ANG LEE FUNG

A STUDY ON THE CHARACTERISTICS OF CHITOSAN AS AN IMMOBILIZATION MATRIX FOR BIOSENSORS

2007 M.Sc.

A STUDY ON THE CHARACTERISTICS OF CHITOSAN AS AN IMMOBILIZATION MATRIX FOR BIOSENSORS

ANG LEE FUNG

UNIVERSITI SAINS MALAYSIA

2007

A STUDY ON THE CHARACTERISTICS OF CHITOSAN AS AN IMMOBILIZATION MATRIX FOR BIOSENSORS

by

ANG LEE FUNG

Thesis submitted in fulfillment of the requirements for the degree of Master of Science

November 2007

ACKNOWLEDGEMENTS

I would like to convey my gratitude to my venerable supervisor Assoc. Prof. Dr. Peh Kok Khiang and co-supervisor Assoc. Prof. Dr. Tham Sock Ying for their invaluable guidance, encouragement, help and patience as well as the stimulating discussion during the entire research period that lead to the completion of this project.

I wish to express my sincere appreciation and gratitude to the staff of the School of Pharmaceutical Sciences, Universiti Sains Malaysia, for their kind assistance and guidance, especially to Assoc. Prof. Dr. Yvonne Tan Tze Fung.

I also extend my gratitude and regards to my beloved parents who have always encouraged and supported me in all respects. Special thanks are also extended to all my lab mates especially Mr. Yam Mun Fei, Ms. Tung Wai Hau, Ms. Yo Li Chen and Mr. Lim Vuangao for their generous support and superb cooperation.

Last but not the least, I would like to express my sincere thanks and appreciation to Malaysia Toray Science Foundation (MTSF) and Your Honorable Tan Sri Dato' (Dr) Katsunosuke Maeda for supporting my study.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

LIST OF ABBREVIATIONS

ABBREVIATIONS	MEANING
% v/v	Percent “volume in volume” expresses the number of milliliters of an active constituent in 100 milliliters solution.
% w/v	Percent “weight in volume” expresses the number of grams of an active constituent in 100 milliliters of solution.
% w/w	Percent “weight in weight” expresses the number of grams of an active constituent in 100 grams of solution.
AA	Ascorbic acid.
ABTS	2,2’-azino-bis(3-ethylbenzthiazoline)-6-sulfonic acid.
$ABTS^{+}$	Oxidized 2,2’-azino-di-(3-ethylbenzthiazoline)-6-sulfonic acid.
Ag/AgCl	Silver/silver chloride.
ANOVA	Analysis of variance.
c	Concentration by volume.
cm	Centimeter.
Cys	L-cysteine.
D	*Dextro* (in configurational sense only).
DD	Degree of deacetylation.
DTGS	Deuterated tri-glycine sulfate.
E	Elongation at break.
e.g.	(*example gratia*) for example.
Eq.	Equation.
et al.	(*et alii*) and others, and other people: for three or more co-authors or co-workers.
F_A	Molar fraction of acetylated units.

FAD	Flavin adenine dinucleotide.
$FADH_2$	Reduced flavin adenine dinucleotide.
FCHIT	Chitosan sample purchased from Fluka.
FDUVS	First derivative ultraviolet spectrophotometry.
FET	Field-effect transistor.
g	Gram(s).
g/ml	Gram per milliliter.
GFC	Gel filtration chromatography.
GlcN	D-glucosamine.
GlcNAc	*N*-acetyl-D-glucosamine.
GOD	Glucose oxidase.
GOD-FCHIT	Immobilized GOD-FCHIT membrane.
GOD-FCHIT/PT	Enzyme electrode of GOD-FCHIT.
GOD-SCHIT	Immobilized GOD-SCHIT membrane.
GOD-SCHIT/PT	Enzyme electrode of GOD-SCHIT.
GPC	Gel permeation chromatography.
H	Vertical distance (mm) from ZCP to each GlcNAc solution spectrum.
h	Hour.
H_1	*H* values of the pure GlcNAc solution.
H_2	*H* values of the different percentages of GlcNAc solutions.
HAc	Acetic acid.
HPLC	High-pressure liquid chromatography.
I	Steady state current.
i.e.	(*id est*) that is.

I_{max}	Maximum current.
IR	Infrared.
kDa	Kilo Dalton.
kg	Kilogram(s).
kg/m^3	Kilogram per cubic meter.
L	Path length of light.
L	*Levo* (in configurational sense only).
LA	Lactic acid.
lim	Limit.
ln	Natural logarithms.
M_1	Weight of the solvent or chitosan solution (g), obtained from weight of pycnometer containing solvent or solution – weight of empty pycnometer.
M_2	Weight of water (g), obtained from weight of pycnometer containing water – weight of empty pycnometer.
MA	Maleic acid.
mg	Milligram.
mg/dl	Milligram per deciliter.
mg/ml	Milligram per millimeter.
min	Minute.
ml	Milliliter.
ml/cm^2	Millimeter per square centimeter.
mm	Millimeter.
mm^2	Square millimeter.
MPa	Mega Pascal.
M_v	Viscosity-average molecular weight.

MW	Molecular weight.
N	Newton.
nA	Nanoampere.
nm	Nanometer.
NMR	Nuclear magnetic resonance.
no.	Number.
PCM	Acetaminophen.
pH	*pondus Hydrogenii* (acid-base scale; log of reciprocal of hydrogen ion concentration).
pI	Isoelectric point.
pK_a	The negative logarithm of the dissociation constant.
POD	Peroxidase.
Pt	Platinum electrode.
R^2	Correlation coefficient.
rpm	Rotation per minute.
RSD	Relative standard deviation.
s	Second.
S.E.M	Standard error mean.
S/N	Signal-to-noise ratio.
SCHIT	Chitosan sample purchased from Sigma.
SD	Standard deviation.
SEA	Specific enzyme activity.
SEC	Size exclusion chromatography.
SEM	Scanning electron microscopy.
TS	Tensile strength.
UA	Uric acid.

UV	Ultra violet.
UV/VIS	Ultra violet-visible.
V	Volt(s).
V_{max}	Maximal velocity.
vs.	Versus.
W	Mass of chitosan sample used.
ZCP	Zero crossing point of aqueous acetic acid.

LIST OF SYMBOLS

SYMBOLS	MEANING
K_M^{app}	Apparent Michaelis-Menten constant.
%	Percent.
[η]	Intrinsic viscosity.
<	Greater than.
>	Less than.
$\Delta G^{\circ\ddagger}$	Free energy of activation.
°C	Centigrade degrees.
µl	Microliter.
µmol	Micromole.
A	Absorbance.
a	Mark-Houwink constant.
A	Correction factor for the thrust of the air, $0.0012M_{2.}$
E	Molar extinction coefficient.
K	Mark-Houwink constant.
M	Molar.
mM	Millimolar.
N	Normal (equivalents of solute per liter of solution, as applied to concentration).
n	Number of run.
t	Efflux time of chitosan solution flow in viscometer (s).
t_0	Efflux time of the solvent flow in viscometer (s).
U	Unit of enzymatic activity.
α	Alfa.

