water or in weak acidic medium. This solution is then added drop wise under constant stirring to the solutions containing other counter ions. Due to the complexation between oppositely charged species, chitosan undergo ionic gelation and precipitate to form spherical nanoparticles (Racovita *et al*., 2008). Much researches have been focused on the preparation of chitosan nanoparticles using inotropic gelation method (Grenha *et al*., 2005; Lopez-Leon *et al*., 2005; Gan and Wang, 2007; Tang *et al*., 2006; Biro *et al*., 2008).

In coacervation/precipitation method, the chitosan solution is spraying into sodium hydroxide, NaOH-methanol or ethanediamine alkaline solutions using compressed air, which in turn originates coacervated chitosan droplets in the form of nanoparticles (Shi *et al*., 2011a). Emulsion-droplet coalescence method involves both emulsion cross linking and precipitation. A stable emulsion containing the aqueous chitosan solution in oil and a second emulsion, containing a NaOH solution, is produced. By mixing the both emulsions under high speed stir, droplets of each emulsion collide at random, coalesce and finally precipitate as small size particles (Shikata *et al*., 2002).

In the reverse micelles method, a surfactant is dissolved in organic solvent to prepare reverse micelles. The aqueous phase containing the chitosan is added to this emulsion with constant vortexing and the nanoparticles forms in the core of the reverse micelles (Agnihotri *et al*., 2004).

In template polymerization, chitosan is firstly dissolved in an acrylic monomer solution under magnetic stirring. Due to the electrostatic interaction, the negatively charged acrylic monomers align along the chitosan molecules. After complete dissolution of chitosan, the polymerization is started by adding the initiator ($K_2S_2O_8$) under stirring at 70°C. The complete polymerization leads to the appearance of an opalescent solution, indicating the nanoparticles formation (Fang *et al*., 2009; Shi *et al*., 2011a).

Molecular self assembly is based on cationic and hydrophobic properties of chitosan. This method is characterized by diffusion followed by specific association of molecules through non-covalent interactions, including electrostatic and/or hydrophobic associations (Ichikawa *et al*., 2005).

1.8. Chitosan Nanoparticles Applications

Chitosan nanoparticles are natural materials with excellent physico-chemical, antimicrobial and biological properties, which make them a superior environmentally friendly material and they possess bioactivity that does not harm humans. Due to these unique properties, chitosan nanoparticles are being used in a vast array of widely different products and applications, ranging from pharmaceutical, tissue engineering and food packaging to biosensing, enzymes immobolization and waste water treatment.

The potential use of chitosan nanoparticles as carriers has led to the development of many different colloidal delivery vehicles. The main advantages of this kind of systems lie in their capacity to cross biological barriers, to protect macromolecules, such as peptides, proteins, oligonucleotides and genes from degradation in biological media and to deliver drugs or macromolecules to a target site with following controlled release (Lopez-Leon *et al*., 2005; Shi *et al*., 2011a). Chitosan nanoparticles have frequently used as a controlled release drug carrier for gene transfer in artificial organs and for immune prophylaxis. In addition, chitosan nanoparticles have been used to improve the strength and washability of textiles and to confer antibacterial effects (Panyam and Labhasetwar, 2003; Mansouri *et al*., 2004). Several researches have been demonstrated that chitosan nanoparticles are able to improve drug bioavailability, modify pharmacokinetics and protect the encapsulated drugs (Janes *et al*., 2001; Shi *et al*., 2011b).

In tissue engineering, chitosan nanoparticles improve transmucosal permeability enhancing transport through the paracellular pathway due to good bio and mucoadhesive properties of the nanoparticles and to an induced structural reorganization of tight junction-associated proteins (Peppas and Huang, 2004).

During the past decade, there was an increasing interest to develop and use bio-based active films which are characterized by antimicrobial and antifungal activities in order to improve food preservation. Chitosan based films have attracted serious attention in food preservation and packaging technology. This is mainly due to the excellent film forming and gas barrier properties of chitosan and its high antimicrobial activity against pathogenic and spoilage microorganisms, including fungi and bacteria (Malmiri *et al*., 2011). Addition of chitosan nanoparticles into the coating and film formulations, improve film tensile properties and their permeability towards water vapor and simple gases (e.g., oxygen and carbon dioxide) (Moura *et al*., 2009).

Electrochemical biosensor has been considered as the best choice for the *in situ* monitoring of active compounds (e.g., phenolic) by virtue of its high sensitivity, simple instrumentation, low production cost and promising response speed (Lu *et al*., 2010). Excellent membrane forming ability of chitosan nanoparticles and their small response time and high sensitivity and stability (due to their high surface to volume ratio), low cost and hydrophilicity making them suitable for biosensor applications that are mostly concerned with working of enzymes for detection mechanisms (Nakorn, 2008).

