IMMOBILISATION OF ELECTRIC EEL ACETYLCHOLINESTERASE ON NANOFIBRES ELECTROSPUN FROM A NYLON AND CHITOSAN BLEND

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ABSTRACT

Organophosphates and carbamates are potent inhibitors of the neurotransmitter acetylcholinesterase. This inhibition results in the blocking of nerve signal transference into the post synaptic neuron leading to loss of muscle action and death. Because of the universal mechanisms of signal transduction in animals, these inhibitors have been widely used as agricultural pesticides as well as chemical warfare agents (nerve agents). Health issues associated with pesticide usage result from the fact that both the pesticides and their breakdown products often end up in water and food sources as well as in the soil. As a result, there has been an increase in the number of studies aimed at the detection of these pesticides in the environment. One popular research area is enzyme based biosensor construction. Some important criteria for consideration during the construction of biosensors are the importance of a suitable solid support as well as the enzyme immobilisation method. Recently, there has been increased interest in using nano-scale material e.g. using nanoparticles as enzyme support material. This is largely due to their advantages such as large surface area to volume ratio as well as reduced mass transfer resistance.

Electrospinning is a straight forward and cost effective method for producing nanofibres from any soluble polymer(s). The applications of electrospun nanofibres have been reported in clinical studies, biofuel production as well as bioremediation. In this study two polymers were selected: nylon for its mechanical stability and chitosan for its biocompatibility and hydrophilicity, for the fabrication of electrospun nanofibres which would function as immobilisation support material for acetylcholinesterase.

The first objective of this study was to electrospin nanofibres from a nylon-6 and chitosan blend solution. A binary solvent system consisting of formic acid and acetic acid (50:50) successfully dissolved and blended the polymers which were subsequently electrospun. Scanning electron microscopy characterisation of the nanofibres showed that (i) a nylon-6: chitosan ratio of 16%: 3% resulted in the formation of bead free nanofibres and (ii) the fibres were collected in non-woven mats characterised by different size nanofibres with average diameters of 250 nm for the main fibres and 40 nm for the smaller nanofibres. Fourier transform infra-red (FT-IR) analysis of the nanofibres indicated that a new product had been formed during the blending of the two polymers.

The second aim of the study was to carry out a facile immobilisation of electric eel acetylcholinesterase via glutaraldehyde (GA) cross-linking. Glutaraldehyde solution 5% (v/v) resulted in the immobilisation of 0.334 mg/cm^2 of acetylcholinesterase onto the nanofibres. The immobilisation procedure was optimised with reference to acetylcholinestease and cross-linker concentrations, incubation time and the cross-linking method. A comparative investigation into the optimum pH and temperature conditions, pH and thermal stabilities, substrate and inhibition kinetics was then carried out on free and immobilised acetylcholinesterase. The final objective of this study was to determine the storage stabilities of the immobilised and free enzymes as well as the reusability characteristics of the immobilised acetylcholinesterase.

Several conclusions were drawn from this study. Acetylcholinesterase was successfully immobilised onto the surface of nylon-6:chitosan nanofibres with retention of its activity. There was a shift in the pH optimum of the immobilised acetylcholineseterase by 0.5 units towards a neutral pH. Although both free and immobilised acetylcholinesterase exhibited the same optimum temperature, immobilised acetylcholinesterase showed enhanced thermal stability. In terms of pH stability, immobilised acetylcholinesterase showed greater stability at acidic pH whilst free acetylcholinesterase was more stable under alkaline pH conditions. Relative to free acetylcholinesterase, the immobilised enzyme showed considerable storage stability retaining ~50% of its activity when stored for 49 days at 4°C. Immobilised acetylcholinesterase also retained > 20% of its initial activity after 9 consecutive reuse cycles. When exposed to fixed concentrations of carbofuran or demeton-S-methyl sulfone, immobilised acetylcholinesterase showed similar inhibition characteristics to that of the free enzyme.

The decrease in enzyme activity observed after immobilisation to the nanofibres may have been due to several reasons which include some enzyme molecules being immobilised in structural conformations which reduced substrate access to the catalytic site, participation of the catalytic residues in immobilisation and enzyme denaturation due to the reaction conditions used for acetylcholinesterase immobilisation. Similar observations have been widely reported in literature and this is one of the major drawbacks of enzyme immobilisation. In conclusion, nylon-6:chitosan electrospun nanofibres were shown to be suitable supports for facile acetylcholinesterase immobilisation and the immobilised enzyme has potential for use in pesticide detection.

Future recommendations for this study include a comparative study of the GA cross-linking method for AChE immobilisation which will lead to more intensely bound enzyme molecules to prevent non-specific binding. An investigation into the effect of inhibitors on stored immobilised AChE, as well as reactivation and reuse studies, may also be useful for determining the cost-effectiveness of reusing immobilised AChE for pesticide detection in environmental water samples. Several models have been designed for the determination of the kinetic parameters for immobilised enzymes. These take into account the mass transfer resistance as well as the overall charge of the immobilisation matrix. The use of these models to analyse experimental data will give a clear understanding of the effects of immobilisation on enzyme activity

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LIST OF ABBREVIATIONS

AA	Acetic acid
ACh	Acetylcholine
AChE	Acetylcholinesterase
BSA	Bovine serum albumin
ChAT	Choline acetyltransferase
ChO	Choline oxidase
CP	Carbamate pesticide
DEAE	Diethylaminoethyl
DD	Degree of deacetylation
DDT	Dichlorodiphenyltrichloroethane
DTNB	5,5’-dithiobis-2-nitrobenzoic acid
FA	Formic acid
GA	Glutaraldehyde
GC/MS	Gas chromatography coupled to mass spectrometry
HPLC	High performance liquid chromatography
MWNT	Multiwall carbon nanotubes
OP	Organophosphate pesticide
OPH	Organophosphate hydrolase
PAN	Polyacrylonitrile
PS	Polystyrene
PVA	Polyvinyl alcohol
TNB	2-nitro-5-thiobenzoic acid
UV	Ultraviolet
WRC	Water Research Commission (of South Africa)

PLAGIARISM DECLARATION

I declare that this thesis is my own, unaided work. It is being submitted for the degree of Master of Science of Rhodes University. It has not been submitted before for any degree or examination at any other university.

Tendai Mafuma

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Grahamstown

RESEARCH OUTPUTS

Conference Presentation

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Chapter 1

Chapter 1: LITERATURE REVIEW AND INTRODUCTION

1.1 Water pollution

Pollution is defined as the direct or indirect introduction of substances into the environment that have potential to cause deleterious effects to living organisms as well as human health (Monserrat *et al.,* 2007). Pollutants may be introduced into aquatic systems via various point and non-point sources. According to Section 502(14) of the Clean Water Act, the term “point source” refers to any discernible, confined and discrete conveyance from which pollutants are or may be discharged (Internet 1). “Non-point sources” on the other hand, are diffuse and include run-off from roads, parking lots and residential areas, metal leaching from dumping sites, fertilizers, pesticides and atmospheric deposition of metals. The identification and prevention of non-point sourced pollution is difficult, since this form of pollution is often diffuse. As a result, non-point source pollution is the leading cause of environmental health problems in aquatic ecosystems (Buntsama and Casey-Lefkowitz, 1999). Intensive agricultural practices are essential to achieve high crop and livestock yields; however, they often utilise pesticides and fertilisers which present potential risks to water sources. These compounds can readily penetrate groundwater sources and run-off during rainfall adding to the level of contaminants in surface waters (Wauchope, 1978; USEPA, 2004).

Agriculture is one of the few activities where chemicals are intentionally utilised and released into the environment with the objective of killing organisms. The term "pesticide" is a composite term that includes all chemicals used to kill or control pests. These pesticides may include herbicides (weeds), insecticides (insects), fungicides (fungi), nematocides (nematodes), and rodenticides (vertebrate poisons) (Tomlin, 2000). Although highly beneficial, pesticide usage has some detrimental effects. Some pesticides have the potential to disrupt predator-prey relationships, reduce biodiversity and interfere with reproductive health. Although water pollution includes both agricultural and non-agricultural compounds, it is difficult to separate the ecological and human health effects of agricultural from those of non-agricultural compounds. There is overwhelming evidence that pesticide usage in agriculture has a major impact on water quality and leads to serious environmental consequences (Ongley, 1996).

Agrochemical usage for pest control in South Africa has increased substantially over the past decades, particularly in the fruit and vegetable producing sectors. London and Myers (1995) investigated the agrochemical usage patterns in specific farming sectors in the southern region of South Africa, where they identified extensive and varied pesticide use depending on agricultural sector. Worldwide sales of pesticides increased substantially in both 1995 and 1996 (Naidoo and Buckley, 2003). It was predicted that pesticide sales would continue to increase. South Africa alone saw an increase of nearly 31% in pesticide sales between 1994 and 2000 (Naidoo and Buckley, 2003). Based on population growth and subsequent food demand, the need for pesticides will increase (Naidoo and Buckley, 2003).

1.1.1 Pesticides

Pesticides are classified according to the type of pest they are used to control e.g. fungicides for fungi. In addition to this, they can be classified according to their chemical properties. Pyrethroid pesticides are derived from naturally occurring pyrethrins obtained from the oleoresin of dried *Chrysanthemums*. Pyrethrins are characterised by strong lipophilic acids, which allow rapid penetration of the pesticides into the insects leading to paralysis of the nervous system through interference with the ion exchange systems (Reigart and Roberts, 1999). Naturally occurring pyrethrins are photosensitive, thus synthetic pyrethrins have been developed and modified to increase their stability in the environment. Most formulations of synthetic pyrethrins are often combined with synergists such as MGK-264 which enhance both their pesticidal effects and persistence in the environment (Casida, 1980; Gosselin *et al.,* 1984). Organochlorine insecticides are a large class of multipurpose chlorinated hydrocarbon chemicals which have been banned due to their adverse health and environmental effects, persistence as well as their tendency for bioaccumulation (e.g. DDT and chlordane). Many organochlorine pesticides are endocrine disrupting chemicals, meaning they have subtle adverse effects on the body's hormonal systems. Organophosphate and carbamate pesticides affect the nervous system by inhibiting acetylcholinesterase (AChE), the enzyme that regulates acetylcholine (ACh), a neurotransmitter found in a wide range of organisms. Pesticide development over time is driven by increasing pest resistance to pesticides, the search for pesticides with improved specificity and reduced negative effects on human health, as well as the need for 'greener' pest control methods (Table 1).

Table 1: Chronology of pesticide development (adapted from Stephenson and Solomon, 1993)

Period	Type of pesticide	Effect on humans	**Environmental effects**
1800-1920's	Nitrophenols, chlorophenols, petroleum oils	Very toxic	Toxic to aquatic organisms
1945-1955	Polychlorinated organics e.g. DDT, chlorinated cyclodienes	Toxic - Neurotoxic, carcinogenic	Persistent, good selectivity, bioaccumulation, ecological effects
1945-1970	Organophosphates e.g. malathion	Poisonous and toxic – interfere with AChE	Low persistence, some ecological effects
1970-1985	Synthetic pyrethroids	Seizures and irritability,	Not selective
1985-	Genetically engineered organisms	Less toxic	Effect on microbial ecology
1990's -	Intergrated pest management	Less toxic	More environmentally friendly, less dangerous to environment

1.2 Organophosphate and carbamate pesticides

Organophosphate pesticides (OPs) are phosphoric acid esters or thiophosphoric acid esters that are widely used in the agricultural industry. The original compounds developed in the 1930s and 1940s were highly toxic to mammals; however, since then, OPs have been developed and manufactured to be less toxic to mammals, but remain toxic towards the target organisms. When applied to crops or directly to the soil as systemic insecticides, OPs generally persist from a few hours to several months. The primary molecular mechanism of these neurotoxic compounds, which are structurally similar to the nerve gases soman and sarin, is the irreversible inhibition of acetylcholinesterase (AChE). AChE is essential for the functioning of the central nervous system in humans and insects (Mileson *et al.,* 1998) and is discussed later in this chapter.

Although more than 100 different OPs are available commercially, the examination of their respective structures has revealed that virtually all OPs have a similar structure (Figure 1a). They generally consist of a central phosphorous atom double bonded to either oxygen or sulphur, side groups R^1 (methyl or ethyl) and R^2 (methoxy, ethoxy, ethyl, phenyl or amino group) and the leaving group X, which imparts the largest number of variations in OP structure. OP and carbamate pesticides selectively inhibit AChE activity (Valbonesi *et al.,* 2003). The P=O bonded OPs phosphorylate the hydroxyl group on the serine residue in the AChE active site preventing it from hydrolysing ACh (Fukuto, 1990). This inhibition results in the build-up of the neurotransmitter in the synapses causing the continuous stimulation of the muscle/nerve and eventually resulting in exhaustion and tetany (Fukuto, 1990; Chapalamadugu, 1992).

Carbamate pesticides (CPs) are derivatives of methylcarbamic acids (Figure 1b) that inhibit AChE via the carbamylation of the active site's serine hydroxyl. They generally consist of a central carbon atom double bonded to an oxygen atom and two side groups R^1 and $HN(R^2)$ where R^2 is either an aromatic or aliphatic group (Figure 1b). R^1 can be a methyl, aromatic or a benzimidazole depending on whether the carbamate is an insecticide, herbicide or fungicide, respectively (Fukuto, 1990). Unlike AChE phosphorylation, carbamylation is unstable and AChE is regenerated relatively quickly.

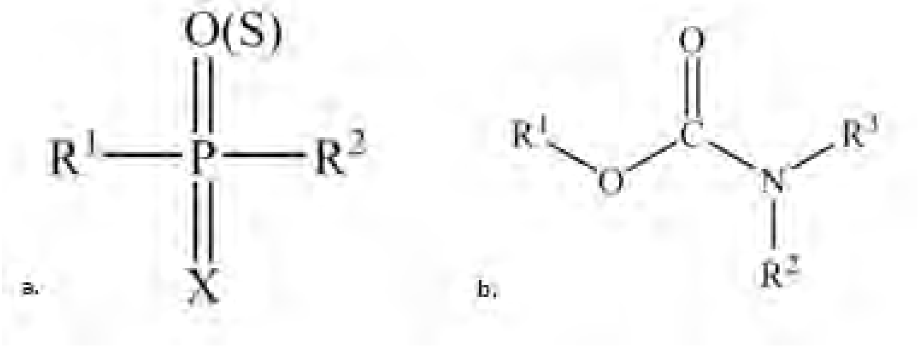


Figure 1: General structure of OPs (a) and CPs (b), (Fukuto, 1990).

Continual and widespread usage of OPs and CPs can be attributed to the fact that they are inexpensive relative to alternatives such as organochlorines; these pesticides also have broad-spectrum applications. Moreover, pests that develop resistance to organochlorine pesticides are not resistant to OPs (Friis, 2010). With the increasing accessibility and synthesis of these compounds, as well as broad applications of OPs, there is also a proportional growth in accidental exposures and fatalities. The need to detect these compounds in the environment has increased drastically and has resulted in accelerated research to develop quick, selective and sensitive methods of detecting OPs and CPs in the environment.

1.3 Current methods of detecting OPs and CPs

Several analytical methods have been developed for the detection of OPs and CPs. These include highly sensitive chromatographic techniques like gas chromatography (GC), high performance liquid chromatography (HPLC), portable surface acoustic wave sensors and spectrophotometric sensors (Mendoza, 1973). To date, GC coupled with a mass spectrometer (GC/MS) is the only field verification "approved equipment" (Hill and Martin, 2002) of OPs. However, this technique involves compound extraction, concentration and clean-up steps, which make them tedious, time consuming, expensive and not suitable for in-field analysis. Moreover, highly trained technicians and specialized laboratories are required. Another drawback is the fact that GC/MS does not measure the toxicological effect of the pesticides (Diehl-Faxon *et al.,* 1996). Alternative methods for pesticide detection make use of enzyme activation e.g. organophosphate hydrolase (OPH) activation by organophosphates, AChE inhibition as well as immunoassays (Jaffrezic-Renault, 2001). Enzymes are biological catalysts that promote the transformation of chemical species in living systems. They have found extensive use in biotechnology, primarily due to characteristics such as specificity, mild reaction conditions and capacity for regulation (Voet *et al.,* 2006). The mechanism of most pesticides is via the inhibition of a specific enzyme, with the degree of enzyme inhibition being proportional to the concentration of the pesticide. This property has allowed the development of alternative detection systems such as biosensors and colorimetric assays to determine the presence of pollutants. Biosensors based on the principle of enzyme inhibition have been applied to a wide range of significant analytes such as OPs, CPs and their derivatives (Mendoza, 1973; Amine *et al.,* 2006) (Table 2). Enzymes used include the cholinesterases butyrylcholinesterase (BChE) and AChE, organophosphorous hydrolase (OPH), and alkaline phosphatase (ALP) (Singh *et al.,* 1999). Since they are sensitive to a wide range of compounds, enzyme-based detection systems are considered as potential early warning systems.