β	Beta.
δ	Delta.
η	Viscosity of the solution or liquid.
η_0	Viscosity of solvent.
η_{inh}	Inherent viscosity.
η_{red}	Reduced viscosity.
η_{rel}	Relative viscosity.
η_{sp}	Specific viscosity.
μA	Micro ampere.
ρ	Density of chitosan solution.
ρ_0	Density of solvent.

LIST OF APPENDICES

KAJIAN CIRI-CIRI KITOSAN SEBAGAI MATRIKS IMMOBILISASI BAGI BIOSENSOR

ABSTRAK

Dua jenis kitosan (FCHIT dan SCHIT) telah diselidik sebagai matriks immobilisasi bagi pembuatan biosensor glukosa. Kelikatan-purata berat molekul bagi FCHIT and SCHIT telah ditentukan iaitu 981.80 kD dan 398.61 kD masing-masing. Darjah deasetilasi yang ditentukan dengan FDUV spektrofotometri didapati sebanyak 82.44% dan 77.20% masing-masing. Ciri-ciri fizikal larutan dan membran kitosan telah dikaji dengan melarutkannya di dalam pelbagai jenis pelarut asid organik (asid asetik, asid laktik dan asid maleik). Kedua-dua jenis kitosan paling larut dalam asid asetik akueus, diikuti dengan asid laktik dan akhir sekali asid maleik. Membran kitosan yang disediakan dalam asid asetik adalah fleksibel, lutsinar, rata dan cepat kering. Membran tersebut mempamerkan kekuatan mekanikal dan panjangan-pada-takat-pecah yang baik serta nyata sekali lebih tinggi daripada yang disediakan dalam asid laktik dan asid maleik. Hasil kajian analisis FTIR dan mikrograf SEM menunjukkan interaksi intermolekular antara kitosan dan glukosa oksidase (GOD). Aktiviti katalitik yang lebih tinggi telah diperhatikan pada GOD-FCHIT daripada GOD-SCHIT dan juga melalui ikatan-silang dengan glutaraldehid daripada penjerapan. Muatan enzim yang lebih tinggi daripada 0.6 mg boleh mengurangkan aktiviti. Reaksi terhadap glukosa paling tinggi diperhatikan pada membran dengan ketebalan 0.21 ml/cm^2 bagi GOD-FCHIT/PT, manakala pada membran dengan ketebalan 0.35 ml/cm^2 bagi GOD-SCHIT/PT. Keadaan eksperimen yang optimum untuk menganalisis glukosa

pada pH 6.0 melalui biosensor didapati ialah 35°C dengan keupayaan gunaan pada 0.6 V. Dalam keadaan itu, masa reaksi pada 85 s dan 65 s telah diperhatikan bagi GOD-FCHIT/PT dan GOD-SCHIT/PT masing-masing. Michaelis-Menten tetap yang nyata didapati 12.7370 mM bagi GOD-FCHIT/PT dan 17.6920 mM bagi GOD-SCHIT/PT. Ini menunjukkan bahawa GOD-FCHIT/PT mempunyai afiniti yang lebih besar bagi enzim itu. Lagipun, GOD-FCHIT/PT menunjukkan kepekaan yang lebih tinggi (52.3666 nA/mM glukosa) apabila dibandingkan dengan GOD-SCHIT/PT (9.8579 nA/mM glukosa) pada S/N>3. Kebolehan mengulang dan kebolehan menyalin yang lebih baik telah dicapai oleh GOD-FCHIT/PT dibandingkan dengan GOD-SCHIT/PT dalam sukatan glukosa. GOD-FCHIT/PT didapati menunjuk aktiviti enzim yang tertinggi di kalangan elektrod yang diselidik selama 2 bulan dalam kajian. Takat gangguan dihadapi oleh GOD-FCHIT/PT dan GOD-SCHIT/PT adalah tidak berbeza dengan nyata sekali. Walaupun biosensor dengan selaput Nafion dapat mengurangkan gangguan isyarat dengan nyata sekali, ia juga dapat mengurangkan reaksi terhadap glukosa dengan signifikan. Perlaksanaan biosensor dalam penentuan glukosa dalam serum tikus telah ditaksir. Keputusan ketepatan dan dapat kembali yang lebih baik telah diperolehi oleh GOD-FCHIT/PT. Maka, GOD-FCHIT/PT menunjukkan perlaksanaan yang lebih baik apabila dibandingkan dengan GOD-SCHIT/PT. Sebagai kesimpulan, membran kitosan mempunyai potensi untuk dijadikan suatu matriks yang sesuai bagi perkembangan biosensor glukosa.

A STUDY ON THE CHARACTERISTICS OF CHITOSAN AS AN IMMOBILIZATION MATRIX FOR BIOSENSORS

ABSTRACT

Two chitosan samples (FCHIT and SCHIT) were investigated as an enzyme immobilization matrix for the fabrication of glucose biosensor. The viscosity-average molecular weight of FCHIT and SCHIT were determined to be 981.80 kD and 398.61 kD respectively. Their degree of deacetylation determined by FDUV spectrophotometry were 82.44% and 77.20% respectively. The physical properties of chitosan solution and membrane were studied by dissolving the chitosan in different organic acids (acetic acid, lactic acid and maleic acid). Both the chitosan samples were most soluble in aqueous acetic acid, followed by lactic acid and maleic acid. Chitosan membranes prepared from acetic acid were flexible, transparent, smooth and quick-drying. They exhibited good mechanical strength and elongation at break and the values were significantly higher than those prepared in lactic acid and maleic acid. FTIR spectra and SEM micrographs showed the existence of intermolecular interactions between chitosan and glucose oxidase (GOD). Higher catalytic activities were observed on GOD-FCHIT than GOD-SCHIT and for those crosslinked with glutaraldehyde than through the adsorption technique. Enzyme loading higher than 0.6 mg could decrease its activity. The highest response for glucose was observed at 0.21 ml/cm^2 membrane thickness for GOD-FCHIT/PT and 0.35 ml/cm^2 membrane thickness for GOD-SCHIT/PT. The optimum experimental conditions for analyzing glucose at pH 6.0 using the biosensors were found to be at 35 °C with an applied potential of 0.6 V. Under such conditions, response