Arsenic, molybdenum, lead and copper widely used in industries and are released through industrial waste water. Sorption processes are found to be capable of adsorbing large number of metal ions from contaminated waste water. In many studies such as adsorption of metals (e.g., copper and zinc), polymer supported nanoparticles has been prepared and used for selective removal of metal compounds and target metal contaminants (Xu and Du, 2003). Chitosan due to its high hydrophilicity and presence of a large number of hydroxyl and amino groups with high activity as adsorption sites is found to be more efficient for the removal of metals such as uranium, copper, vanadium and molybdenum (Guibal *et al*., 1994; Ngah *et al*., 2011). Nowadays, chitosan nanoparticles have been used effectively in waste water treatment to remove toxic metal ions such as arsenic (Anto and Annadurai, 2012).

Nano carriers can be effectively controlled by the application of nanotechnology. The catalytic efficiency and stability property of immobilized enzymes can be greatly improved. Furthermore, immobilization rate of enzyme can be improved using chitosan nanoparticles (Tang *et al*., 2006; Wu *et al*., 2009; Kalkan *et al*., 2012).

1.9. Chitosan Nanoparticles as Enzyme Immobilization Support

The results of immobilization, including the performance of immobilized enzymes, strongly depend on the properties of supports, which are usually referred to as material types, compositions and structures (Wang *et al*., 2009). Chitosan is known as an ideal support material for enzyme immobilization because of its many characteristics like improved resistance to chemical degradation and avoiding disturbance of metal ions to enzyme (Vazquez-Duhalt *et al*., 2001; Yang *et al*., 2010).

Table 4. Some of immobilized enzymes onto chitosan nanoparticles

Enzyme	Support	Preparation method	Reference
Alcohol dehydrogenase	Fe_3O_4–chitosan nanoparticles	Covalent binding with glutaraldehyde	Li *et al*. (2008)
β-d- Galactosidase	Fe_3O_4–chitosan nanoparticles	Covalent binding with glutaraldehyde	Pan *et al*. (2009)
β -Galactosidase	Chitosan nanoparticles	Precipitation, emulsion cross linking, ionic gelation	Biro *et al*. (2008)
Glucoamylase	Fe_3O_4–chitosan nanoparticles	Ionic adsorption	Wang *et al*. (2012)
Glucose oxidase	Chitosan nanoparticles	Covalent binding	Nakorn (2008)
Laccase	Chitosan nanoparticles	Reversed phase suspension	Fang *et al*. (2009)
l-Laccase	Fe_3O_4–chitosan nanoparticles	Ionic adsorption and covalent binding	Kalkan *et al*. (2012)
Lipase	Fe_3O_4–chitosan nanoparticles	Cross linking with trypolyphosphate	Wu *et al*. (2009)
Neutral proteinase	Chitosan nanoparticles	Ionic gelation	Tang *et al*. (2006)
Pullulanase	Fe_3O_4–chitosan nanoparticles	Photochemistry in aqueous suspension	Zhang *et al*. (2009)
Trypsin	Linolenic acid-modified chitosan nanoparticles	Covalent binding with glutaraldehyde	Liu *et al*. (2005)

Enzymes have been immobilized onto chitosan supports with different particle size. For practical purposes, carrier beads with size falling into millimeter range are mainly used (Guo *et al*., 2003; Krajewska, 2004; Shi *et al*., 2011b).

Under the scale of nano, nanomaterials have characteristics, such as magnetism and large surface area to volume ratio. These characteristics are in favour of immobilization of the enzymes (Tang *et al*., 2006). Recent studies indicated that the performance of enzyme immobilization onto chitosan nanoparticles is higher than that of the biocatalyst immobilized onto chitosan microparticles (Biro *et al*., 2008; Nakorn, 2008). The result can be related to better distribution of the enzyme onto the support due to high specific surface area and numerous active functional groups available for fixing the enzyme molecules (Tang *et al*., 2006).

Activity of immobilized enzymes strongly affects by size and size distribution of the support particles. Specific surface available to bind the applied enzyme and the contacting area with the substrate during a reaction mainly depends on particle size. Therefore, a lot of efforts were made to minimize the particle size and maximize the specific surface area (Biro *et al*., 2009). In addition, the physical characteristics of nanoparticles such as enhanced diffusion and particle mobility can impact inherent catalytic activity of attached enzymes (Jia *et al*., 2003).