Table 2: Enzyme based biosensors for pecticide and other chemical compounds.

Inhibitor	**Ezymes (s)**	**Support matrix**	**Type of boisensor**	**Sample**	**References**
Oxalic acid	AChE	Cross-linking with GA vapour	Amperometric	Real water sample	Joshi *et al.* (2005)
Paraoxon	AChE	MWCN	Amperometric	NA	Ciucu *et al.* (2003)
Carbofuran and aldicarb	AChE and ChO	Retention with dialysis membrane	Amperometric	Spiked river water samples	Bachmann *et al.* (2000)
Paraoxon and carbofuran	AChE variants	Entrapped in PVA-SbQ polymer	Potentiometric	Real water samples	Suwansa-ard *et al.* (2005)
Carbofuran and carbaryl	AChE	Covalent binding on silica gel	Optical	Spiked vegetable samples	Xavier *et al.* (2000)
Proxpur and carbaryl	AChE	Linkage on CPG	Amperometric	Real water sample	Joshi *et al.* (2005)
Aldicarb, carbaryl and carbofuran	AChE	Crosslinking with GA	Amperometric	NA	Nunes *et al.* (2004)
Chloropyrifos-ethyl	AChE	Immobilization on TCNQ	Amperometric	Grape and wine samples	Del Carlo *et al.* (2002)
Parathion	Parathion hydrolase	Immobilisation on a CPE with polyethylenimine and GA	Amperometric	Spiked river water	Sacks *et al.* (2000)
Aldicarb	AChE and ChO	Poly(2-hydroxyethymethacrylate) membrane	Potentiometric	NA	Kok *et al.* (2002)
Trichlorfon	HRP, ChO and ChE	Immobilization on SPE	Potentiometric	NA	Ciucu *et al.* (2002)

PVA-SbQ: polyvinyl alcohol bearing styrylpyridinium groups, TCNQ: 7,7,8,8-tetracyanoquinodimethane, PPD: poly-*o*-phenylenediamine, AChE: acethylcholinesterase , ChO: choline oxidase, OPH:organophosphorus-hydrolase, BuChE: butyrylcholinesterase, HRP: horseradish peroxidase, GA: glutaraldehyde.

1. 4 Acetylcholinesterase (AChE)

Acetylcholinesterase (EC 3.1.1.7) belongs to the class of serine hydrolase enzymes and is one of two cholinesterases that have been identified in a wide range of organisms including a number of non-neuronal tissues in animals, fungi, bacteria, and plants (Wessler *et al.,* 2001; Horiuchi *et al.,* 2003). The first studies of this enzyme were carried out using purified extracts from the electric organ of electric eel (*Electrophorus electricus*). The electric organ of these fish is derived from muscle tissue and studies have shown that region of contact between individual cells that make up this organ and the nerve which transmits the impulse to trigger the electric discharge is similar to the neuromuscular junction (Hagiwara *et al.,* 1965). Electric eels are considered to be a readily obtainable source of AChE (Mendoza, 1973). Electric eel AChE exists as a tetrameric glycoprotein containing saccharides related or identical to sialic acid, *N*-acetylglucosamine, *N*-acetylgalactosamine, mannose and/or glucose, and, galactose. The enzyme is normally characterised by lipid moieties attached to the protein chains, which anchor the enzyme to the cell membrane. The active site is characterised by an amino acid triad of serine-histidine-glutamate found in a deep cleft (Duval *et al.,* 1992). AChE occurs at the neuromuscular junctions throughout the central and peripheral nervous system of all vertebrates (Rickwood and Galloway, 2004) and functions in the rapid hydrolysis of the neurotransmitter acetylcholine (ACh) into the inactive products acetyl and choline. In the presynaptic neuron, choline-acetyltransferase (ChAT) catalyses the synthesis of acetylcholine (ACh) from choline and acetyl-coenzyme A. This is then packaged into synaptic vesicles. Action potentials trigger the release of ACh into the synaptic cleft, where it interacts with the muscarinic (M1) receptor site on the postsynaptic membrane causing stimulation of the nerve or muscle (Figure 2). Since the products from ACh hydrolysis do not stimulate the postsynaptic membrane, AChE acts as a regulatory agent of nervous transmissions by reducing the concentration of ACh in the synapse (Voet *et al.,* 2006).

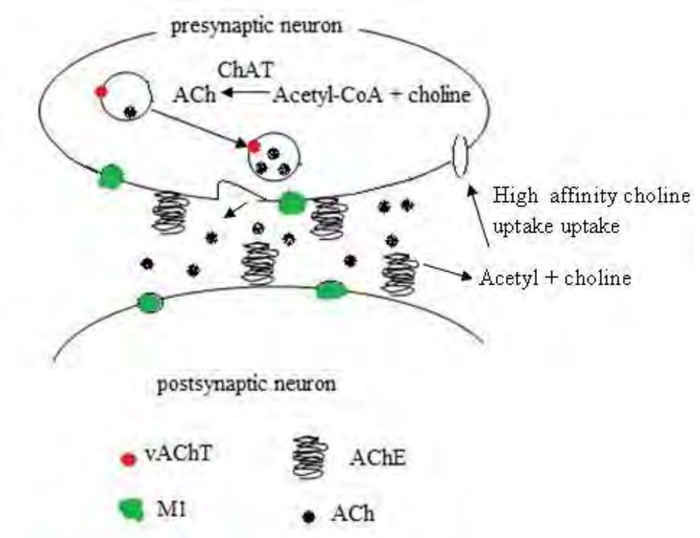


Figure 2: Acetylcholinesterase activity at the synaptic cleft catalyses the hydrolysis of acetylcholine from the presynaptic neuron to produce acetyl and choline thereby, facilitating transient activation of cholinergic receptors (modified from Voet *et al.*, 2006).

AChE inhibition by OPs and CPs has been shown to have the same mechanism to that of AChE acetylation during ACh hydrolysis. The difference, however, is that whilst acetylated AChE is unstable and easily breaks down to give acetyl and choline; phosphorylated or carbamylated. AChE is highly stable and in some cases phosphorylation is irreversible (Aldridge, 1950). Many current OP detection research studies using AChE are focused on biosensor development, which is based on AChE inhibition. A comparison of the catalytic rate of AChE at a given time to a baseline or pre-exposure level provides means for the detection of OPs and CPs (Gabrovska *et al.,* 2008). The inhibition of AChE activity can be measured by the change in pH associated with the hydrolysis of acetylcholine or through the oxidation of the choline using choline oxidase and measuring the rate of oxygen consumption or hydrogen peroxide production. Other AChE inhibition sensors include the measurement of oxidation current of the electroactive thiocholine iodide using amperometric techniques (Neufeld *et al.,* 2000). In addition, cholinesterase activity can be monitored spectrophotometrically using dithio-bis-nitrobenzoic acid (DTNB) (also known as Ellman's reagent) and the artificial substrate acetylthiocholine. Hydrolysis of acetylthiocholine by AChE yields acetic acid and thiocholine, which then reacts with DTNB producing a coloured compound detectable at 412 nm (Ellman *et al.,* 1961; Nguyen *et al.,* 1998).

In spite of the previously mentioned advantages and applications, the use of enzymes is limited by factors such as high cost, instability and non-reusability (Rothwell *et al.,* 2010). These disadvantages have resulted in intensive research in the enzyme immobilisation technology field in order to improve their practical applications. The term "immobilised enzymes" refers to enzymes that are physically confined to a defined region of space with retention of their catalytic activities, and which can be used repeatedly and continuously (Brena and Batista-Viera, 2006). Immobilised biocatalysts are more stable and easier to handle compared with their free counterparts. Furthermore, several studies have demonstrated that immobilised enzymes have increased activity in environments in which they would normally be inactive e.g. elevated temperatures and organic solvents (Ngo and Yam, 1978; Zaidi *et al.,* 1995; Stoilova *et al.,* 2010). Current applications of immobilised biocatalysts include energy production by biological processes, the selective treatment of specific environmental pollutants, and continuous analysis of compounds with a high sensitivity and specificity, and medical uses such as enzyme therapy (Mahmoud and Helmy, 2009).

1. 5 Immobilisation methods

Enzyme immobilisation methods (Figure 3) can be broadly classified as (i) physical, where weak interactions between the support and enzyme exist, (ii) chemical, where covalent bonds are formed with the enzyme (Tischer and Wedekind, 1999), (iii) enzymatic and (iv) genetic engineering (Josten, 1999; Shpigel,1999). Physical enzyme immobilisation methods can further be broken down into four methods, which are: adsorption, gel entrapment, microencapsulation and formation of Langmuir-Blodgett films (Figure 3).

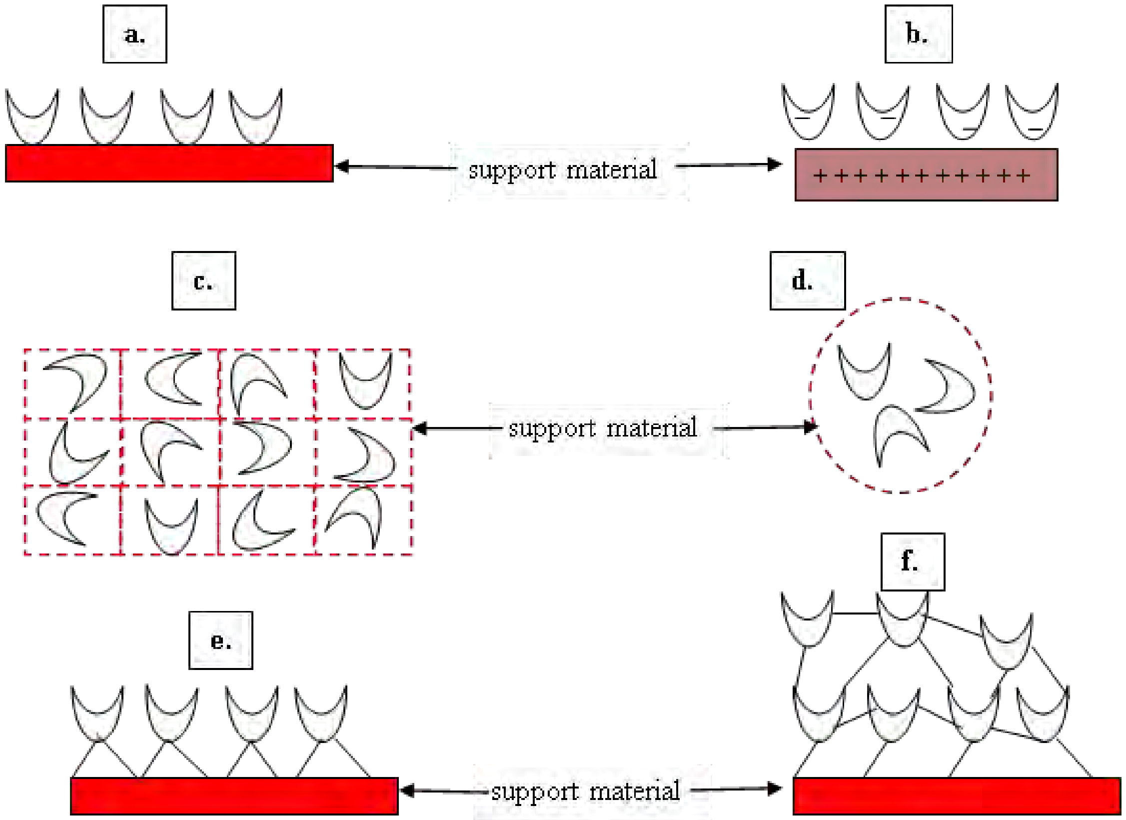


Figure 3: Methods of protein immobilisation (a) adsorption, (b) ionic interactions, (c) entrapment, (d) encapsulation, (e) covalent attachment and (d) cross linking). Adapted from Costa *et al.* (2004).

Non-covalent adsorption onto surfaces can occur through different types of interactions including van der Waals forces, hydrogen bonding and ionic interactions. Efficient enzyme immobilisation through van der Waals forces requires both the support material and the enzyme to have large hydrophobic surface areas. In most enzymes; however, hydrophilic residues are exposed on the surfaces whilst the hydrophobic residues are hidden. Van der Waals forces have therefore largely been used for the immobilisation of lipases as they are characterised by the presence of hydrophobic superficial regions. This is due to lipase activity being at the oil/fat and water interface. Novozyme 435, a commercially available *Candida* *antarctica* lipase B, adsorbed onto a hydrophobic microporous polymer, was used to demonstrate the advantages of the immobilised enzymes for the resolution of cyanohydrin acetates in toluene (Heinsman *et al.,* 2003). Since most enzymes display hydrophilic amino acid residues on their surface and others are characterised by glycosylation (which imparts increased hydrophilicity), enzymes can also be immobilised onto hydrophilic carriers such as cellulose, Avicel, Celite and silica gel via hydrogen bonding (Cao, 2005).

A relatively new but not widely reported method of enzyme immobilisation is the use of mixed self-assembled monolayers (SAM_{mix}). These are normally characterised by two different species of alkane-thiols with different carbon chain lengths which form a biocompatible matrix for enzyme immobilisation. The main advantage offered by the use of SAM_{mix} is the absence of cross-linking agents, which may hamper enzyme activity, as well as the faster mass transport. In a study by Cancino and colleagues (2013), SAM_{mix} was used for the immobilisation of AChE; the results showed that the modification of polycrystalline gold surfaces with self-assembled monolayer of short and long-chain alkane-thiols was an efficient strategy for electrochemical pesticide detection. However, the flaw in this method is that due to the absence of cross-linkers, physical factors such as agitation may cause desorption of the enzymes.

Ionic interactions can be used for the physical confinement of enzymes. Depending on the pH of the solution and the isoelectric point, the surface of the enzymes may have a net charge. Ionic binding is based on the electrostatic interactions between differently charged groups of the immobilisation matrix and the enzymes. Different ion exchangers can act as support materials. Depending on the predominant charge on the enzyme, the ion exchanger can be either negatively charged, e.g. carboxylate, or positively charged e.g. protonated amino groups (Cao, 2005). The main advantage of immobilisation through physical methods is that the enzymes do not require any pre-treatment or chemical modifications. Significant disadvantages, however, include enzyme leakage from the support and non-specific binding.

The entrapment method for immobilisation consists of the physical trapping of the active components into a film, gel or fibre. This method differs from adsorption and covalent binding in that enzymes are free in solution, but restricted in movement by the lattice structure of a support (Bickerstaff, 1991; Cao, 2005). The porosity of the lattice is controlled to prevent enzyme leakage and simultaneously allow free movement of substrate and product.

This method can be achieved by mixing an enzyme or active molecule with a polymer and then cross-linking the polymer to form a lattice structure that traps the enzyme. The main advantage of enzyme immobilisation via entrapment is that enzyme loading is very high. Disadvantages include enzyme leakage from the support and diffusion limitations. Encapsulation can be achieved by enveloping enzymes in semipermeable membranes (Groboillot *et al.,* 1994). Materials such as nylon and cellulose nitrate have been used to construct microcapsules varying from 10-100 μm in diameter. On the nano scale level, Menaa and colleagues (2009 and 2010) reported the use of sol-gel glasses for the study of encapsulation and stabilisation proteins. Sol-gel is a chemically inert glass prepared from silica materials. It is highly porous and can be moulded into any desired shape. Enzymes can also be immobilised via microencapsulation where enzyme solutions are enclosed within spherical semi-permeable polymer membranes with controlled porosity (Costa *et al.,* 2004). Encapsulation has been suggested as the best method for enzyme immobilisation without structural disruptions. It also offers the possibility of co-immobilisation where enzymes may be immobilised in any desired combination depending on application.