times of 85 s and 65 s were observed for GOD-FCHIT/PT and GOD-SCHIT/PT respectively. The apparent Michaelis-Menten constant (K_M^{app}) was found to be 12.7370 mM for GOD-FCHIT/PT and 17.6920 mM for GOD-SCHIT/PT. This indicated that the GOD-FCHIT/PT had greater affinity for the enzyme. Moreover, GOD-FCHIT/PT showed higher sensitivity (52.3666 nA/mM glucose) when compared with GOD-SCHIT/PT (9.8579 nA/mM glucose) at S/N>3. A better repeatability and reproducibility were achieved by GOD-FCHIT/PT than GOD-SCHIT/PT in the glucose measurement. GOD-FCHIT/PT was found to give the highest enzymatic activity among the electrodes under investigation. The extent of interference encountered by GOD-FCHIT/PT and GOD-SCHIT/PT was not significantly different. Although the Nafion coated biosensor significantly reduced the signal due to the interferents under study, it also significantly reduced the response to glucose. The performance of the biosensors in the determination of glucose in rat serum was evaluated. Comparatively better accuracy and recovery results were obtained for GOD-FCHIT/PT. Hence, GOD-FCHIT/PT showed a better performance when compared with GOD-SCHIT/PT. In conclusion, chitosan membrane has the potential to be a suitable matrix in the development of glucose biosensor.

CHAPTER 1: INTRODUCTION

1.1 CHITOSAN

1.1.1 General Introduction and Functional Properties

Chitosan, a linear binary heteropolysaccharide, is composed of β-1,4-linked glucosamine (GlcN) with various degrees of *N*-acetylation of GlcN residues (Kittur *et al*., 2003). Chitosan occurs naturally in some microorganisms, yeast and fungi (Illum *et al*., 2001). Its occurrence is much less widespread than that of chitin. Chitin is a linear chain consisting of *N*-acetyl-D-glucosamine (2-acetamido-2-deoxy-β-D-gluconopyranose) joined together by β(1→4) linkage (Krajewska, 2005). It is a non-toxic, biocompatible and biodegradable natural polymer of high molecular weight (~500,000 kDa) (Yadav & Bhise, 2004). It is the second most common polysaccharide occurring in nature after cellulose. Chitin is found in abundance in shells of exoskeletons of insects, shells of crustaceans and fungal cell wall (Illum *et al*., 2001; Tangpasuthadol *et al*., 2003; Aberg *et al*., 2004).

Chitosan is prepared by alkaline *N*-deacetylation of chitin (Kittur *et al*., 2003; Berger *et al*., 2004) using concentrated sodium hydroxide (NaOH) solutions at high temperature for a long period of time. Another approach to produce chitosan is by enzymatic *N*-deacetylation under relatively mild conditions (Prashanth *et al*., 2002; Wang *et al*., 2004). The commercially available chitosan is mostly derived by alkaline *N*-deacetylation from chitin of crustaceans because it is easily obtainable from the shells of crabs, shrimps, lobsters and krill (Amorim *et al*., 2003; Cervera *et al*., 2004a; Krajewska, 2005).

Figure 1.1 shows the two-step process in the production of chitosan. It involves extraction of chitin and removal of calcium carbonate ($CaCO_3$) with dilute hydrochloric acid from shells of crustaceans and deproteination with dilute aqueous sodium hydroxide. The second step is deacetylation of chitin by treating it with 40-50% aqueous sodium hydroxide at 110-115 °C for several hours without oxygen. Chitosan is produced when the degree of deacetylation (DD) is greater than 50% (Steenkamp *et al*., 2002). However, it was also reported that chitin with a DD of 75% or above is known as chitosan (Cervera *et al*., 2004a).

The two polymers, chitin and chitosan have similar chemical structure and are analogues of the homopolymer cellulose where the respective acetamido and amino groups replace the hydroxyl group at carbon-2 as shown in Figure 1.2. The difference between chitin and chitosan is in the acetyl content of the polymer where they can be distinguished by their solubility.

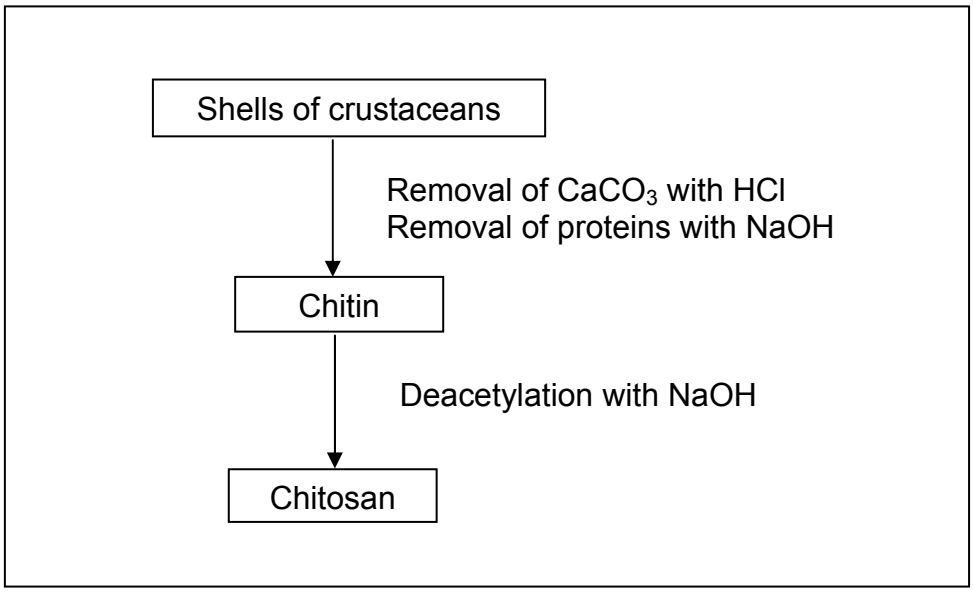

Figure 1.1. Production of crude chitosan.