Biro *et al*. (2008) indicated that the activity of ß-galactosidase immobilized onto chitosan nanoparticles was higher than that of the biocatalyst immobilized onto chitosan micro and macroparticles. Research studies have also indicated that the stability of immobilized enzymes onto chitosan nanoparticles drastically affects by method of chitosan nanoparticles preparation and type of surfactant (Tang *et al*., 2006; Biro *et al*., 2008).

Immobolization of enzymes onto biopolymers nanoparticles has shown some benefits like improving their stability to pH and temperature, resistance to proteases and other denaturing compounds, as well as an adequate environment for their repeated use or controlled release (Fang *et al*., 2009). In the last decades, immobilized enzymes onto nanoparticles have been also considered for biosensors and food packaging applications (Nakorn, 2008; Azeredo, 2009). Applications of chitosan nanoparticles in immobilization of some enzymes are summarized in **Table 4**.

Liu *et al*. (2005) studied trypsin immobilized on linolenic acid-modified chitosan nanoparticles using glutaraldehyde as cross linker. Their results indicated that the kinetic constant value (K_m) of trypsin immobilized on nanoparticles (71.9 mg mL^{-1}) was higher than that of pure trypsin (50.2 mg mL^{-1}). Tang *et al*. (2006) used Chitosan nanoparticles as support to immobilize and protect activity of neutral proteinase. Their results indicated that chitosan nanoparticles could improve 13.17% of neutral proteinase activity than that of free neutral proteinase. Nakorn (2008) used glucose oxidase immobilized onto chitosan nanoparticles in biosensor for glucose determination. The coated electrode with immobilized glucose oxidase exhibits a rapid and sensitive current response for the changes of glucose concentration in the prepared solutions and indicates the excellent electrocatalytic behaviour of the electrode. Li *et al*. (2008) immobilized *Saccharomyces Cerevisiae* Alcohol Dehydrogenase (SCAD) to magnetic Fe_3O_4-chitosan nanoparticles. For reduction of phenylglyoxylic acid by immobilized SCAD, the kinetic analysis data indicated that the immobilized SCAD retained 48.77% activity of its original activity. Furthermore, the immobilized SCAD enhanced thermal

stability and good durability in the repeated use after recovered by magnetic separations. Fang *et al*. (2009) prepared magnetic chitosan nanoparticles and then immobilized laccase onto them. The immobilized laccase exhibited an appreciable catalytic capability (480 units · g^{-1} support) and had good storage stability and operation stability. The K_m of immobilized and free laccase for ABTS (2, 2' -azino-bis (3-ethyl benzthiazoline-6-sulfunate)) were 140.6 and 31.1 μM in phosphate buffer (0.1 M, pH 3.0) at 37°C, respectively. Pan *et al*. (2009) developed a novel and efficient immobilization of β-d-galactosidase by using magnetic Fe_3O_4–chitosan nanoparticles. The immobilized β-d-galactosidase showed the same or even higher activity in wider ranges of temperature and pH than that of its free form. In addition, the immobilized enzyme could be stored for a long time with little activity loss. Furthermore, the immobilized enzyme retained 92% of its initial activity after successively utilization for 15 cycles. Wu *et al*. (2009) prepared magnetic Fe_3O_4–chitosan nanoparticles and immobilized lipase onto them. The immobilization of lipase onto the nanoparticles showed good loading ability and little loss of enzyme activity and the stability of the catalyst was very good. In fact, it only lost 12% of enzyme activity after five batches. Zhang *et al*. (2009) prepared magnetic chitosan beads (with mean particle size of 50 ± 3 nm) and immobilized pullulanase on them. Results indicated that the kinetic constant value (K_m) of immobilized pullulanase was three times higher than that of free pullulanase. However, the thermal and operational stabilities of immobilized pullulanase were improved greatly. Kalkan *et al*. (2012) immobilized l-laccase onto chitosan-coated magnetic nanoparticles by adsorption and covalent binding after activating the hydroxyl groups of chitosan with carbodiimide or cyanuric chloride. The results indicated that the immobilized enzyme retained more than 71% of its initial activity at the end of 30 batch uses. Wang *et al*. (2012) immobilized glucoamylase onto Fe_3O_4–chitosan nanoparticles. The results from characterization and determination remarkably indicated that the immobilized glucoamylase obtained presents excellent storage stability, pH stability, reusability, magnetic response and regeneration of supports.