Chemical immobilisation methods include: (i) covalent attachment, (ii) cross-linking with use of a multifunctional, low molecular weight reagent, and (iii) co-cross-linking with other neutral substances, e.g. protein. Covalent binding is based on the formation of covalent bonds between the support material and some functional groups of the amino acids on the external surface of the enzyme. Protein functional groups which are generally involved in covalent binding are the amino (NH_2) group of lysine or arginine, carboxyl (CO_2H) group of aspartic acid or glutamic acid, hydroxyl (OH) group of serine or threonine, and sulphydryl (SH) group of cysteine (Hanefeld *et al.,* 2009). Other functional groups such as sugars can also be used for immobilisation (Cao, 2005; Basso *et al.,* 2007). Covalently bound enzymes are tightly bound to the support material thereby minimising enzyme leaching. However, binding can result in the chemical and conformational modification of enzymes, which may lead to reduced activity. Direct fixation onto support surfaces also presents several disadvantages, including potential harmful interactions between enzyme and surface of the support matrix, shielding of enzyme functional groups and reduced substrate accessibility due to steric hinderance and electrostatic repulsion. Several studies (Ngo and Yam, 1978; Isgrove *et al.,* 2001) have reported the use of short or long spacers for attachment of reactive groups. Spacer molecules give a greater degree of mobility to the coupled biocatalysts so that its activity can, under certain circumstances, be higher than if it is bound directly to the support. Although many studies have reported that long spacers allow wider conformational flexibility to the protein, there is also supporting evidence indicating that shorter spacers confer thermal stability to proteins by restricting their mobility and preventing unfolding (Basso *et al.,* 2007). Bi-functional agents such as hexanediamine and glutaraldehyde are often used as spacers. Isgrove and co-workers (2001) reported the use of chitosan as an amplifying spacer arm.

The cross-linking method is based on the formation of covalent bonds between the enzyme or active molecules, by means of bi- or multifunctional reagents. Several cross linking agents such as glutaraldehyde (GA), cyanogen bromide and carbodiimide have been used for immobilisation. However, GA is the most commonly used cross-linking agent as it is the simplest, most gentle coupling method (Chae *et al.,* 1998). A study of AChE immobilisation via drip-coating on functionalised MWCT was carried out by Khan and Ghani, (2012). In this study, it was reported that the success of this method is hampered by the continuous loss of enzyme during the measurement process. This was attributed to the weak non-specific interactions for which GA crosslinking was suggested as a method for reducing enzyme loss.

Coupling with GA is beneficial because the reaction proceeds in aqueous buffer solution under conditions close to physiological pH, ionic strength, and temperature. Enzyme immobilisation via GA cross linking involves several functional groups such as the lysine ε-amine, phenol hydroxyl group of tyrosine, thiol of cysteine and the histidine imidazole group. This cross linking occurs through nucleophilic attacks within the pH range of 2.0 and 11.0. GA reacts with the thiol group only in the presence of a primary amino group (Onyezili, 1987) and it has also been shown that the reactivity of proteins with GA is reversible outside of the pH 7.0 – 9.0 range. Enzyme cross-linking often involves the ε-amino group of lysine residues which has a pK_a of 10.54 (Voet *et al.,* 2006). However, within the pH range of 7.0 and 9.0 where GA irreversibly reacts with proteins, the reactive groups of lysine are protonated and unable to carry out nucleophilic attacks on GA.The assumed reaction mechanism is that the few amines present between pH 7.0 and 9.0 are sufficient to react with GA, driving the acid-base equilibrium to deprotonation, thereby providing more groups for subsequent reactions. The reactions suggested for the GA reaction with proteins include a Schiff base reaction as well as Michael type addition (Walt and Agayn, 1994; Migneault *et al.,* 2004). Enzyme immobilisation via GA cross-linking can be achieved either through the formation of a three-dimensional network as a result of intermolecular cross-linking and the binding to an insoluble carrier (e.g., nylon and fused silica). Since cross-linking normally involves covalent bonds, immobilised biocatalysts in this way frequently undergo conformational changes resulting in the loss of activity and this observation has been attributed to enzyme rigidity imparted by cross-linking.

Immobilised enzymes have been widely used in the production and processing of various products. In the biomedical field, immobilised enzymes have been used for antibiotic production as well as studies in drug metabolism whilst the industrial applications of immobilised enzymes include biodiesel production. Immobilised proteins and enzymes have also shown potential applications in the analysis of environmental samples as well as bioremediation (Table 3).

Table 3: Examples of immobilised enzymes in/on various materials for different applications

Enzyme/protein	Matrix	Method (s)	Application	References
Alkaline phosphatase	Glass carbon	Entrapment	Biosensors and enzyme linked immune assays Induction of bone and cartilage minerilisation	Filmon *et al.,* 2000
Acetylcholinesterase	Electrospun polyacrylonotrile nanofibres	Covalent bonding , ionic interactions, entrapment and cross linking	Organophosphate pesticides biosensor	Stoilova *et al.,* 2010
Bilirubin oxidase	Agarose	Covalent binding	Bioreactor for removal of bilirubin from blood	Lavin *et al.,* 1985
Glucose oxidase	Multiwall carbon nanotubes (MWNT)	Sol–gel encapsulation and glutaraldehyde cross linking	Glucose biosensor	Shi *et al.,* 2011
Papain	Electrospun hybrid chitosan/nylon-6	Covalent binding	Papain purification	Zhang *et al.,* 2010

1. 6 Immobilisation matrices

The properties of immobilised enzyme preparations are determined by both enzyme and the support material properties. These interactions impart specific chemical, biochemical, mechanical and kinetic characteristics (Sheldon, 2007). A matrix can enhance the operational stability of the immobilised enzyme system. Although a wide range of supports have been employed for enzyme immobilisation, there are certain characteristics that should be present in any material considered for immobilisation thus it is recognised that there is no universal support. Ideal support properties need to include physical resistance to compression, hydrophilicity, inertness towards enzymes, ease of derivatisation, biocompatibility, resistance to microbial attack, and availability at low cost (Bickerstaff, 1991). The type of support can be classified according to its chemical composition into synthetic organic polymers, natural organic polymers and inorganic solid supports (Table 4). Synthetic polymers such as nylon, polystyrene (PS) and polyacrylonitrile (PAN) are derived from petroleum oil, whilst natural polymers such as cellulose and chitosan are found in nature and can be extracted.

Table 4: Examples of organic (natural and and synthetic) and inorganic polymers used in enzyme and protein immobilisation

Organic		Inorganic
Natural	Synthetic	
• Polysaccharides e.g starch, chitin and chitosan, dextran, agar and agarose. • Proteins e.g. gelatin, collagen, albumin, ferritin	• Polyacrylamides, Polystyrene, Polyacrylate, Polyamides and, Polyethyleneglycol (PEG)	• Minerals • Fabricated materials e.g. non-porous and controlled pore glass, porous silica, iron oxide and stainless steel

Synthetic polymers have been widely used for enzyme immobilisation due to the favourable characteristics which include high stability (chemical and mechanical) and relatively low cost. Although their chemical inertness is often considered an advantage, this characteristic is also considered a major drawback as they have limited carboxyl and amino residues for covalent attachment. Other disadvantages of synthetic polymers include imperfect biocompatibility and hydrophobicity. Several (physical and chemical) modification procedures have been developed for the enhancement of synthetic polymers binding capacity. Physical modification includes the activation of surfaces through ultraviolet (UV) radiation (Shrearer *et al.,* 2000), plasma activation and plasma deposition (Dai *et al.,* 1997; Chu *et al.,* 2002). Physical modification strategies are rarely used because they often result in surface destruction. Chemical modification has been achieved by treating the polymers with different reagents such as sodium hydroxide (NaOH), hydroxylamine and weak acids (Beeskow *et al.,* 1997; Michielsen, 1999). On nylon surfaces, chemical functionalisation has been achieved by the reaction of terminal amine, carboxylic acid group or repeating amide groups to introduce hydrophilic functional groups essential for enzyme immobilisation (Ngo and Yam, 1978). Marinov and coworkers (2009) reported the preparation of NaOH functionalised PAN membranes with integrated gold nanoparticles for AChE immobilisation. Results from their studies demonstrated that chemically modified membranes were more effective for AChE immobilisation.

Nylon is a convenient immobilisation matrix, because it is relatively inexpensive, inert, non-toxic and is available in different forms. Unmodified nylon surfaces are hydrophobic and this can lead to a substantial amount of nonspecific hydrophobic binding of ligands and subsequently lead to reduced activity and the leaching of immobilised enzyme from the support matrix. The earliest uses of nylon for immobilisation of biomolecules involved the partial acid hydrolysis of the nylon surface to generate amino groups and carboxyl groups, which could be coupled to proteins with glutaraldehyde. Ngo and Yam (1978) successfully immobilised AChE on nylon nets where the nylon surfaces had been modified through *O*-phosphorylation, *O*-alkylation and hydrolytic cleavage. Furthermore, their results showed that the introduction of a spacer between the nylon nets and the enzyme increased the half-life of the immobilised enzyme. Alkylating agents such as dimethyl sulphate (DMS) were also used as nylon functionalizing agents for enzyme immobilisation. In spite of the very high toxicity and possible carcinogenicity of these alkylating agents they have been widely applied (Sundaram and Hornby, 1970; Ngo and Yam, 1978).

A variety of natural polymers, mainly water-insoluble polysaccharides (cellulose) and proteins (gelatin) have been widely used as supports for immobilising enzymes. The first industrial application of an immobilised enzyme in a biotransformation was the production of L-amino acids via the resolution of racemic acylamino acids using aminocyclase from *Aspergillus oryzae* that had been immobilised by ionic adsorption on DEAE-Sephadex. Polysaccharide polymers have gained a lot of interest in biotechnology due to their abundance in nature and cheap availability. Chitin, considered the second most abundant polysaccharide in the world, is a major constituent of crustacean shells, insect exoskeletons and the cell walls of fungi (microfibrils providing strength and stability). The main commercial sources of chitin are crustaceans such as crab and shrimp shells, which are abundantly supplied as waste products of the seafood industry. Chitin; however, is not readily soluble in common solvents and thus it is often converted to its more deacetylated derivative, chitosan (Peter, 1995).

Chitosan, a linear polysaccharide composed of β(1-4)-linked-2-amino-2-deoxy-D-glucopyranose, is identified by its degree of deacetylation (DD), which is the percentage of free amine groups along the chitosan backbone. Although water-insoluble like chitin, the presence of amino groups on chitosan renders it soluble in acidic solutions below pH 6.5, thus it is preferred over chitin for a wide range of applications. The non-toxic, biocompatible and biodegradable properties of chitosan are well documented. Chitosan is also characterised by a unique set of characteristics including physiological inertness, anti-bacterial properties and a high affinity for proteins. Increasingly, over the last decade, chitin and chitosan based materials have been examined and a number of potential products have been developed for areas such as wastewater treatment and agriculture. In biotechnology, they may find application as chromatographic matrices, membranes for membrane separations, and as enzyme/cell immobilisation supports (Peter, 1995).

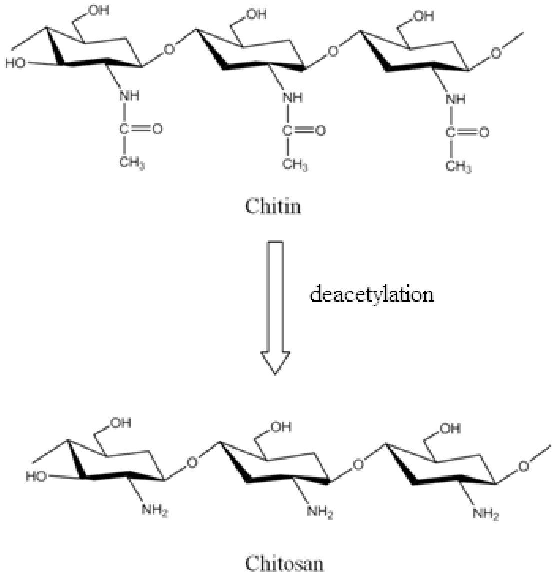


Figure 4: Schematic representations on the structures of chitin and chitosan. Deacetylation of chitin yields chitosan. (Modified from Voet *et al.*, 2006).

Natural and synthetic polymers can be used for enzyme immobilisation in their pure states. Furthermore, enzyme immobilisation matrices with novel characteristics can be prepared by blending polymers. This is usually done to introduce functional groups that would otherwise be absent.

The shape of the carrier can be classified into two types, i.e. irregular and regular shapes such as beads, fibres, hollow spheres, thin films, discs and membranes. Selection of the geometric properties for an immobilised molecule is largely dependent on the application. In addition to the aforementioned ideal characteristics of support material for enzyme immobilisation, the size of the support material is of paramount importance. A reduction in size of support materials has been shown to greatly enhance the efficiency of immobilised enzymes. This has been attributed to several factors, including high enzyme load per unit mass on non-porous materials, and reduced diffusional resistance on porous materials. Many studies have thus reported the use of micrometer-sized support materials for enzyme immobilisation (Xu and Klibanov, 1996; Govardhan, 1999; Eldin *et al.*, 2000). Recently, there has been increasing interest in the use of nano-scale supports for enzyme immobilisation. These are considered superior to other materials due to their extremely high surface area-to-volume ratios (Kim *et al.,* 2006). Zacchigna *et al.* (1998) showed that the effective enzyme loading on nanoparticles can be over 10 (wt) % with particles less than 100 nm. However, some nano-structured materials have disadvantages that are difficult to overcome (Wang *et al.,* 2009). For example, although nanoparticles and nanotubes are known to remarkably decrease mass transfer limitation, nanoparticle recycling is more difficult. On the contrary, nanofibres have great potential to overcome these problems.

A number of manufacturing processes have been explored to fabricate micro or nano-scale fibrous matrices, including drawing, self-assembly, template-directed synthesis, phase separation, and electrospinning. Drawing is a process where fibres are literally drawn from a micro-droplet of solution from small molecules (Ondercuhu and Joachim, 1998), forming nanofibres with a length of hundreds of microns. In template synthesis, a polymer solution is pushed through a template with nanopores and the extruded nanofibres will solidify in a suitable solution (Ondercuhu and Joachim, 1998). The diameter of the nanofibres is directly determined by the dimensions of the pores of the template. The main mechanism of phase separation nanofibre formation is the separation of phases due to physical incompatibility (Ondercuhu and Joachim, 1998). Many steps are involved in the process: polymer dissolution, gelation, solvent extraction and freeze-drying, resulting in porous nanofibres. Electrospinning has been widely accepted as the simplest and least expensive means to fabricate ultrafine fibres, and has been employed to fabricate nanofibres from a variety of synthetic or natural polymers (Larrondo and Manley, 1981).

1. 7 Electrospinning

The electrospinning technique is a well-known process for making continuous micron to nano-size fibres in the form of a nonwoven mat. Electrospun nanofibrous membranes possess several attributes that make them very attractive in immobilisation technology. These include high porosity and interconnectivity, which endows them with a low hindrance for mass transfer. Moreover, the nanofibre surfaces can be modified to benefit enzyme activity. Electrospun materials, by nature, exhibit an extensive surface area. Although each nanofibre provides the surface for hosting enzymes, the collection of randomly arranged nanofibres usually forms a non-woven mesh (or membrane) with reusability (Doshi and Reneker, 1995).

Nanofiber products have potential uses in a wide range of filtration, medical and textile applications (Figure 5). In the field of filtration**,** nanofibers have significant applications because of their high porous structure with high surface area and they have been shown to be suited for for filtering submicron particles from air or water their main advantages over more traditional filter media are longer filter life, filtration efficiency, easier maintenance and lower weight (Thavasi *et al.,* 2008). Nanofibers are also used in medical applications, which include, drug and gene delivery, artificial blood vessels, artificial organs, and medical facemasks. Potential medical applications include efforts to fabricate electrospun polymer nanofiber scaffolds for nerves, tissues, skin and bone. Among their other applications are the studies related to nanofiber membranes sensors, polymer batteries and military fabrics. Military fabrics for chemical and biological protection have been enhanced by laminating a layer of nanofiber between the body side layer and the carbon fibers (Venugopal and Ramakrishna, 2005).