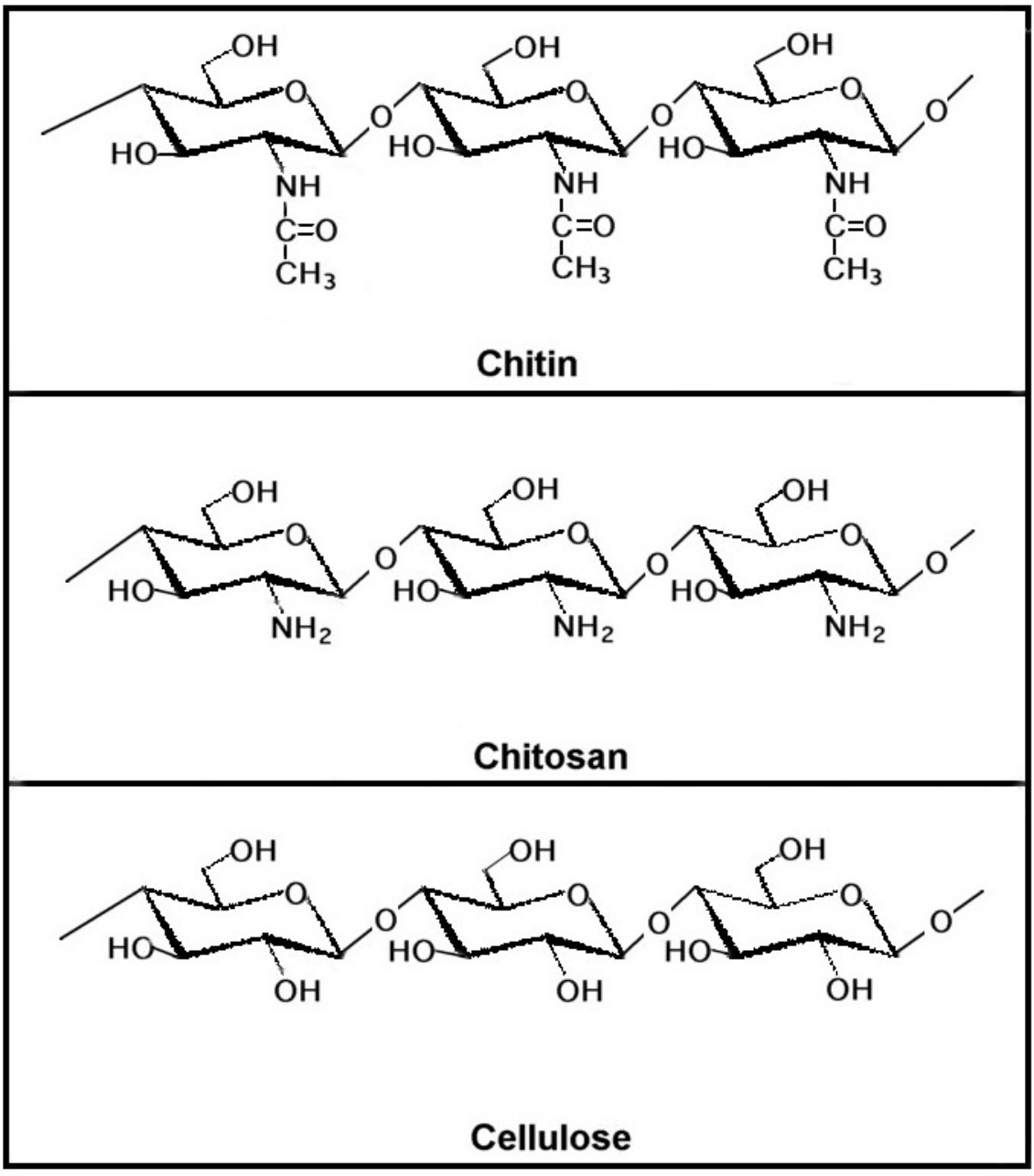

Figure 1.2. Structure of chitin, chitosan and cellulose.

The degree of deacetylation (DD) and molecular weight (MW) are two fundamental parameters that can affect the properties and functionality of chitosan (Berger *et al*., 2004; Baxter *et al*., 2005; Cho *et al*., 2006). These properties include solubility (Rege & Block, 1999; Hwang & Shin, 2000; Duarte *et al*., 2002), viscosity (Yadav & Bhise, 2004), reactivity such as heavy metal ion chelation and proteinaceous material coagulation (Sabnis & Block, 2000; Duarte *et al*., 2002; Gamage & Shahidi, 2007), loading (enzyme-loaded) properties (Alsorra *et al*., 2002) and film properties such as tensile strength, elasticity, elongation and moisture absorption (Lipscomb, 1995; Tan *et al*., 1998; Nunthanid *et al*., 2001).

With the apparent pK_a value of the amino group of about 6.5 (Taqieddin & Amiji, 2004), chitosan is only soluble in aqueous acidic solutions and insoluble in water and alkaline solutions (Krajewska, 2004). When dissolved, the amino groups ($–NH_2$) of the glucosamine are protonated to $-NH_3^+$ (Wang *et al.*, 2006). The cationic polyelectrolyte readily forms electrostatic interactions with other anionic groups (Fee *et al*., 2003). In an acidic environment the majority of polysaccharides are usually neutral or negatively charged (Chen & Tsaih, 1998; Hwang & Shin, 2000). The cationic chitosan molecule interacts with negatively charged surfaces and anionic systems leading to modification of the physicochemical characteristics of these systems (Illum *et al*., 2001; Xu *et al*., 2005), ultimately giving rise to its unique functional properties.

1.1.2 Membrane Properties

The mechanical property is one of the parameters considered in the selection of the membrane in any application (Chen & Hwa, 1996). Tensile testing provides an indication of the strength and elasticity of the membrane. Tensile strength is a measurement of breaking strength applied per unit of cross-sectional area. Elongation at break however, is a measure of the ductility of a membrane, a characteristic that defines the ability of a membrane to deform before failure occurs. Therefore, elongation is a type of deformation, which is simply a change in shape under stress. Low values for elongation at break imply brittleness in the membrane (Macleod *et al*., 1997). A membrane is considered brittle when it cannot deform very much or stretch very far before it breaks. Therefore, tensile strength and elongation at break take into account the response of membranes to an external stress.

1.1.3 Molecular Weight and Methods of Characterization

The total length of the chitosan polymer formed by repeating units of D-glucosamine is an important characteristic of the molecule. Hence, the molecular weight (MW) is a key feature for its functional properties (Wang *et al*., 2004). Nunthanid *et al*. (2001) reported that increase in molecular weight of chitosan increased the tensile strength, elongation as well as moisture absorption of the films. Chen and Hwa (1996) explored the effect of MW of chitosan with the same degree of deacetylation (DD) on the tensile strength, elongation at break, enthalpy and permeability properties of the chitosan membrane. They showed that tensile strength, elongation at break and enthalpy of membrane prepared from high MW chitosan were higher than those

of low MW chitosan. However, the permeability of membrane prepared from high MW chitosan was lower than that prepared from low MW chitosan. Higher MW chitosan was reported to have good film-forming properties because of intra- and intermolecular hydrogen bonding (Cervera *et al*., 2004b). Furthermore, high MW chitosan could affect the ability of chitosan to retard drug release. Fukura *et al.* (2006) reported the use of high and low MW chitosan as matrix tablet retardants and as drug release enhancers for poorly water-soluble drugs respectively. The latter might be due to an improvement in wettability resulting from better solubility of low MW chitosan in water. The effect of MW of chitosan on its antibacterial activity has also been explored. Increasing the MW of chitosan increased the antibacterial activity (Zhang *et al*., 2003).