1.10. Challenges in Applications of Nanoparticles to Immobilize Enzymes

Application of nanoparticles in enzyme immobilization has been associated with two main disadvantages. The first is lower storage stability of immobilized enzyme onto nanoparticles as compared to that of the immobilized enzyme onto microparticles. This can be explained by the fact that nanoparticles are aggregated during the storage. In fact, if the nanoparticles are not stabilized, they try minimizing their surface energy by clustering together (Ren *et al*., 2011). Biro *et al*. (2008) indicated that the stability of ß-Galactosidase immobilized onto chitosan nanoparticles was lower than that of the biocatalyst immobilized onto chitosan micro and macroparticles. The second disadvantage of using nanoparticles to immobilize enzyme is difficult separation of them from the reaction mixture at the end of the biochemical process, due to their small size (Prakasham *et al*., 2010). In this case, suitable methods are necessary to apply to facilitate the separation of the catalyst particles. Therefore, the general use of nanosized catalysts in industrial biotechnology needs considerable research efforts yet (Biro *et al*., 2009).

To overcome these issues, nanosized magnetic particles have received increasing attention because of their larger specific surface area for the enzymes immobilization, their super paramagnetic nature for the reduction of self aggregation and easy separability from the reaction mixture by the application of a magnetic field (Wang *et al*., 2009; Demir *et al*., 2011). Magnetite (Fe_3O_4), silica, Zinc Oxide (ZnO), cellulose and chitosan are some of magnetic materials and their nanoparticles have been used to immobilize of various enzymes (Ansari and Husain, 2012).

Chitosan can be used as a material for magnetic carriers, since it has a variety of functional groups which can be tailored to specific application (Wu *et al*., 2009; Zhang *et al*., 2009). Additionally, glutaraldehyde cross linking is shown to be the simple and efficient method to immobilize enzymes. Glutaraldehyde can react with several functional groups of proteins and supports, such as amino groups. Each of glutaraldehydes is expected to form Schiff bases upon nucleophilic attack by the primary amino groups in enzymes and chitosan and due to the linkage formed by the Schiff base reaction, the enzyme stability is improved (Xie and Wang, 2012). Several methods have been developed to synthesize magnetic chitosan nanoparticles, such as microemulsion polymerization, emulsion polymerization and *in situ* polymerization (Zhang *et al*., 2009).

As clearly observed in **Table 4**, magnetic Fe_3O_4–chitosan nanoparticles have been used as support to immobilize the most of enzymes. In fact, magnetic Fe_3O_4 nanoparticles tend to aggregate in liquid

media due to the strong magnetic dipole–dipole attractions between particles. Thus, chitosan with specific functional groups have been used as stabilizer to modify and increase their stability (Pan *et al*., 2009). In addition to, coating magnetic Fe_3O_4 nanoparticles with chitosan can protect the nanoparticles against corrosion an also offers flexibility, favourable functional groups and features (e.g., their ability to fold into a globular state or structure) for various applications (Zhang *et al*., 2009).

Magnetic nano and micron sized particles with specific modifications are widely used in biomedical applications such as diagnostics, magnetic separation and purification of biomolecules. In fact, magnetic nanoparticles coated with biopolymers and immobilized with ligand were found to have promising characteristics for the application of these adsorbents in bioseparation processes, particularly in antibody, nucleic acids and enzymes purification (Berensmeier, 2006; Altintas *et al*., 2007).

Hritcu *et al*. (2009) prepared of magnetite/chitosan composite nanoparticles using co-precipitation followed by ionic gelation. The products can be used in bio-separation methods after attaching an application specific ligand to the surface amino groups. The production of core-shell Fe_3O_4-gold-chitosan nanocomposites has attracted much attention over the past several years as they can be used in biotechnological and biomedical areas, including biotargeting for cancer treatment, drug delivery, biodetection and downstream processing (e.g., the purification and bioseparation of biomolecules) (Salehizadeh *et al*., 2012).

2. CONCLUSION

Macro, micro and nanosized chitosan particles are suitable as carriers for enzyme immobilization. Chitosan nanoparticles due to their highest specific surface area are much proper for immobilization of higher amount of enzymes. As compared to chitosan macro and microparticles, higher activity values of immobilized enzyme onto chitosan nanoparticles is explained again by better distribution of the enzyme onto the larger surface area of nanoparticles. Higher magnetic property of chitosan nanoparticles reduces self aggregation of immobilized enzymes onto them and increases the stability of immobilized enzymes. Further studies will be needed explore the kinetic of immobilized enzymes onto chitosan nanoparticles. The immobilization of enzymes onto nanoparticles and the subsequent attachment of the nanoparticles onto an electrode is also an attractive alternative in biosensor researches and developments, especially in the case of magnetic nanoparticles which can be removed from the electrode by the action of magnetic fields.

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