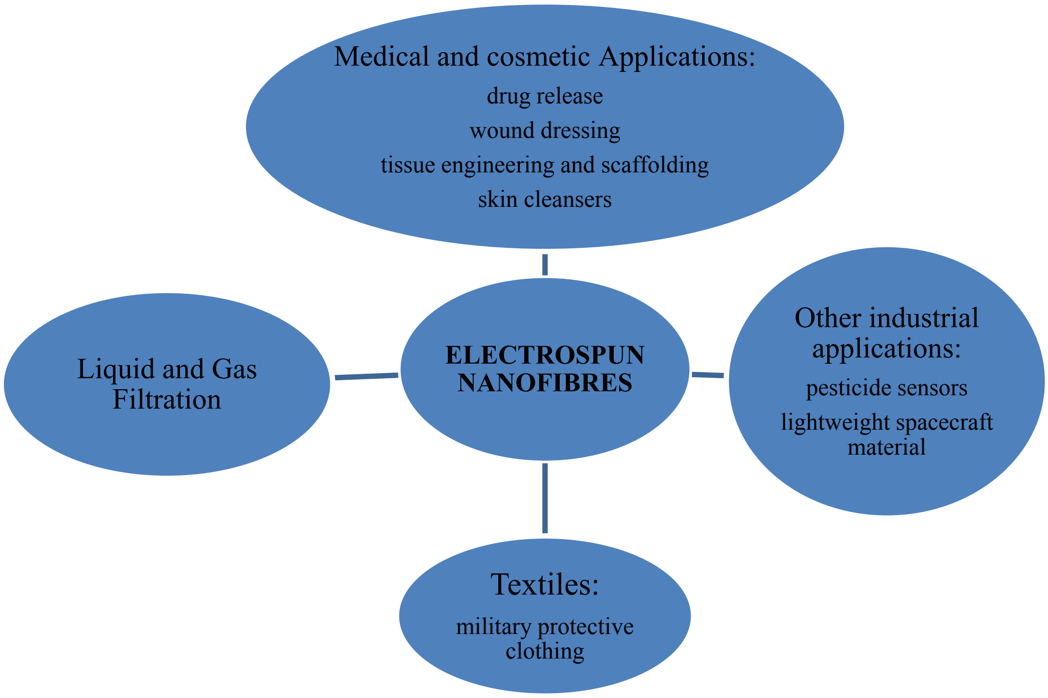


Figure 5: Applications of nanofibres generated through electrospinning

A typical electrospinning setup includes a reservoir of polymer solution with a metallic capillary connected to a voltage and a metallic collector (Figure 6). The hemispherical shape of the droplet at the needle tip is destabilized by the accumulated charges on the surface, and is converted to a Taylor's cone when high voltage is applied to a polymer solution. At a critical value of the voltage, the electric forces overcome the surface tension on the droplet and a jet of ultra-fine fibres is produced from the tip of the Taylor cone (Megelski *et al.*, 2002).

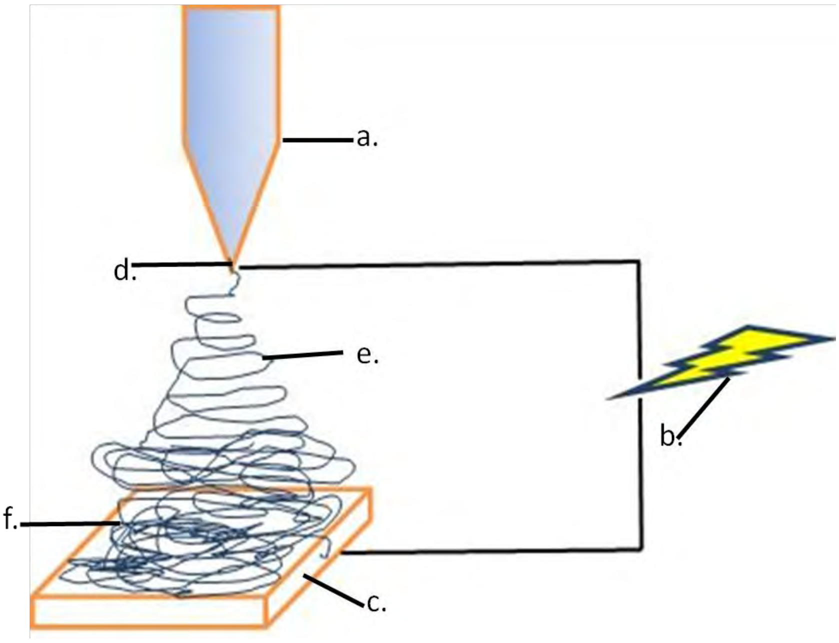


Figure 6: A polymer solution (a) is pumped through a syringe into a needle. Due to the electric field (b) between the needle and a grounded collector (c), a Taylor cone (d) is formed at the needle tip and a jet (e) travels towards the collector where nanofibres are collected (f) (Adapted from Megelski *et al.*, 2002).

Several methods for fibre production via electrospinning are known and these are (i) melt electrospinning, (ii) solvent electrospinning, (iii) islands in the sea electrospinning and (iv) melt blowing electrospinning. In melt electrospinning the molten polymer is pumped through the needle. An advantage of melt blowing electrospinning is that no solvent is used, thus no emissions of solvent fumes occur. A condition for melt electrospinning is that the polymer has an acceptable melting temperature in order to keep the energy cost under certain limits (Larrondo and Manley, 1981). This process, however, results in irreproducible nonwovens. The average diameter of the resulting nanofibres using this technique is at present limited to around 700 nm. In solvent electrospinning on the other hand, the polymer is dissolved in a suitable solvent. On its path to the collector plate the solvent will evaporate and solid fibres are formed on the collector plate. Because of the evaporation of the solvent, much smaller fibres can be obtained in comparison to melt electrospinning.

Solvent electrospinning can be used for virtually every soluble polymer, including polymer blends. Fibres electrospun from water soluble polymers such as polyethylene oxide (PEO), polyvinyl alcohol (PVA) and polyacrylic acic (PAA) rapidly decompose on exposure to water. This property is highly beneficial to biomedical applications such as skin grafting, but further modifications such as cross-linking are required for technical applications such as filtration. Organo-soluble polymers such as polystyrene (PS), polyacrylonitrile (PAN) and polyamides are widely used in solvent electrospinning because they are soluble in a wide range of solvents. However, the flammability, toxicity and corrosiveness of the solvents (e.g. formic acid) are major disadvantages. Although many studies have reported the electrospinning of pure polymer solutions, there has been an increase in the number of publications reporting the electrospinning of hybrid materials prepared from natural and synthetic polymers. Methods for obtaining these materials include copolymerisation, blending, coating and grafting. The electrospinning of copolymers allows for the generation of fibres with enhanced properties including surfaces with functional groups to allow effective enzyme immobilisation (Cao, 2005).

Enzyme immobilisation on electrospun nanomaterials is well documented. Wu *et al.* (2005) reported the successful immobilisation of cellulase in electrospun polyvinyl alcohol (PVA) membranes. Enzyme activity of immobilised cellulase was over 65% of that of the free enzyme. Immobilised enzymes have also been shown to be more stable in conditions that have previously been shown to be hostile. Jia *et al.,* (2002) demonstrated that α-chymotrypsin immobilised on electrospun functionalised PS had non-aqueous activity three orders of magnitude higher than its native counterpart suspended in organic solvents. Several studies have also reported the storage stability and reusability of enzymes immobilised on electrospun nanofibres. Lipase immobilised on PS retained >90% of its initial activity after storage in buffer for 20 days. Moreover, the enzyme retained 70% of its specific activity after 10 repeated use cycles. Free lipase on the other hand lost 80% of its initial activity after storage for 20 days (Li *et al.,* 2007). In addition to reusability and storage stability, it has been reported that enzyme immobilisation enhances the pH and thermal stabilities of enzymes (Li *et al.,* 2007). For effective enzyme immobilisation, functional groups have to be introduced onto most support matrices in order to overcome their chemical inertness.

Enzymes have also been immobilised on fibres produced from electrospinning of copolymers (Jia *et al.,* 2007; Stoilova *et al.,* 2010 and Zhang *et al.,* 2010).

1.8: Immobilisation of acetylcholinesterase on electrospun nanofibres

In several studies, acetylcholinesterase (AChE) has been successfully immobilised on electrospun nanofibres prepared from various polymers. In a study by Moradzadegan *et al.* (2010) PVA nanofibers were prepared via electrospinning of a mixture of AChE, BSA as an enzyme stabilizing additive and PVA. Stoilova *et al.* (2010) covalently immobilised AChE successfully onto electrospun mats of styrene–maleic anhydride copolymers that had been functionalised by modification with two types of spacers: a polymer with a flexible hydrophilic polyether chain and a rigid low-molecular-weight spacer (*p*-phenylenediamine). The results from this study showed that AChE immobilised via the hydrophobic chain exhibited the highest activity. Chitosan modification of electrospun nanofibers has also been used to enhance the binding properties on nanofibers for AChE immobilisation, (Stoilova *et al*., 2010).

Chapter 2

Chapter 2: RESEARCH MOTIVATION AND HYPOTHESIS

2.1 PROBLEM STATEMENT

In the face of a growing human population and increasing urbanisation, the demand for pesticides is predicted to continue rising as farmers battle to increase yields on increasingly less farm surface area. Although pesticides are beneficial for productivity in the agricultural sector, their adverse effects on human health as well as the environment are well documented. Due to their non-persistence in the environment as well as relatively low cost, OPs and CPs are among the most widely used pesticides in South Africa. Their principal negative impact on health across many animal species includes neurotoxicity; thus there is a need for a rapid and reliable means of detection for these compounds. Several studies have reported the use of AChE as a biomarker for pollution and for the construction of OP and CP biosensors. The degree of AChE inhibition is a common technique used for the detection of OP and CP in water sources. In as much as the advantages of using AChE are widely documented, the shortcomings of enzymatic assays are also reported in literature. The enzymatic shortcomings reported in literature include the non-reusability of enzymes in solution and low storage stability, which contribute to higher operation costs. Therefore, enzyme immobilisation has been suggested as a strategy for enhancing enzyme storage stability as well as allowing enzyme reuse.

2.2 HYPOTHESIS

Nanofibres produced from electrospinning of a polyamine-6/chitosan blend can be used for the immobilisation of AChE.

2.3 AIMS AND OBJECTIVES

The aim of this study was to electrospin nanofibres suitable for AChE immobilisation. A blend of nylon-6 and chitosan was prepared in a suitable solvent and electrospun into non-woven nanofibres onto which electric eel AChE was immobilised.

The specific objectives were:

1. To determine a suitable solvent system for dissolving nylon and chitosan to form a homogenous solution that can be electrospun.
2. To electrospin and characterise nylon-6:chitosan nanofibres.
3. To immobilise electric eel AChE onto nanofibres.
4. To determine kinetic parameters of the immobilised AChE.
5. To determine the storage, thermal, and pH stabilities of the immobilised enzyme as well as the reusability of the immobilised AChE.

Chapter 3

Chapter 3: ELECTROSPINNING

3.1 INTRODUCTION

Various parameters are involved in the electrospinning process; these all have an influence on the stability of the process as well as the obtained nanofibres. The parameters that play a role can be divided in three groups: (i) polymer solution, (ii) process and (iii) ambient parameters (Cui *et al.,* 2007; Beachly and Wen, 2009). Polymer solution parameters include the type and molecular weight of the polymer and the solvent. A prerequisite for electrospinning is that a suitable solvent should be available for dissolving the polymer (Wannatong *et al.,* 2004). In some cases a combination of solvents is necessary to prepare a solution that can be electrospun. Thus, the influence of the solvent is important and needs to be considered.

Formic acid (FA) and acetic acid (AA) are low molecular carboxylic acids (Figure 7) that are fully miscible with each other. AA has a relatively low dielectric constant of 6.6 ε_0 at 298 K and it dissolves in both polar and non-polar compounds. The use of AA as a single solvent for electrospinning is not widely reported but it is widely used in multi-solvent systems. FA on the other hand is widely used either as a single solvent or as part of multi-solvent systems for electrospinning (Supaphol *et al.,* 2005). Although a combination of formic acid and acetic acid has been shown to be promising for polyamide 6 (Daels *et al.,* 2010), this combination is still less exploited. A mixture of FA and AA was essential for steady state electrospining of nylon-6 with a minimum concentration of 30 v% formic acid and 10 v% acetic acid. This might be attributed to the relatively high dielectric constant of formic acid (57.2 ε_0 at 298 K) and the relatively low dielectric constant of acetic acid.

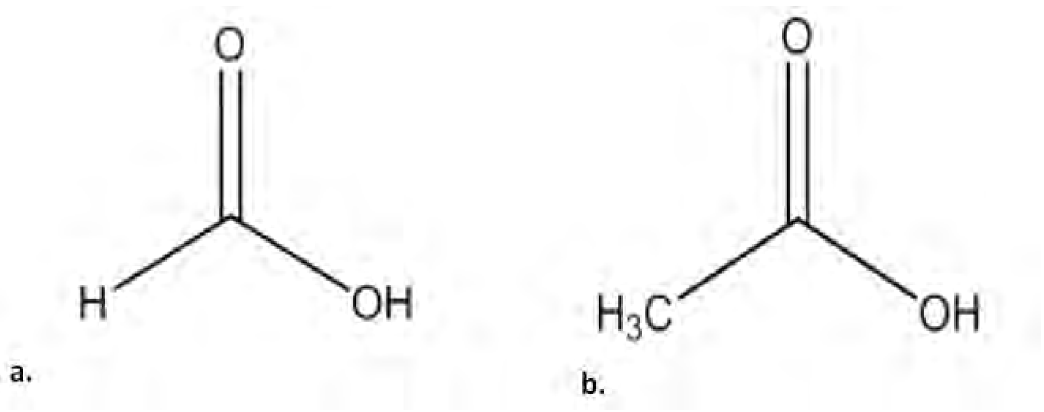


Figure 7: Structural formula of formic acid (a) and acetic acid (b). Formic acid and acetic acid resemble each other chemically and are fully miscible.

Besides the characteristics of the polymer solution, the electrospinning process itself is controlled by different parameters. These parameters are less crucial relative to the polymer solution parameters, but they are important for the optimisation of the electrospinning process as they have a significant influence on the properties of the fibres. The three main types of process parameters are applied voltage, flow rate, tip to collector distance and these are dependent on each other. For example, the applied voltage needs to be adjusted according to the applied flow rate and tip to collector distance to ensure stable or steady state electrospinning (Bhardwaj and Kundu, 2010).

The major requirement for enzyme immobilisation via chemical methods is the presence of functional groups that can form covalent bonds between the enzyme and the support material (Li *et al.,* 2007; Hanefeld *et al.,* 2009). Polyamide 6, commonly known as nylon-6, possesses excellent physical and mechanical properties. Nylon-6 has been successfully electrospun and the nanofibres used in applications such as solid phase extraction and filtration (Xu *et al.,* 2010; Chigome *et al.,* 2011). However, the use of electrospun nylon-6 nanofibres as enzyme immobilisation matrices has been limited mostly by their inertness and the subsequent need to be functionalised (Nirmala *et al.,* 2011). On the other hand, natural polymers such as chitosan are biocompatible and have functional groups that allow enzyme immobilisation. Although several studies have reported the successful electrospinning of chitosan (Cooper *et al.,* 2011; Jacobs *et al.,* 2011), the applications for these nanofibers are limited due to the polymer's poor mechanical and thermal properties.

One way to overcome the disadvantages of both synthetic and natural polymers is by the blending of synthetic and natural polymers. By combining synthetic and natural polymers together, hybrid polymers are produced and these possess the bio-functionalities of natural polymers together with easy modification and mechanical strength of synthetic polymers (Pasparakis *et al.,* 2010). Blending of two or more polymers to obtain materials with new and unique properties has become one of the most important researched topics in the field of polymers. These blends (synthetic and natural polymers) represent a new class of materials which have attracted a substantial amount of attention especially in application as biomaterials. Composite nylon-6:chitosan nanofibres were fabricated via electrospinning and characterised by Zhang *et al.,* (2009) who found that the nanofibres were well oriented and had good incorporation of chitosan. Infrared spectrometry indicated that the amino groups of chitosan still existed in the blended nanofibres. Nirmala *et al.* (2011) prepared nanofibres from a blend of chitosan and polyamide-6 nanofibres by a single solvent via electrospinning which were used for culturing human osteoblasts. Both studies concluded that a new composite product was created and attributed the system’s stability to new interactions such as the hydrogen bond formation between the nylon-6 polymers and chitosan structures.

In this chapter, the fabrication of nanofibres from a blend of nylon-6 and chitosan via electrospinning is reported. Electrospinning was optimised with reference to polymer concentrations, flow rate, distance between tip and collector as well as applied voltage and the nanofibres were characterised via scanning electron microscopy (SEM) and Fourier Transform infra-red spectroscopy (FT-IR).

3.2 MATERIALS AND METHODS

3.2.1 Electrospinning

Nylon-6 (16 % w/v and 13 % w/v) and chitosan were dissolved in formic acid:acetic acid (FA:AA) (50:50). Initially, chitosan (1% and 3% with respect to nylon concentrations) was dissolved in FA:AA on a magnetic stirrer for 2 hours. Nylon-6 pellets were then added to the solution and left on the magnetic stirrer until completely dissolved. Electrospinning was performed at room temperature (21 ± 2°C). The polymer blend was placed in a 25 mL syringe with a metal needle of 0.3 mm in diameter. A power supply (ES4OP-20W/DAM) was used to provide a high voltage (25 kV) to the syringe needle tip. Fibres were collected on a negatively charged (-5 kV) collector wrapped with aluminium foil with a tip to collector distance of 6 cm and polymer flow rate of 1 mL/h (Figure 8).