Due to the harsh deacetylation in commercial processing of native chitin involving both alkaline *N*-deacetylation and acidic depolymerization, commercial chitosan are available in the MW range of 50 to 2,000 kDa (Rege & Block, 1999). MW of chitosan can be further lowered by acidic depolymerization (Berger *et al*., 2004) and prolonged reaction time of deacetylation (Blair *et al*., 1987).

MW of chitosan can be measured by gel permeation chromatography (Chen & Hwa, 1996; Pochanavanich & Suntornsuk, 2002; Kumar *et al*., 2004), size-exclusion chromatography coupled to multi-angle laser light scattering (Fee *et al*., 2003), high-performance liquid chromatography (Wu *et al*., 1976), light scattering (Rao, 1993; Chen & Tsaih, 1998) or viscometry (Maghami &

Roberts, 1988; Chen & Hwa, 1996; Schipper *et al*., 1996; Sabnis & Block, 2000; Berth & Dautzenberg, 2002). Among these techniques, viscometry is the most commonly used method for determining the MW of polymers (Wang *et al*., 2004). Use of light-scattering instrument usually requires prior experience and unknown sources of dust in the sample can often corrupt the data. Although gel permeation chromatography (GPC), size exclusion chromatography (SEC), high-pressure liquid chromatography (HPLC) and gel filtration chromatography (GFC) are by far the most versatile and useful techniques for the determination of MW in a polymer sample, these would involve the use of expensive instruments.

1.1.3(a) Viscometry

Polymers dissolved in solution may have polymer-solvent interactions, and generally results in an increase in viscosity (Sekhon & Singh, 2004). The viscosity of polymers is dependent on molecular weight (MW). The higher the MW of polymer, the more viscous the polymer solution will be (Choi *et al*., 2005). When a polymer has a higher MW, it has a bigger hydrodynamic volume, that is, the volume of a polymer coil when it is in solution. The solvent molecules will be bound more strongly to the polymer with increasing hydrodynamic volume, leading to a decrease in the motion of the polymer in the solvent. Hence, the viscosity of a polymer solution is proportional to the MW of the polymer. Therefore, by measuring the viscosity of a polymer solution, the MW of the polymer can be conveniently determined.

The viscosity of a fluid is a measure of its resistance to flow (Harding, 1997). Several important viscosity functions are used in viscosity studies. The relative viscosity, $\eta_{rel} = \eta/\eta_0$, is the dimensionless ratio of solution viscosity, η, to solvent viscosity, η_0. The specific viscosity is given by $\eta_{sp} = \eta_{rel} - 1$. The reduced viscosity, $\eta_{red} = \eta_{sp}/c$, is the increase in fluid viscosity per unit polymer solute concentration, c. The unit of reduced viscosity is ml/g (or dl/g). A related term is the inherent viscosity, $\eta_{inh} = (\ln\eta_{rel})/c$. Owing to the effects of non-ideality and/or associative phenomena, both η_{red} and η_{inh} are concentration dependent. The limit as $c \rightarrow 0$ of both η_{red} and η_{inh} is defined as the intrinsic viscosity $[\eta]$, presumably so named because it is an intrinsic function of the dissolved/dispersed macromolecule (Harding, 1997):

$$[\eta] = \lim_{c\rightarrow 0}(\eta_{red}) = \lim_{c\rightarrow 0}(\eta_{sp}/c)$$

$$[\eta] = \lim_{c\rightarrow 0}(\eta_{inh}) = \lim_{c\rightarrow 0}\{(\ln\eta_{rel})/c\}$$

Extrapolation of zero polymer concentration will eliminate polymer intermolecular interactions. When the polymer concentration is expressed in g/ml, the units of $[\eta]$ will be ml/g. The plots used to find the intrinsic viscosity are called the Huggins plot (η_{red} versus c) which usually has a positive slope and Kraemer plot [$\ln(\eta/\eta_0)$ versus c] which has a negative slope. The curves of both plots should be linear with a common intercept, which is the intrinsic viscosity (Harding, 1997).

The intrinsic viscosity measured in a specific solvent is related to the viscosity-average molecular weight, M_v, by the Mark-Houwink equation,

$$[\eta] = KM_v^a$$

where *K* and *a* are Mark-Houwink constants, whose values depend on the polymer type and the solute-solvent system (Laka & Chernyavskaya, 2006). For chitosan, they are affected by the degree of deacetylation, pH, ionic strength and temperature (Mao *et al*., 2004; Wang *et al*., 2004) but are independent of MW over a wide range of values (Prashanth *et al*., 2002; Kittur *et al*., 2003; Wang *et al*., 2004). The exponent '*a*' is a function of polymer geometry, and is equal to 0, 0.5~0.8 and 1.8 for sphere, random coil and rod shape respectively. These constants can be determined experimentally by measuring the intrinsic viscosities of several polymer samples for which the MW can be determined by an independent method such as light scattering (Wang *et al*., 1991).

1.1.4 Degree of Deacetylation

The chemical composition of different types of chitosan is characterized by the F_A value (molar fraction of acetylated units) or the degree of deacetylation [DD = 100(1- F_A)%] (Trzciński *et al*., 2002). DD is the mole fraction of the glucosamine residue (GlcN) in the polymer chain (Shigemasa *et al*., 1996), indicating the proportion of free amino groups (reactive after dissolution in weak acid) on the polymer. This parameter is important since it indicates the cationic charge on the molecule after dissolution in dilute acid.

Chitosan with high DD has high positive charges resulting in high reaction activity because the relatively active primary amino groups of chitosan are readily available for chemical modifications (Pochanavanich & Suntornsuk, 2002; Wang *et al*., 2004). Depending on its MW, the increase in DD of chitosan

could change the tensile strength of the membranes. Chitosan membranes become more brittle and absorb less moisture at higher DD (Nunthanid, 2001). Kim *et al*. (2006) reported that low DD chitosan films have lower water vapour permeability and total soluble matter as well as higher tensile strength compared with high DD chitosan films.

The *N*-deacetylation of chitin is almost never complete without inducing degradation of the polysaccharide backbone (Prashanth *et al*., 2002; Cervera *et al*., 2004a). The DD values close to 100% is rarely achieved with the relatively mild and simple alkaline *N*-deacetylation method (Yong *et al*., 2000). The DD of commercially available chitosan generally ranges from 60 to 90%, depending on the manufacturing process (Rege & Block, 1999). Anyway, DD can be lowered by reacetylation (Berger *et al*., 2004). Hwang *et al*. (2002) reported that the MW of chitosan drastically decreased and DD increased with an increase in temperature, reaction time and NaOH concentration.