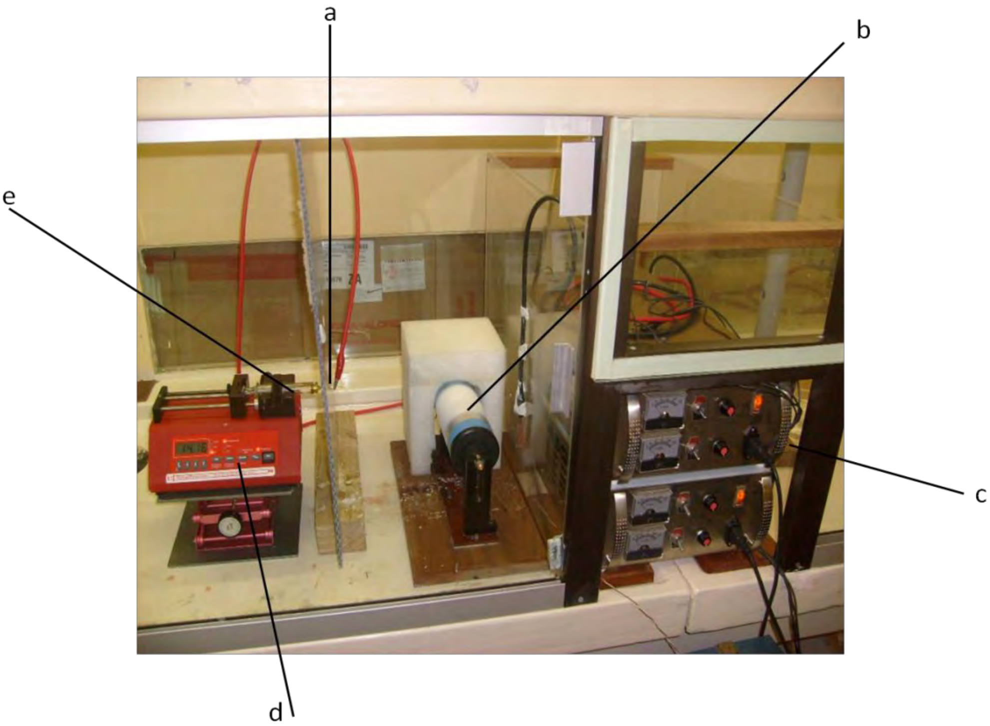


Figure 8: Electrospinning set-up in the laboratory, (a) is the needle to which a positive voltage is supplied, (b) the rotating collector, (c) the voltage supply, (d) the pump and (e) the syringe containing the polymer solution.

3.2.2 Characterisation

The morphology and diameter of the electrospun nanofibres were determined by scanning electron microscopy (SEM) (Zeiss Evo MA 15). Prior to scanning samples were sputter coated for 90 s with gold using a JEOL JFC-1200 fine coater. The diameters of fibres were analyzed using image visualization software analySIS. FT-IR spectra of the electrospinning nanofibres and the films were recorded with a Nicolet 17DSX FT-IR Spectrometer.

3.3 RESULTS

Process and solvent conditions for electrospinning nylon-6 blended with chitosan were investigated and the optimum conditions are reported in Table 5. The morphology of the electrospun nanofibres was determined via SEM (see Figure 9) and FT-IR analysis (see Figure 10) was used to determine whether a new product had been formed by the blending of nylon-6 and chitosan.

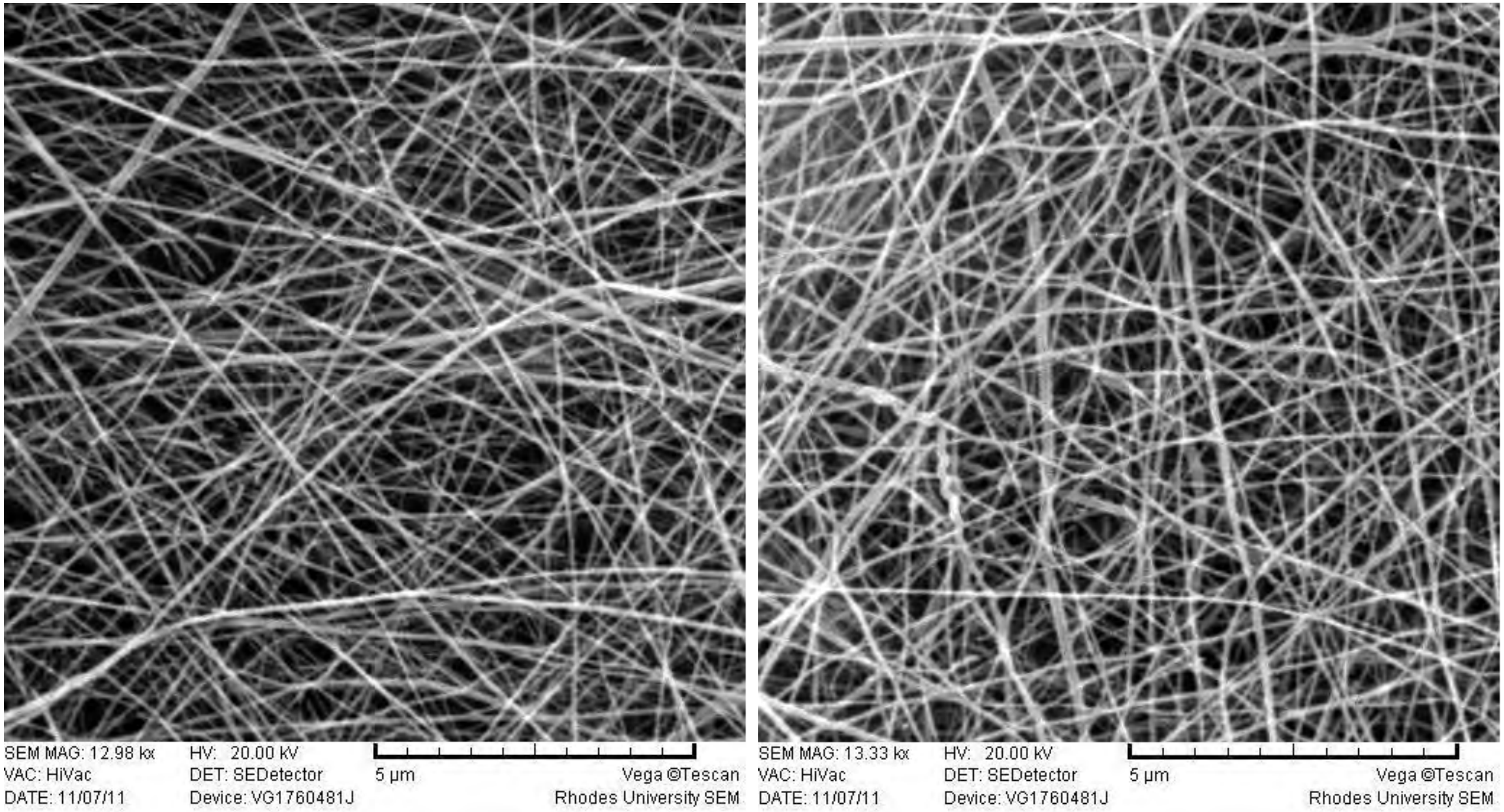
Table 5: Optimised electrospinning conditions for a nylon-6 and chitosan blend

Parameter	
Flow rate	1 mL / hour
Tip to collector distance	6 cm
Applied voltage	25 kV
Solvent ratios	50:50 (v/v)
Voltage on collector	-5 kV

Different combinations of process conditions for electrospinning chitosan blended with nylon-6 were investigated and optimised. Under these conditions, smooth nanofibres were formed with uniform diameter along their length.

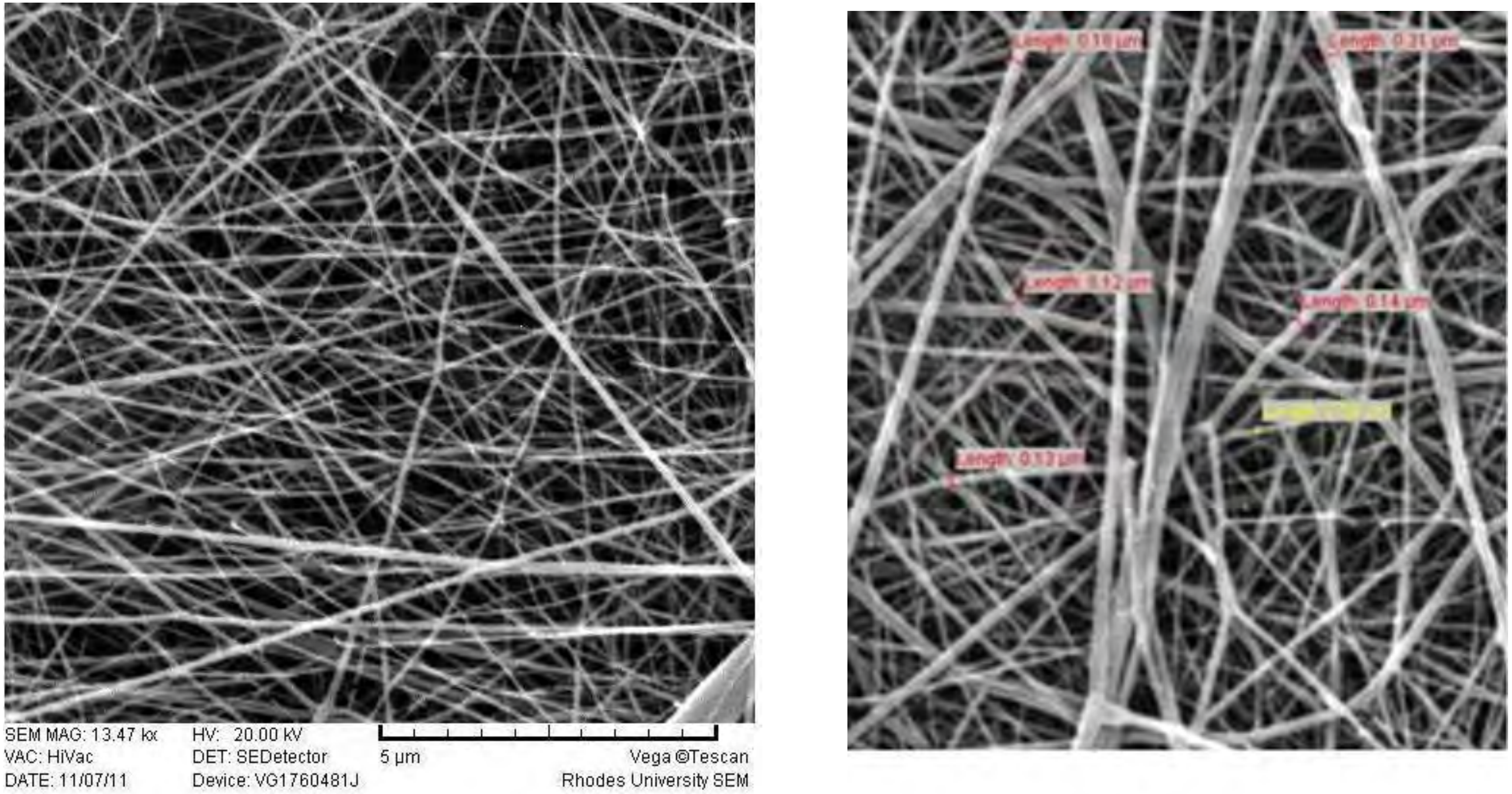
3.3.1 Characterisation of the nanofibers using SEM

The surface morphology of the electrospun nanofibres was determined using the scanning electron microscope (SEM) as shown in Figures 9 a-f. Typical magnifications ranged between 12000 × and 13000 ×. For determining the diameters of the nanofibres, Olympus Imaginf Software and analySIS software were used.



a. **b.**

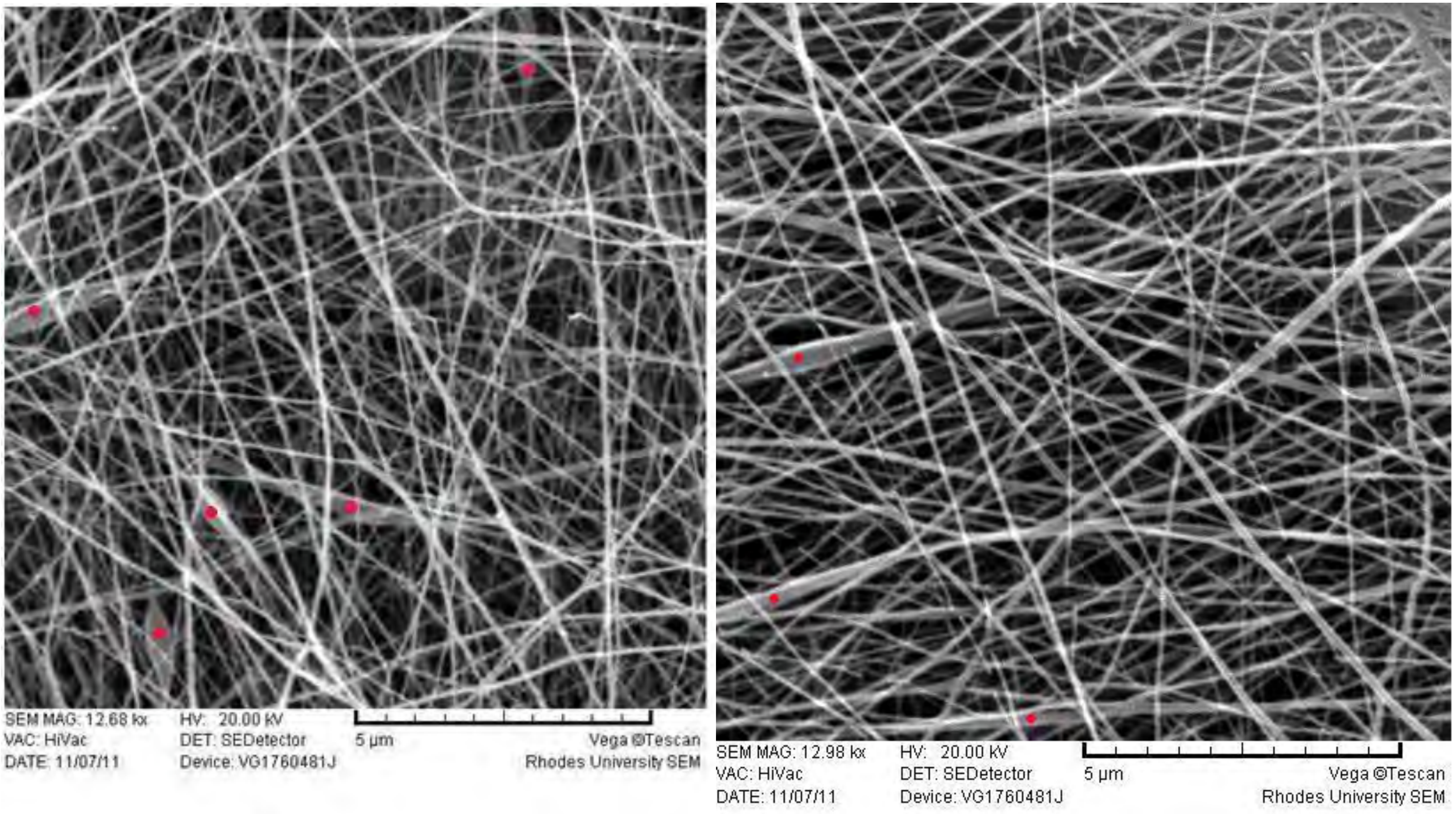
Figure 9: SEM micrographs of electrospun nylon-6:chitosan nanofibers from different wt % of nylon-6 and chitosan (a) 16% nylon-6, (b) 16% nylon-6:1% chitosan.



c.

d.

Figure 910: SEM micrographs of electrospun nylon-6:chitosan nanofibers from different wt % of nylon-6 and chitosan (c) 16% nylon-6:3% chitosan, (d) enlarged view of (c) to allow diameter measurement.



e. **f.**

Figure 9: SEM micrographs of beaded electrospun nylon-6:chitosan nanofibers from different wt % of nylon-6 and chitosan, * shows beading (e) 13% nylon-6:1% chitosan and (f) 13% nylon-6: 3% chitosan.

SEM analysis showed the nanofibres were successfully formed from both the pure nylon and the hybrid nylon and chitosan solutions. Addition of chitosan resulted in modifications in the morphology of the nanofibres. At low nylon-6 concentrations (13%), addition of chitosan resulted in the formation of beaded nanofibres whilst at high nylon-6 concentrations (16%) bead free nanofibres were formed. To determine whether any new bonds had been formed within the nylon-6:chitosan nanofibres, FT-IR analysis (Figure 10) was carried out.

3.3.2 FT-IR analysis

The interactions between nylon-6 and chitosan within the electrospun fibers were analyzed by FT-IR over the wave number range of 650-4000 cm^{-1}. FT-IR is a well-defined method to detect the intermolecular interactions between two polymers.

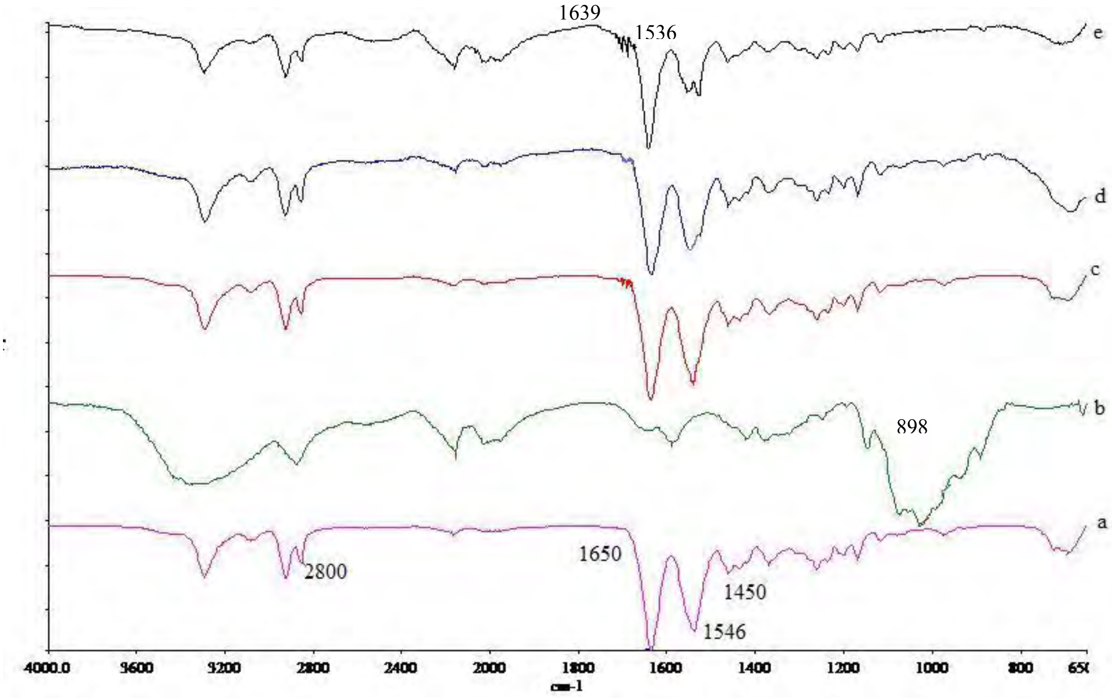


Figure 10: FT-IR spectra of (a) pure 16 % nylon-6 nanofibres; (b) raw chitosan and (c-e) 16 % nylon-6:chitosan nanofibres with different chitosan concentration of 1 % wt, 2 % wt and 3 % wt respectively.

A comparison of the FT-IR spectral scans of the nylon-6 nanofibres (Figure 10 a) and of raw chitosan (Figure 10 b) and nanofibres electrospun from different ratios of nylon-6 and chitosan (Figure 10 c – e) showed that there was interaction between nylon-6 and chitosan.

The nylon-6 amide bond at 1650 cm^{-1} was slightly displaced towards a lower energy number with increasing chitosan content.

3.4 DISCUSSION

The major problem associated with electrospinning polymer blends is the availability of either a single solvent or several miscible solvents that will dissolve both polymers. Polyamides have been electrospun with many different single solvent systems such as formic acid, *m*-cresol and sulphuric acid. In addition, solvent combinations such as formic acid/*m*-cresol, formic acid/ethanol, formic acid/dichloromethane and formic acid/acetic acid have also been used (Supaphol, 2005). However, it is clear that most of the studies implement solutions of formic acid because formic acid is cheaper and less dangerous to human health than the other solvents. Chitosan, on the other hand, is known to dissolve in organic acids e.g. dilute aqueous acetic acid, formic acid, lactic acids as well as in mixtures of water with methanol, ethanol or acetone. Based on several studies that reported the dissolution of either pure nylon-6 or pure chitosan in formic acid, an attempt was made to prepare a homogenous blend of these polymers by dissolving them in formic acid. The results showed that, in addition to forming gelatinous lumps, chitosan did not dissolve completely, thus the solution could not be electrospun. These results were different from those reported in literature (Nguyen *et al.,* 2007). However Matsumoto *et al.* (1991) reported that the homogeneity of chitosan solutions is affected by its degree of deacetylation (DD). Supaphol (2005) reported the combination of formic acid and acetic acid for dissolving nylon-6. De Vrieze *et al.* (2009) also reported the use of varying ratios of formic acid and acetic acid (ranging from 50:50 to 66:33) as suitable for the steady state electrospinning of nylon 6. Formic acid and acetic acid resemble each other chemically (Figure 7) and are fully miscible with each other. A solvent system prepared from FA: AA (50:50) was thus used to prepare a homogenous blend of nylon-6 and chitosan. This solvent system for dissolving nylon and chitosan has, to our knowledge, not been reported.

Composite membranes of nylon-6:chitosan nanofibres with different weight ratio of nylon-6 to chitosan were fabricated by electrospinning. The morphology and diameter of the nanofibres were influenced by the concentration of the solution and weight ratio of the blending component materials. The SEM images of electrospun 16% nylon-6:chitosan composite nanofibres for different chitosan concentrations with 0, 1 and 3 wt%, respectively, are shown (Figure 9a-d). The nanofibres had smooth surfaces and uniform diameters along their lengths. After the addition of 1% chitosan (Figure 9b), some fibres were observed to have uneven surfaces. A combination of main fibres and fine nanofibres that formed a 'mesh like' structure was observed. The diameters of the main nanofibres ranged between 180 and 300 nm, whilst those of the fine nanofibres were an order of magnitude smaller than the main fibres ranging between 10 and 60 nm. Results from other studies in which nanofibres were produced via electrospinning of nylon-6:chitosan blend did not report the formation of high aspect ratio nanofibres. However, these observations were similar to those reported by Nirmala *et al.* (2011). In addition, the density of the main fibres increased with increasing chitosan %.

Formation of fine nanofibrous structures which increased the surface area to volume ratio may be due to the strong applied voltage created between the electrodes. According to Nirmala *et al.* (2011), when mixed with FA, reactive functional groups on nylon-6 may participate in reactions that produce nylon with poly-electrolytic characteristics. This solution is capable of attracting counter ions from the solvent resulting in a highly charged polymer that is forced to eject from the syringe and fibres are collected as ultra-fine mats between the main fibres (Nirmala *et al.,* 2011). Studies by Jia and colleagues (2007) showed that an increase in the chitosan content of a PVA/chitosan blend resulted in a decrease in the diameter of the electrospun nanofibres. This was attributed to the presence of ionisable amino acids that are characteristic of chitosan. Under acidic conditions, the amino groups are ionised thereby increasing the charge density on the surface of the jets formed during electrospinning. An increase in the charge density results in higher elongation forces being imposed on the jets and ultimately formation of fibres with small diameters (Zong *et al.,* 2002).

The morphology and diameter of electrospun nanofibres is highly dependent on parameters such as solution concentration and applied voltage (Fong *et al.,* 1999). However several studies have shown that the solution concentration or the viscosity of the electrospinning solution is one of the most effective variables that determine fibre morphology (Zong *et al.,* 2002). Sometimes electrospun fibres exhibit bead-on-string structures, which have been generally considered to be undesirable by-products or defects. The electrospinning of 13% nylon:1 % chitosan blends resulted in the formation of beaded nanofibers (Figure 9f). It was observed that at low (13%) nylon-6 concentration, (i) a mixture of beads and fibres was obtained and (ii) as the chitosan content increased the beads became spindle shaped which corresponded to literature (Deitzel *et al.,* 2001). Liu and Hsieh (2002) showed that when low molecular weight nylon solutions were subjected to high electric fields, jet stability was not achieved, and, as a result, the Taylor cone broke into smaller jets and formed beads; these observations were also reported by Deitzel *et al.* (2001). In this study, when the concentration of nylon in the hybrid blend was increased from 13 % (w/v) to 16% (w/v) beading was not observed (Fig 9b). Furthermore, increasing the chitosan content from 1% (w/v) to 3% (w/v) improved the morphology of the nanofibres (Figure 9c).

The decrease in bead formation observed when solution concentration was increased has been attributed to the increased degree of chain entanglement with a rise in the molecular weight, ultimately causing an increase in viscosity and therefore higher visco-elastic force, causing elongation of fibres (Ryu *et al.,* 2003; McKee *et al.,* 2004). Therefore, there should be an optimum solution concentration for the electrospinning process, to prevent beading at low concentration. Moreover, at high solution viscosity the formation of continuous fibres was prohibited because of the inability to maintain the flow of the solution at the tip of the needle (Sukigara *et al.,* 2003). Although several studies have reported the production of bead free nanofibres from pure nylon-6 concentrations ranging from as low as 13 %, bead free nanofibres could not be produced from a >16 % nylon blend with chitosan. This could be attributed to the high charge density of chitosan chains which disrupted the cohesion between the polymer chains.

When two polymers are blended for the fabrication of nanofibres, not only can the structure of the two materials be detected but the inter-molecular interaction can also be determined. Fourier transform infra-red (FT-IR) spectroscopy was used to investigate the changes in the functional group interactions that occurred during the blending of nylon-6 and chitosan for electrospinning (Figure 10). The characteristic bands for amide groups and methylene segments of nylon-6 are shown in Figure10a. Their assignments are: 3200-3400 cm^{-1} (H-bonded and N–H stretch vibration), 3080 cm^{-1} (N–H in-plane bending), 1650 cm^{-1} (amide I, CO stretch) and 1546 cm^{-1} (amide II, C–N stretch and CO–N–H bend). A typical FT-IR spectrum of the raw chitosan (Figure 10b) shows two middle strong bands at 1655 and 1590 cm^{-1}. These may be attributed to the carbonyl C=O or amide I band and amide II absorption band, respectively. A broad band in the range of 3400–3100 cm^{-1} is attributed to N–H and H-bonding stretching vibrations and intermolecular hydrogen bonding of the polysaccharide molecules (Zhang *et al.,* 2009). The observed FT-IR spectra for raw chitosan was different from those reported in literature and this can be justified by reports by Kim *et al.* (2006) which showed that the FT-IR spectra for chitosan was dependant on its source and DD.

According to Nirmala *et al.* (2011) characteristic peaks for composite nylon-6:chitosan nanofibres are found within the 500 – 1750 cm^{-1} region. The intensity of the peaks for the amide bands II and III (located at 1536 and 1462 cm^{-1}, respectively) decreased slightly with increasing chitosan content. Figures 10 (c-e) show the FT-IR spectra of nanofibers electrospun from 16 % nylon-6 blended with different weight ratios of chitosan. Nylon-6 consists of amide groups separated by linear chains of methylene units. These groups provide intermolecular hydrogen bonding between neighbouring polymer chains (Zhang *et al.,* 2009). However, hydrogen bonds formed between two different macromolecules are stronger than those between molecules of the same polymer. According to Kuo *et al.* (2006), mixing chitosan with nylon-6 disrupts the hydrogen bonding in the C=O-H-N amide plane of the blending polymers. This leads to a switch in the dipole direction to reduce the energy and the reformation of the amide plane with the chitosan molecules inserted.

The most common interactions in the blends are hydrogen and ionic bonding and the formation of dipole and charge-transfer complexes. The increase in the frequency of the N–H band suggested that addition of chitosan resulted in the decrease of the hydrogen bonding between polyamide chains. Furthermore, it was observed that the absorption associated with –OH and –NH stretching vibrations (3000 cm^{-1} to 3400 cm^{-1}) that is characteristic of chitosan, shifted to a lower wave number when nylon-6 was added. This observation was also reported by Jia *et al.* (2002) who attributed it to the formation of H-bonding between PVA and chitosan. Other studies have also reported the presence of a prominent peak at 1639 cm^{-1} in the IR spectra of the blended nylon-6 and chitosan. This band is primarily dependent on the stretching vibrations on the C=O and C-N groups, thus these can be attributed to the amino groups in the polymer blend (Zhang *et al*., 2009).

This study indicated that intermolecular interactions occurred between nylon-6 and chitosan and the intermolecular interactions varied with varying chitosan content in the fibres. This was in agreement with literature where it has been shown that new hydrogen bonding networks can be formed between nylon and chitosan resulting in a hybrid polymer with improved hydrophilicity for application in biotechnological processes (Zhang *et al*., 2010). It was concluded that a new composite product was prepared and could be electrospun to produce nanofibres. A few studies have reported the electrospinning of nylon and chitosan blends as well as their application. Nirmala *et al.* (2011) reported the biomedical applications of these hybrid nanofibres in which human osteoblastic cells were cultured on the surface of the nanofibres and the results showed that there was a cytotoxic response. In another study, the electrospun nanofibres were further functionalised using Cibacron Blue F3GA (CB), and used as an affinity membrane for papain purification (Zhang *et al.,* 2009). After the successful production of electrospun nanofibres in this study, we proceeded to report the the facile immobilisation of AChE on the nanofibres using a cross-linking method This is presented in the following chapter, Chapter 4.

Chapter 4

Chapter 4: IMMOBILISATION

4.1 INTRODUCTION

Immobilisation of enzymes on insoluble supports is a significant process due to its potential for improving pH and thermal stability, simplifying product purification, and facilitating enzyme recycling (Caramori *et al.,* 2004; Fernandes *et al.,* 2004; Cheng *et al.,* 2006; Blanco *et al.,* 2007). Several methods can be used for enzyme immobilisation, each with their drawbacks, including the potential to leach from the solid support and enzyme conformational changes which may occur due to the immobilisation process. When immobilising enzymes on an insoluble surface, it is important to select a method of attachment aimed specifically at the functional groups that are not associated with the catalytic groups of that enzyme active site. Immobilisation of enzymes has been achieved by the intermolecular cross linking of the enzyme, either to other protein molecules, or to the functional groups on an insoluble support matrix. Generally, cross linking is used in conjunction with some of the other methods mentioned previously (Chapter 1) (Goradia *et al.,* 2005; Lai and Lin, 2005; Panya *et al.,* 2005).

Of the many commercially available cross-linking agents, including cyanogen bromide and carbodiimide, glutaraldehyde (GA) is the most widely utilised. It is used to immobilise proteins to solid supports for use in enzyme reactors, affinity chromatography, immunochemical research, and as a cross-linking agent for biosensors. Two primary procedures for accomplishing immobilisation using GA include: (i) immobilisation onto aminated supports pre-activated with GA or (ii) direct cross-linking of proteins that are bound to aminated cationic supports via ionic interactions (Figure 11). Several different supports have been used in conjunction with GA including nylon, fumed silica, controlled-pore glass, gold (Ag) nanoparticles and carbon nanotubes.

It is well established that the reaction of GA with proteins is mainly through the *ε*-amino groups of lysine residues. In addition to lysine residues, GA also reacts with other amino acids such as tyrosine, tryptophan, histidine and cysteine. GA has been shown to be very reactive towards the α-amino groups of amino acids, N-terminal groups of some peptides, as well as the sulfhydryl groups of cysteine residues. Moreover, GA is capable of reacting with some polysaccharide moieties (Russel and Hopwood, 1976).