Various methods have been reported for the determination of the DD of chitosan. These include pH-metric titrimetry (Avadi *et al*., 2004), linear potentiometric titrimetry (Tolaimate *et al*., 2000), colloid titrimetry (Berth & Dautzenberg, 2002), sodium hydroxide titrimetry (Pochanavanich & Suntornsuk, 2002), hydrogen bromide titrimetry (Domszy & Roberts, 1985; Sabnis & Block, 1997), ninhydrin test (Curotto & Aros, 1993), ^{1}H NMR (Tolaimate *et al*., 2000; Mao *et al*., 2004; Freier *et al*., 2005), CP/MAS ^{13}C NMR (Prashanth *et al*., 2002; Kittur *et al*., 2003; Kumar *et al*., 2004), gel permeation chromatography (Berth & Dautzenberg, 2002), pyrolysis-gas chromatography

(Muzzarelli *et al*., 1980; Lal & Hayes, 1984), infrared spectroscopy (Sabnis & Block, 2000; Amorim *et al*., 2003; Mao *et al*., 2004;), near infrared spectroscopy (Rathke & Hudson, 1993), first derivative ultraviolet spectrophotometry (Muzzarelli & Rochetti, 1985; Tan *et al*., 1998; Khan *et al.* , 2002), ultraviolet spectrophotometry (Aiba, 1986), pyrolysis-mass spectrometry (Mattai & Hayes, 1982) and circular dichroism measurements (Domard, 1987).

Although many methods are available for the determination of DD, it is essential to choose a simple, rapid, user-friendly, cost effective and reliable method that could tolerate the presence of impurities, especially the common contaminant protein. Methods that measure directly the amine or acetyl amine groups on the glycoside unit of chitosan would be preferred (Tan *et al*., 1998). Sophisticated methods such as circular dichroism, NMR (nuclear magnetic resonance) and thermogravimetry are not only costly for routine analyses but require highly trained and skilled personnel (Tan *et al*., 1998). Infrared and near infrared spectroscopy are primarily solid-state methods, and may yield inaccurate results during the weighing of the hygroscopic chitosan sample. Moisture content hence needs to be eliminated and the sample purity must be determined separately. Furthermore, variation can be found in the results obtained using different baselines with these methods (Shigemasa *et al*., 1996; Tan *et al*., 1998). On the other hand, the hydrogen bromide titrimetry is limited by the presence of protein contaminants remaining in the sample during the extraction process, which resulted in lower DD values (Khan *et al*., 2002). Tan *et al.* (1998) also reported the protein contaminants commonly present in crude chitosan samples affecting the results of NMR, linear potentiometric titrimetry

and ninhydrin test. Titrimetry, NMR spectroscopy and gel permeation chromatography methods depend on the sample solubility (Shigemasa *et al*., 1996).

The first derivative ultraviolet spectrophotometry (FDUVS) was reported as the simplest and most convenient method among all the presently available methods (Tan *et al*., 1998). The method requires only very small amount of sample, simple reagents and instrumentation. There is no interference problem from protein contamination. Therefore, the FDUVS method was selected to determine the DD of chitosan samples in the present study.

1.1.5 Applications of Chitosan

Chitosan is increasingly important in the areas of biomedical, agriculture, cosmetics, environmental control, waste-water treatment and food processing. In biomedical applications, chitosan has been employed as absorption enhancer of hydrophilic drugs across mucosal surfaces (Fee *et al*., 2003), accelerator for wound healing (Muzzarelli, 1977; Minagawa *et al*., 2007), wound dressing (Martindale, 2000), haemodialysis membranes (Mallete *et al*., 1983; Nasir *et al*., 2005), contact lenses (Ravi-Kumar, 2000), artificial skin (Ravi-Kumar, 2000; Freier *et al*, 2005) and surgical sutures (Nakajima *et al*., 1986; Tachibana *et al*., 1988). Chitosan has also been used in drug delivery systems (Illum *et* al., 2001; Wang *et al*., 2001; Mi *et al*., 2002; Hsiue *et al*., 2003; Nie *et al*., 2006), ophthalmology (Ravi-Kumar, 2000), tissue engineering (Zhong *et al*., 2000; Anseth *et al*., 2002) and for enzyme immobilization (Zhou *et al*., 2002; Hsieh *et al*., 2003; Wang *et al*., 2005).

The excellent membrane forming, high mechanical strength and adhesion ability coupled with non-toxic and biocompatible characteristics make chitosan an ideal immobilization matrix for the fabrication and construction of biosensors (Yao *et al.*, 2003; Wang *et al.*, 2005; Lin *et al.*, 2007). In addition, chitosan is capable of adsorbing metal ions and various organic halogen substances thus prevent the enzyme used in biosensors from damage (Wang *et al*., 2005). Moreover, chitosan can form thermally and chemically inert film that is insoluble in water (Wang *et al*., 2005). Yang *et al.* (2004b) reported the enzyme immobilized on chitosan showed high activity due to its considerable protein-binding capacity. Apart from this, the ability to form a transparent thin film is another virtue for chitosan to be used in optical sensor (Zhao *et al*., 1998; Zhou *et al*., 2002).

In agriculture, chitosan is used primarily as a plant growth enhancer, a preservative coating and biofungicide that boosts the ability of plants to defend against fungal infections (Oester *et al*., 2000). In the cosmetic area, chitosan is used as a fungicidal and fungistatic agent in moisturizer, body creams, hair lotion and bath lotion (Ravi-Kumar, 2000). Moreover, chitosan is effective in treating acne. It is able to inhibit certain bacteria that cause inflammation associated with acne (Oester *et al*., 2000).

Chitosan-based formulations have major applications in wastewater treatment due to the coagulating, flocculating and metal-chelating properties of chitosan originating from the high density of amino groups on its polymer chains (Krawjewska, 2005). Chitosan is used as non-toxic flocculent in the treatment

of organic polluted wastewater and as a chelating agent or for the removal of toxic (heavy and reactive) metals from industrial wastewater. Furthermore, proteinaceous material from industrial wastewater can be removed through coagulation mechanism (Krajewska, 2005).

Chitosan has been found to be safe for oral consumption. In food industry, chitosan-based materials have been used as antimicrobial agents, beverage clarification additives, flavour extenders, colouring and texture stabilizers (Krajewska, 2005). Apart from these uses, chitosan is well known as a fat binder (Hennen, 1996). It is an amino polysaccharide that has the ability to bind lipids in the stomach before the lipids are absorbed through the digestive system into the blood stream. Recent years, Hayashi and Ito (2002) reported the antidiabetic action of chitosan. Accordingly, daily administration of chitosan solutions as drinking water prevented the progression of non-obese and obese type-2 (non-insulin dependent diabetes) diabetes mellitus through normalization of hypertriglycaeridemia, hyperglycaemia and hyperinsulinism.