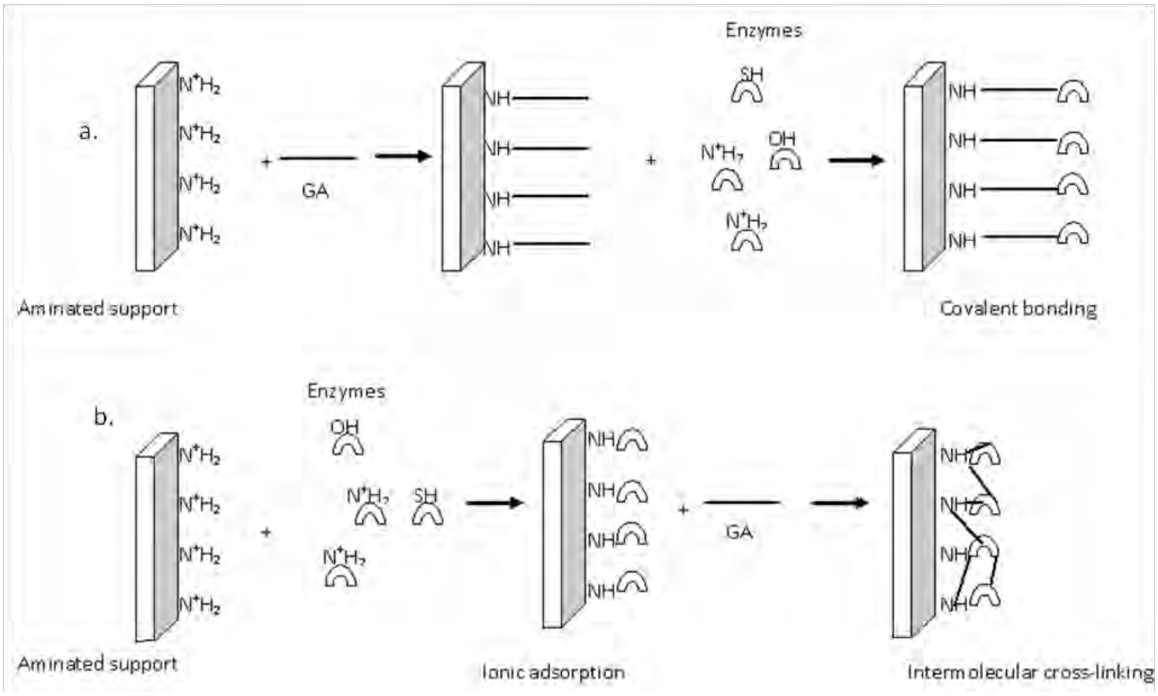


Figure 11: Two main strategies that exist for GA cross-linking of proteins onto surfaces with amine groups. Aminated supports can be pre-activated by GA prior to the addition of the proteins (a) or proteins are initially adsorbed onto the support via ionic interactions after which they are cross-linked to the support matrix with GA (Betancor *et al.*, 2011).

Several studies have reported the immobilisation of enzymes on nylon or chitosan matrices using both cross-linking methods (Goldstein *et al.,* 1974; Marinov *et al.,* 2009; Sousa *et al.,* 2001). AChE was co-immobilised with choline oxidase (ChO) onto a platinum (Pt) surface using GA and the activity of immobilised enzymes was evaluated in the presence of dimethyl-2,2-dichlorovinyl phosphate (DDVP). The cross-linking involving GA significantly increased the enzyme attachment to the transducer, thus electron exchanges could occur more directly (Singh *et al.,* 1999). Gulla and colleagues (2002) also reported the immobilisation of AChE in nylon net using GA. However, the disadvantages associated with enzyme immobilisation via GA cross-linking include loss of substrate affinity and reduced activity.

In this chapter, the immobilisation of electric eel AChE onto electrospun nylon-6:chitosan hybrid nanofibres via GA cross linking is reported. The immobilisation procedure was optimised in terms of GA concentration, incubation time and AChE concentration.

4.2 MATERIALS AND METHODS

4.2.1 Effect of GA on soluble AChE activity

The effect of GA concentration on AChE enzyme activity was determined according to the micro-Ellman method (Ellman *et al.,* 1961) using ATChI as substrate and 5,5-dithiobis- (2-nitrobenzoic acid) (DTNB: Ellman's reagent). The enzyme reaction product interacted with DTNB to produce 2-nitro-5-thiobenzoic acid (TNB), a yellow-coloured solution. The reaction was followed spectrophotometrically at 412 nm every 30 seconds for 10 minutes using a Powerwave$_x$ microtiter plate reader using KC Junior software. In a microtitre plate, 20 µl AChE, 10 µl DNTB, GA (1 – 25%) and 0.1 M phosphate buffer (pH 7.5) were incubated for 10 minutes. Buffer volume was adjusted accordingly to allow for a total reaction volume of 200 µl for the desired GA concentrations. The reaction was then initiated by the addition of 10 µl ATChI and the change in absorbance monitored. All assays were performed in triplicate with 1 unit (1U) of enzymatic activity defined as the amount of enzyme that catalysed 1 µmol of TNB per minute.

4.2.2 Enzyme immobilisation

The electrospun nylon-6:chitosan nanofibres (1 cm × 1 cm) were immersed in 5 ml of 5% GA solution for 5 hours at 4°C after which the membranes were rinsed in distilled water. The membranes were then immersed in 0.5 mg/ml solution of AChE in 0.1 M sodium phosphate buffer solution (pH 7.0) for 30 h at 4°C with continuous stirring. To determine the effect of incubation time on enzyme immobilisation, 50 µl of AChE solution were collected from the incubation solution at 1, 5, 12, 24 and 30 hours after incubation. Non-specifically bound protein was removed by washing with 0.1 M sodium phosphate buffer (pH 7.0) and the wash solutions were assayed for AChE activity.

The amount of AChE immobilised (x) was calculated according to the following equation:

$$x = a - (b + a)$$

Where a was the initial amount of AChE in the incubation solution, b was the amount of protein in the pooled wash solution and a was the amount of AChE remaining in the incubation solution after 30 hours.

To determine the amount of AChE immobilised per cm^2 of nanofibre, 5 pieces of 1 cm^2 nanofibres were each immersed in 0.5 mg/ml AChE solution for 30 hours. The amount of AChE immobilised on each nanofibre was then determined and the average amount of AChE immobilised per cm^2 was calculated.

4.2.3 Protein determination

The amount of protein in the different samples was determined according to Bradford method (Bradford, 1976), using bovine serum albumin (BSA) concentrations ranging from 0 mg/ml – 1 mg/ml as the protein standards. Bradford's reagent (250 µl) was added to microtitre plates that contained 5 µl of either the BSA solution or the immobilisation assay samples. The absorbances for the unknowns were recorded at 495 nm and the protein concentrations were determined using a BSA standard curve (see Appendix 1).

4.2.4 Effect of glutaraldehyde concentration on enzyme immobilisation

Nylon-6:chitosan hybrid nanofibres were cut into roughly 1 cm × 1 cm pieces and immersed in GA solutions of concentrations ranging from 1% to 25% at 4ºC for 10 hours. The cross-linked nanofibres were then rinsed 3 times in distilled water and subsequently incubated in 0.5 mg/ml AChE solution for 30 hours with continuous stirring. After incubating for 30 hours, the nanofibres were washed with 0.1 M sodium phosphate buffer (pH 7.0) until there was a negligible amount of protein present in the wash solutions. To determine the amount of protein bound to the nanofibres, the Bradford's protein assay was carried out in triplicate on the wash solutions as well as on the incubation solution. FT-IR analysis of the cross-linked nanofibres and cross-linked nanofibres after AChE immobilisation was carried out as described in Chapter 3.

4.2.5 Comparison of the GA cross- linking methods

AChE was immobilised onto GA cross-linked nanofibres using two methods widely reported in literature. Immobilisation of AChE on GA pre-activated nanofibres was carried out as previously described. In the second method, 1 cm^2 pieces of nylon-6:chitosan nanofibres were suspended in 5 ml of 0.5 mg/ml AChE prepared in 0.1 M sodium phosphate buffer (pH 7.0). The suspension was continuously stirred at 4ºC and aliquots were drawn at 5 hour intervals and assayed for protein concentration and catalytic activity. After 30 hours, the nanofibres were washed in distilled water and incubated in 5% GA solution and stirred for 5 hours at 4ºC. Non-specifically bound AChE was washed off in distilled water.

4.2.6 Effect of initial enzyme concentration on immobilisation yield

AChE solutions (0.1 – 0.6 mg/ml) were prepared in 0.1 M sodium phosphate buffer (pH 7.5) and kept on ice. Nylon/chitosan hybrid nanofibres were cross-linked in 5 % GA solution as described previously and incubated in the AChE solutions for 30 hours. At various time intervals (1, 5, 12, 24 and 30 hours) the amount of protein immobilised on the nanofibres was determined as described above. AChE catalytic activity was also determined for the immobilised enzyme after 30 hours of incubation. Enzyme activity was calculated using the Beer– Lambert law with the known molar extinction coefficient of TNB ($\varepsilon = 13600\ M^{-1}\ cm^{-1}$).

4.2.7 FT-IR analysis

FT-IR analysis of the GA cross-linked nanofibres prior and post AChE immobilisation was carried out as described previously (Chapter 3).

4.3 RESULTS

Nanofibres prepared in Chapter 3 were used as immobilisation matrices for electric eel AChE via GA cross linking. AChE has been immobilised on a range of support matrices for the construction of biosensors (Amine *et al.,* 2006). GA cross linking of AChE onto solid supports has been shown to be a reliable immobilisation strategy and has thus been widely documented. The effect of several factors, including GA concentration on enzyme activity, amount of protein immobilised (Figure 12) and initial AChE concentration (Figure 13) was determined. AChE was also immobilised using the two cross linking methods reported in literature (Figure 14) and the effect of incubation time was determined (Figure 15). Immobilisation was monitored via both protein assays (i.e. Bradford's assayss) and AChE activity assays.

4.3.1 Effect of GA concentration on soluble AChE activity

GA (5% -25% final concentration) was added to the reaction mixtures for free AChE assay to determine the effect of the cross linker on ezyme activity. The residual activity was plotted relative to the activity of AChE that was not exposed to GA (Figure 12).

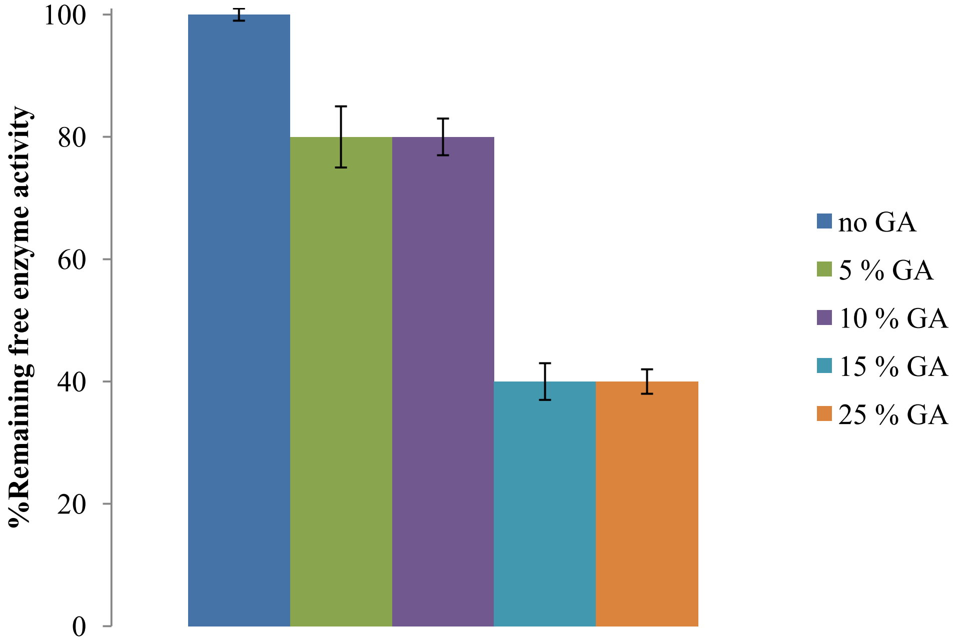


Figure 12: Effect of GA on enzyme activity of free (soluble) AChE. Soluble AChE was incubated with DTNB and GA (5 – 25 %) in 0.1 M sodium phosphate buffer pH (7.5) for 10 minutes after which the reaction was initiated by the addition of acetylthiocholine Data points represent the means± SD (n =3).

Increasing GA concentrations from 0% - 25% resulted in a decrease in the activity of AChE in its soluble form. The activity of the untreated AChE was considered to be 100 % and the addition of GA concentrations of 5% and 10% reduced AChE activity by 20%. Further increases in GA concentrations resulted in ~60 % loss in AChE activity.

4.3.2 Effect of AChE concentration on immobilisation yield

The relationship between the initial AChE concentration in the incubation solution and amount of protein immobilised was determined (Figure 13)

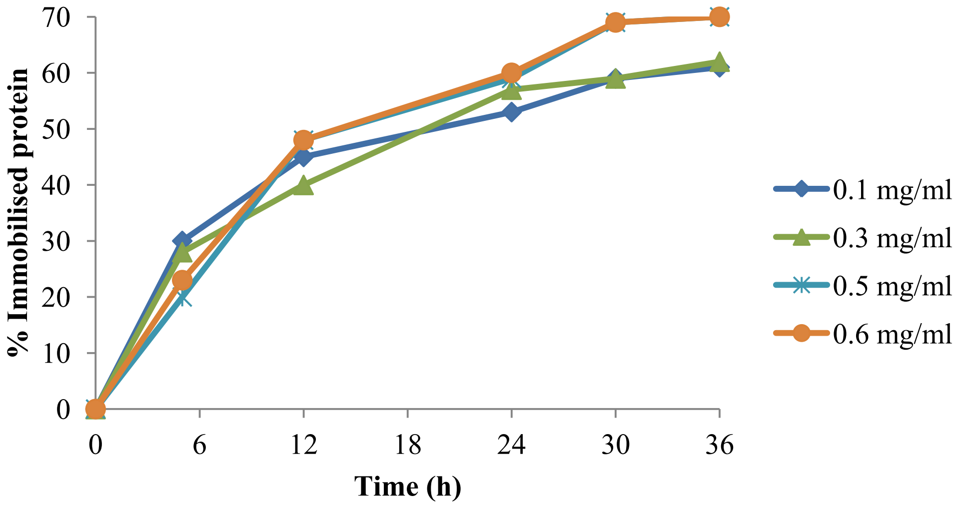


Figure 13: The effect of enzyme concentration on immobilisation yield was determined by incubating nylon-6/chitosannylon-6:chitosan nanofiberfibres in AChE concentrations ranging from 0.1 mg/ml to 0.6 mg/ml for 36 hours. The amount of protein immobilised was then determined via the Bradford's assay.

Increasing the initial concentration of AChE in the incubation solution resulted in an increase in the amount of protein immobilised after 30 hours of incubation. Beyond an initial AChE concentration of 0.5 mg/ml, the curve tended to plateau when the immobilised protein was about 70 %. AChE activity was determined and the values for each initial AChE concentration is tabulated in Table 6 below.

Table 6: Effect of initial AChE concentration on the activity of the immobilised enzyme

AChE concentration (Initial)	0.1 mg/ml	0.2 mg/ml	0.3 mg/ml	0.4 mg/ml	0.5 mg/ml	0.6 mg/ml
Retained protein mg/cm²	0.005	0.009	0.018	0.029	0.033	0.035
Activity (µmolDTNB/cm²/min)	0.73	1.98	3.42	6.13	10.1	10.1
Specific activity (U/mg protein)	15.0	21.3	18.8	18.4	30.3	28.9

Increasing the concentration of AChE in the incubation solution from 0.1 mg/ml to 0.6 mg/ml resulted in an increase in the amount of protein immobilised on the nanofibre surfaces. AChE immobilised on nanofibres incubated in 0.5 mg/ml AChE solution had the highest specific activity.

4.3.3 Effect of GA concentration on AChE immobilisation

The effect of GA concentration on the amount of AChE immobilised onto the nanofibres was determined for GA concentrations ranging from 0% to 25% (v/v). The amount of enzyme immobilised was determined via the Bradford's assay and the results are shown in Figure 14.

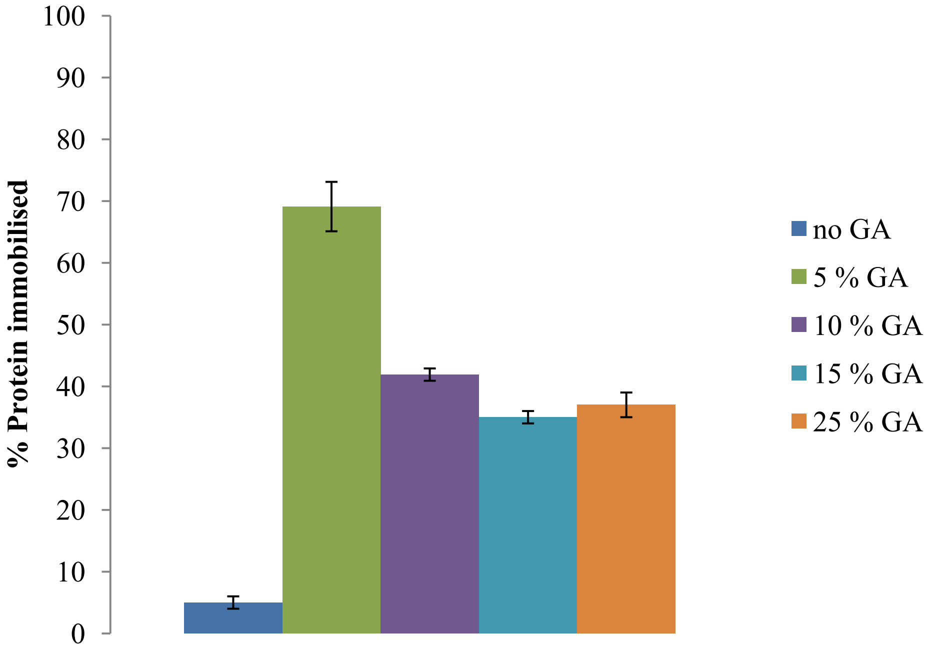


Figure 14: Effect of crosslinker concentration (GA) on protein immobilisation. 0.5 mg/ml AChE, GA pre-activated nanofibres. Data points represent the means± SD (n =3).