1.2 BIOSENSOR

1.2.1 Introduction

A biosensor is commonly described as an analytical device incorporating a biological or biologically derived recognition element, either intimately associated or integrated within a physicochemical transducer to produce a signal proportional to the target analyte concentration (Singhal *et al*., 2002).

The biological component e.g. enzymes, antibodies, nucleic acids and receptors is a biomolecule that contributes to the high specificity of the biosensor in recognizing its target analyte. The analyte is first transformed by the biological component to a quantifiable property and then into an electrical signal by the transducer. Biological components can be distinguished as bioconverting agents or biocapturing agents (Freitag, 1999). Bioconverting agents such as enzymes catalyze oxidation or reduction involving specific substrate(s) to product(s). Antibodies, nucleic acids and receptors are examples of biocapturing agents where their selectivity are dependent on their affinity towards the target analyte. Depending upon the biological recognition elements used, biosensors can be divided into two groups, namely catalytic and affinity biosensors (Tombelli *et al*., 2005).

The choice of biological component depends on the analyte under investigation. What is important is a direct relationship between the biosensor signal and the quantity of the analyte. Since the invention of the first oxygen electrode by Clark and Lyons (1962), enzymes have been the most regularly employed biorecognition elements encountered in catalytic biosensors for the

analysis of small molecules such as glucose which is widely monitored in medicine, biotechnology and food industry (Freitag, 1999).

In the development of any biosensor, some critical performance requirements for a particular application must be considered. A reliable biosensor should respond selectively to an analyte of interest among a range of analytes. Alternatively, the response may be to a group of analytes of similar chemical structure such as carbonyl compounds. Apart from selectivity, a biosensor needs to show high sensitivity. The signal-to-noise ratio must be large, with detectable signals from small changes in analyte (e.g. 0.1 mM or approximately 2 mg/dl glucose) concentration (Wilkins & Atanasov, 1996). The linear dynamic range of the calibration curve should be wide enough for the assay of the analyte. For example, the determination of glucose in blood needs to be at least $1X10^{-4}$ to $5X10^{-2}$ M to cover the range of normal and diabetic blood glucose levels. For the biosensor to be useful, the detection limit has to be better than 10^{-5} M. Besides this, the response time has to be considered when developing a reliable biosensor as this may affect the usefulness of the device for repetitive routine analyses. The response time which refers to the time for the system to reach equilibrium should not exceed 10 min ideally (Eggins, 2002).

Being analytical devices, the measurements by biosensors must be precise where random errors must be below a certain level so that repetitive measurements are reproducible within a certain range. With biosensors, the expected reproducibility between replicate determinations should be at least

±(5-10)% (Eggins, 2002). Accuracy, which describes the proximity to the true value, and affected by systematic errors is another important criterion. Together with precision, they determine whether a method is suitable for a particular task (validation) or whether data generated under the routine use of a bioanalytical method are acceptable (acceptance criteria) (Karnes & March, 1993).

1.2.2 Enzyme Immobilization

The conversion of enzymes from a water-soluble, mobile state to a water-insoluble immobile state fixed onto a support/matrix physically separates the enzyme from the bulk of the solution (Krajewska, 2004; Milosavić *et al*., 2005). Three important aspects must be considered prior to immobilization, namely, a) properties of the free enzyme vs. the immobilized enzyme, b) type of support used and c) methods of support activation and enzyme attachment (Worsfold, 1995).

1.2.2(a) Properties of Free Enzyme vs. Immobilized Enzyme

Enzymes are catalytic proteins which possess high selectivity towards a given substrate. They increase the rate or velocity of a chemical reaction under mild conditions by lowering the free energy of activation ($\Delta G^{\circ\ddagger}$) of the chemical reaction without changing the overall process or equilibrium of a reaction. Although enzymes can catalyze one reaction after another, they may have lower activity after several runs. Unlike inorganic catalysts, enzymes are specific. Most enzymes can break down a particular substrate or synthesize a particular compound. The specific action of enzymes gives minimum unwanted

side-products. The various types of specificity of enzymes are stereo specificity, absolute specificity, group specificity and low specificity.

Immobilized enzyme possesses a number of advantages compared to the free enzyme (Pekel *et al*., 2003). Immobilization of enzymes onto a solid support protects them against oxygen, humidity and biological contaminants (Miertuš *et al*., 1998). The structure is therefore more stable and their handling easier (Naik *et al*., 2005). Immobilized enzyme systems allow reuse of the enzyme and easy recovery of the product, thus minimizing enzyme loss (Seo *et al*., 1998; Akgöl *et al*., 2001; Tsai *et al*., 2003). If immobilization procedure is reversible, the inactive enzyme can be desorbed and the matrix further recharged with the fresh enzyme.

In analytical applications, immobilized enzyme is key to the development of biosensors (Krajewska, 2004). The resultant biosensor must have good sensitivity, selectivity, dynamic range, response time, stability and shelf-life (Sakuragawa *et al*., 1998; Tsai *et al*., 2003). The performance of an enzyme electrode may be affected by the thickness of the enzymic layer, the enzyme loading as well as the conditions for the enzymatic reaction (Bardeletti *et al*., 1991).

Immobilization may have a considerable effect on enzyme kinetics, stability (Xu *et al*., 2001), changes in pH and temperature, Michaelis-Menten constant (K_M^{app}) and maximum reaction rate (V_{max}) for the enzyme-catalyzed reaction (Bartlett *et al*., 1992; Danisman *et al*., 2004). This could be due to structural

changes to the enzyme (Wang *et al*., 2003) with the creation of a distinct microenvironment, different from the bulk solution around the enzyme (Krajewska, 2004). The properties and functions of immobilized enzymes are therefore characterized by three factors that include a) the biochemical properties and the kinetic parameters of the enzyme, b) the chemical as well as mechanical properties of matrices and c) the immobilization methods.

1.2.2(b) Support

The most important factor affecting the performance of an immobilized enzyme is the support material (Krajewska, 2004). Different types of supports have been used to immobilize enzymes namely beads and membranes (Ida *et al*., 2000) using different immobilization techniques. There is no universal support for all enzymes. The types of matrix and conditions for immobilization have to be determined for each enzyme (Bickerstaff, 1997). The following characteristics should be considered when choosing a support for immobilizing an enzyme.

Physical properties

A suitable support must possess ease of assuming different geometrical configurations providing the system with permeability and surface area suitable for a chosen biotransformation (Krajewska, 2004). The surface density of the binding site available to the enzyme determines the maximum binding capacity. The support materials should also have good mechanical stability, rigidity and good flow properties for enzyme stability and activity on storage (Danisman *et al*., 2004; Krajewska, 2004).

Chemical properties

Hydrophilic matrices are generally preferred for enzyme immobilization. They should be inert to enzyme(s), substrate(s) or co-factor(s) and possess available functional groups for direct reactions and chemical modifications (Krajewska, 2004), have high affinity to proteins (Krajewska, 2004), have the ability to be regenerated or reused and are compatible with certain buffers (Fortier *et al*., 1990). They should also have a large surface area with a high content of the reactive groups (Arica *et al*., 2000; Danisman *et al*., 2004). Apart from this, a good support material should be non-degradable and biocompatible without altering the native structure of the enzyme and affecting its biological activity (Luo *et al*., 2004; Taqieddin & Amiji, 2004). In addition, an ideal support should be resistant against bacterial or fungal attack, disruption by chemicals, pH, temperature, organic solvents, or even enzymes such as proteases (Bickerstaff, 1997). They should be non-toxic and biocompatible if the end product is to be used for food, pharmaceuticals or agricultural products (Arica *et al*., 2000; Taqieddin *et al*., 2002; Krajewska, 2004).

1.2.2(c) Methods of Enzyme Immobilization

Methods of enzyme immobilization can be broadly classified as physical or chemical methods (Krajewska, 2004). The four common approaches to enzyme immobilization are a) adsorption, b) entrapment, c) covalent coupling and d) crosslinking (Eggins, 2002).

Adsorption

Adsorption is a simple, economical, reversible and quick way for immobilizing an enzyme with the retention of its activity (Hsu & Tsai, 2001; Yağar & Sağiroğlu, 2002; Debeche *et al.*, 2005). In this procedure, links between the matrix and the protein molecules can be hydrophobic or ionic in nature (Momić *et al*., 2002) with little or no conformational changes of the enzyme (Tang *et al*., 2004). The amount and stability of the immobilized enzyme might be low with no formation of covalent bonds between the support and the amino acid residues on the enzyme surface (Yağar & Sağiroğlu, 2002). Desorption of the enzyme may occur with changes in temperature, pH, solvent, ionic strength, concentration of enzyme or adsorbent (Zhu *et al*., 2005).

Entrapment method

This method is based on the localization of an enzyme within the lattice of a polymer matrix or its enclosure in semi-permeable membranes tight enough to prevent only the biocatalyst but not the substrate(s) or product(s) from diffusing out into the reaction medium. Here the enzymes are entrapped in the interstitial spaces of crosslinked and water-insoluble polymers without formation of bonds or chemical coupling between the enzyme and the gel matrix or membrane (Kennedy & Cabral, 1987).

The advantages of the technique include high viable enzyme concentration and the possibility of co-immobilizing different types of enzymes physically separated from each other. The technique does not alter the conformation of the enzyme where only aqueous solvents are used (Scheller & Schubert,

1992). There are, however, some major drawbacks. Firstly, the diffusional barriers as well as the steric hindrance to high molecular weight substrates make the method unsuitable for enzymes such as ribonuclease, trypsin, and dextranase acting on macromolecular substrates. The large diffusional barriers to the substrate and product may slow down the reaction and the response time of the biosensor. Secondly, some loss of enzyme activity due to the production of free radicals during polymerization or leakage through the wide pores in the gel could occur.

Another approach involves entrapping the enzyme within a hollow fibre of semi-permeable membrane such as cellulose triacetate where the substrate solution flows through the hollow fibre. The advantages of this method include high resistance of the fibres to weak acids and alkalis, solutions of high ionic strength and organic solvents. However, inactivation of the enzyme may occur with the use of water-immiscible liquids, polymer solvents or precipitating agents (Kennedy & Cabral, 1987).

The entrapment method also includes microencapsulation of the enzyme within a semi permeable membrane without any bond formation (Sharma *et al*., 2007). Microencapsulation provides a means of utilizing an enzyme continuously in its native state over a long period of time. The advantages of this immobilization technique include the extremely large surface area for contact between substrate and enzyme within a relatively small volume and the possibility of simultaneous entrapment of several (different) enzymes in a single step (Kennedy & Cabral, 1987). The sequence of enzymatic reactions in

multiple enzyme systems will result in longer response time (Bardeletti *et al*., 1991). Leakage of enzyme from the microcapsule may also take place (Kennedy & Cabral, 1987).

Covalent-binding method

Covalent coupling of the enzyme molecules with the support material lead to very stable preparations. The bond is normally formed between functional groups on the carrier and groups on the enzyme not essential for the catalytic activity (Lim *et al*., 1999; Eggins, 2002). Chemically reactive sites of a protein are usually amino (NH_2) groups from lysine or arginine, carboxyl (COOH) groups from aspartic acid, glutamic acid, hydroxyl (OH) groups from serine, threonine, phenol residues of tyrosine, sulfhydryl (SH) group from cysteine and the imidazole group of histidine (Scheller & Schubert, 1992; Eggins, 2002). Three main factors have to be considered for covalent immobilization of enzymes, namely a) the functional groups of proteins suitable for covalent binding, b) the coupling reactions between the enzyme and the support and c) the functionalized supports suitable for enzyme immobilization (Kennedy & Cabral, 1987).

The immobilization process is conducted in three steps namely activation of the carrier, coupling of the enzyme and removal of adsorbed enzymes from the support (Kennedy & Cabral, 1987). A wide variety of support materials have been used for enzyme immobilization including Sepharose (beaded agarose), cellulose, magnetic particles, silicates derived from China clay or diatomaceous earth and glass. In all cases, the support materials must possess reactive

groups. If they do not, then the support can be activated by chemical means using cyanogen bromide, carbodiimide, glutaraldehyde, aminosilane, diazonium salts, acid chloride, isocyanate and isothiocyanate derivatives. Selection of the crosslinker determines the type of covalent bond that will be formed (Kennedy & Cabral, 1987).

An advantage of this method is that covalent bonding is strong with no release of the enzyme into the solution even in the presence of substrate dissolved in high ionic strength solutions (Kennedy & Cabral, 1987). The covalent bonding between enzyme and carrier not only stabilizes the enzyme during catalytic reactions at higher temperature, it also allows the enzyme to withstand denaturants and organic solvents better (Arica *et al*., 2000). However, a loss in enzymatic activity due to its conformational changes is encountered if amino acids essential for the catalytic activity are involved in the covalent linkage to the support (Scheller & Schubert, 1992) or harsh coupling conditions are used (Afaq & Iqbal, 2001). To protect the active site, the enzyme can be immobilized in the presence of a competitive inhibitor or substrate (Kennedy & Cabral, 1987).

Crosslinking

This approach is based on the production of three-dimensional crosslinked insoluble enzyme aggregates by bi- or multifunctional reagents (Kennedy & Cabral, 1987). The chosen crosslinking agent specifically binds functional groups on the enzyme away from its active site to avoid inactivation, at concentrations suitable for aggregation. The gelatinous nature of the product