In the absence of GA, less that 10% AChE was immobilised on to the nanofibre surfaces. When the nanofibres were treated with 5 % GA, about 70% AChE was immobilised onto the nanofibres. GA concentrations between 10 and 25% resulted in lower amounts of protein being immobilised relative to 5% GA.

4.3.4 Effect of incubation time on amount of AChE immobilised

Electrospun nylon-6:chitosan nanofibres were crosslinked in 5% GA solution after which they were incubated in a 0.5 mg/ml AChE solution and stored at 4ºC. At different time intervals aliquots were collected from the incubation solution and protein concentration determined via Bradford's assay. After 30 hours incubation, the nanofibres were washed with double-distilled water to remove non-specifically bound protein and amount of immobilised protein determined (Figure 15).

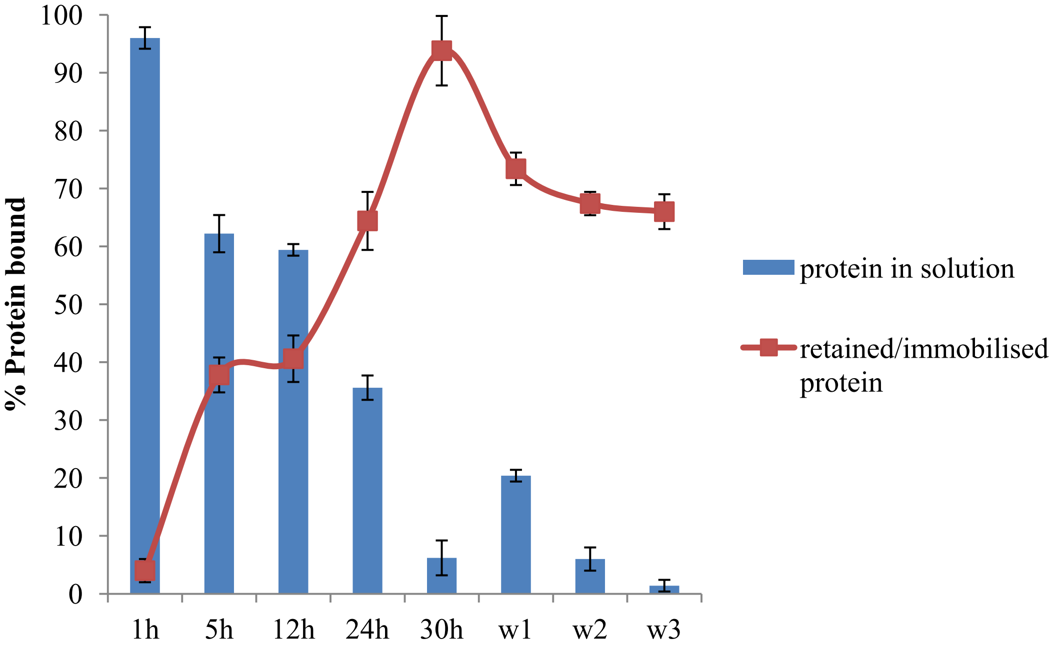


Figure 15: Effect of time on protein immobilisation. After incubation for 30 hours, the nanofiberfibres were washed (w1-w3) and the protein content was determined. Data points represent the means± SD (n =3).

There was an increase in the amount of protein immobilised with increased incubation time (Figure 15). After incubation for 30 hours, >90% of the initial AChE in solution was retained on the nanofibres. Protein assays of the washing solutions showed that some of the protein had been weakly bound to the the nanofibre surfaces.

4.3.5 Comparison of the two GA cross-linking methods

The effectiveness of the two GA cross linking methods was then investigated for the immobilisation of AChE and the results are shown in Figure 16.

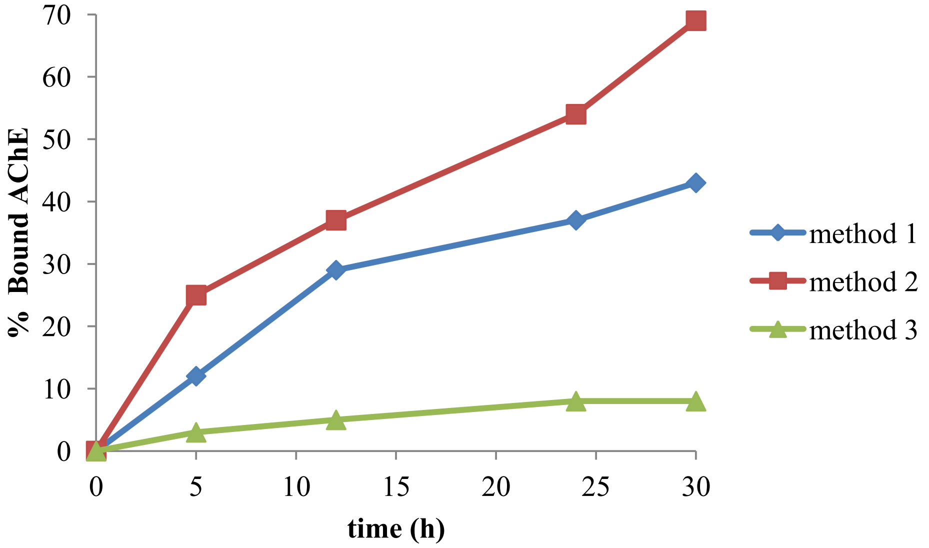


Figure 16: Two different procedures for AChE immobilisation via GA cross linking were used to immobilise AChE. AChE was initially adsorbed onto the nanofibre surface which was then treated with GA (method 1); the aminated nylon-6/chitosannylon-6:chitosan nanofibres were cross-linked and subsequently immersed in AChE (method 2) and with GA untreated nanofibres which were incubated in AChE solution (method 3).

Both GA cross-linking methods for protein immobilisation on aminated solid supports showed positive results for the immobilisation of AChE onto nylon-6:chitosan nanofibres. Figure 16 also shows that even when the nanofibres were not treated with GA, AChE was immobilised. AChE could be immobilised on the hybrid nanofibres via adsorption or ionic interactions.

4.3.6 FT-IR analysis

After immobilisation, FT-IR analysis of the nanofibres was carried out to determine whether any new interactions had been formed between the nanofibres, GA and AChE (Figure 17).

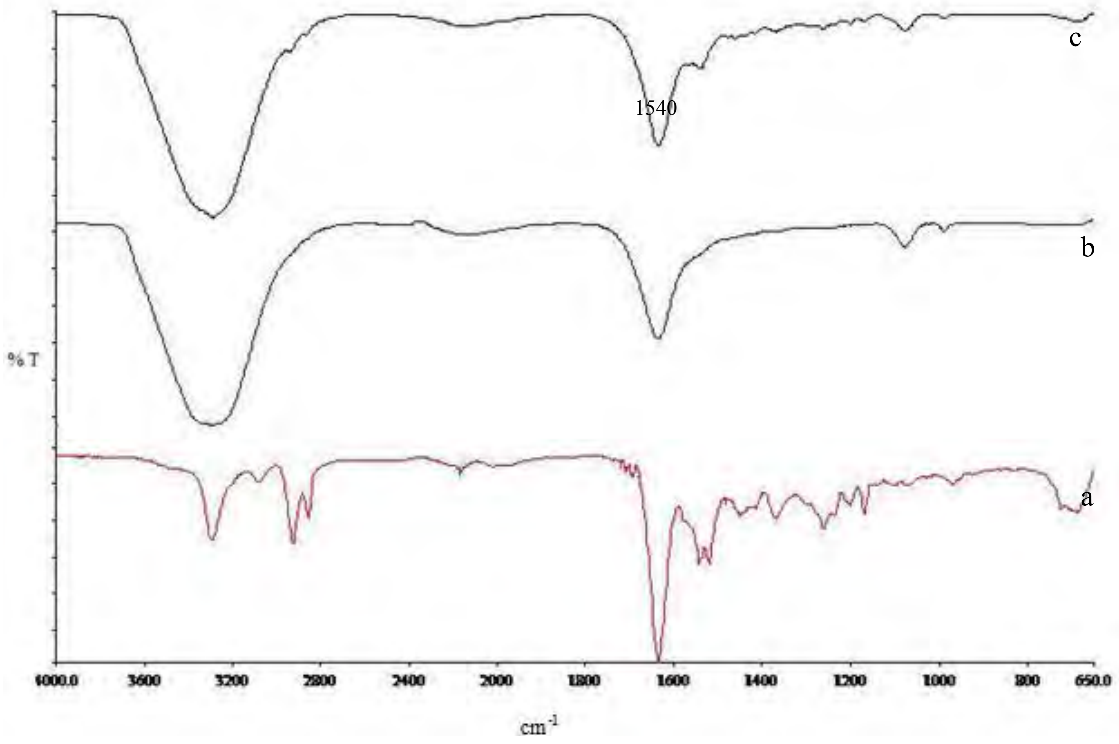


Figure 17: FT-IR spectra of (a) nylon-6:chitosan nanofibres (16%:3%) (b) GA crosslinked nanofibres (c) GA crosslinked nanofibres after incubation AChE immobilisation.

FT-IR analysis of GA cross-linked nanofibres (17 a) showed an increase in the intensity of the OH-stretch at 3400 cm$^{-1.}$ A peak at 1540 cm^{-1} was also observed.

4.4 DISCUSSION

GA is highly reactive towards both hydroxyl and amine groups and acts as a linker between the solid support and the enzyme. Various data on aldehyde reactivity (at pH values between 2.0 and 11.0) have been reported with the following amino acids in the literature: lysine, tyrosine, tryptophan, phenylalanine, histidine, cysteine, proline, serine, glycine, glycylglycine and arginine (Walt and Agayn, 1994; Migneault *et al.,* 2004). The data collected from these studies has been used to rank the ability of GA to react with the functional groups on different amino acids in decreasing order of reactivity as follows: ε-amino, α-amino, guanidinyl, secondary amino, and hydroxyl groups (Migneault *et al.,* 2004). The effect of GA on soluble AChE activity was thus studied to determine whether GA inhibited or activated AChE activity. After incubation of soluble AChE in varying concentrations of GA solution, it was observed that exposure to GA decreased AChE activity. GA concentrations above 10% reduced AChE activity to below 40% of its initial activity (Figure 12). Proteins in solution can be crosslinked to each other to form structures known as Cross Linked Enzyme Aggregates (CLEA) (Hanefeld *et al.,* 2009); these aggregates may have formed upon AChE solution exposure to GA, reducing the substrate accessibility to the active site. Cross linking an enzyme to itself is inexpensive; however, it may be insufficient for industrial and commercial use, as some of the protein material will inevitably be acting as support material leading to relatively low activity.

Since GA has been shown to react with several amino acid residues, loss of AChE catalytic activity can be attributed to alterations in the physical and chemical structure of AChE, resulting in reduced substrate affinity. Although studies have ranked GA reactivity with hydroxyls group as the least reactive/important, with respect to serine hydrolases such as AChE, even minimal GA reactivity with the catalytic hydroxyl would lead to significant loss of enzyme activity. Studies by Capaldi (1973) showed that GA had reactivity towards some beef erythrocyte glycoproteins. It is therefore possible that, in addition to reacting with amino acids to facilitate AChE immobilisation, the immobilisation process may also have occured through glycoprotein cross-linking. The effect of GA concentration on AChE immobilisation was also investigated (Figure 14), and 5 % GA solution resulted in the largest amount of AChE being immobilised. GA concentrations above 10% resulted in at most 40% of the initial AChE being immobilised.

To covalently immobilise AChE, the amines on the nylon-6:chitosan hybrid nanofibres were initially activated by the coupling agent GA followed by reacting an AChE solution with the activated nanofibres. This method of enzyme immobilisation via cross linking has been reported in several studies (Adriano *et al.,* 2005; Gabrovska *et al.,* 2008; Stoilova *et al.,* 2010). In addition to this, another strategy for GA cross linking was also utilised for AChE immobilisation. In this strategy, AChE was initially adsorbed onto the aminated nanofibres followed by subsequent GA cross-linking of the enzyme and support. AChE immobilisation strategies via the two methods were compared to determine the most efficient method to immobilise AChE onto to the nylon-6:chitosan blend nanofibres (Figure 16). Very low amounts (~10%) of AChE were non-specifically adsorbed onto the untreated nanofibres. This observation may have been due to the ionic bonds that formed between the amine groups of chitosan and the carboxylic groups of the enzyme as well as those belonging to aspartic and glutamic acid side chains. Although proteins adsorbed to surfaces are prone to enzyme leaching from the support, the advantage of adsorption with no chemical modification is that the enzyme maintains its physical conformation thus resulting in a reduced loss of activity (Hanefeld *et al.,* 2009).

The molarity of the immobilisation buffer has also been shown to have a significant effect on the rate and success of immobilisation. At low ionic strength the proteins are immobilised through the most negatively charged areas of the protein surface whilst at high ionic strength immobilisation would occur through the region with the most positively charged functional groups. This means that, just by altering the ionic strength during the immobilisation, not only the immobilisation rate is altered, but also the region of the protein that is involved in the immobilisation (Tomar and Prahbu, 1985; Borges *et al.,* 2009).

When the nanofibres were treated with GA and then subsequently incubated in AChE, there was a steady increase in the amount of protein immobilised over time. After 30 hours of incubation, over 60 % of the initial protein in solution was bound. When the immobilisation is carried out on pre-activated supports, the primary amino groups of the enzyme react with the aldehyde groups that have been introduced by modification of the amino groups of the support by glutaraldehyde molecules (Monsan, 1978; Guisan *et al.,* 1997). Protein immobilisation on GA pre-activated supports is usually performed around pH 7–8.5, because of the low stability of glutaraldehyde activated supports at high pH values. However, at these pH values, the reactivity of -amino groups of lysine may be expected to be quite low, and the intensity of the multipoint covalent attachment may be not very high. However, the main advantage of this strategy is that chemical modification of the enzyme is limited to only those groups in the protein that are involved in the immobilisation, which are usually surface residues that are not involved in the protein's catalytic activities (Monsan, 1978).

Immobilisation via adsorption onto a cationic support, such as chitosan, followed by treatment with glutaraldehyde has been found to be a very simple and fast procedure which can be employed to prepare remarkably stable covalently immobilised enzymes. As observed for the GA pre-treated nanofibres, there was also a steady increase in amount of protein immobilised over time using this strategy. However, after 30 hours of incubation, about 70% of the initial AChE in solution had been immobilised. Bradford's protein assay on the residual AChE solution as well as on the wash solutions showed a high protein content but displayed very low AChE activity. This observation may have been due to the leaching of non-specifically bound protein into the bulk of the GA cross linking solution leading to cross linking of the protein molecules to themselves rather than to the nanofibres. When the enzyme is firstly adsorbed on the support, and then treated with glutaraldehyde under mild conditions, all the primary amino groups of the enzyme and support may be activated with one molecule of glutaraldehyde. This may allow the formation of intense cross linkages known as multi-point covalent enzyme-supports, which have been shown to reduce enzyme leaching from supports. On the other hand, multi-point covalent attachments may result in loss in enzyme activity due to conformational changes (Walt and Agayn, 1994).

The effect of varying AChE concentrations in solution ranging between 0.1 – 0.3 mg/ml on the nanofibres total protein loading was investigated. In each experiment, 0.1 mg of 1 cm^2 nanofibres was immersed in 5 ml of AChE immobilisation solution Figure (15). An increase in AChE concentration led to an increase in the amount of protein immobilised. However, another phenomenon was observed; with an increase in the initial AChE concentration, it was observed that for a fixed time, i.e. 12 hours, there was a noticeable decrease in the amount of protein immobilised. This observation may be due to diffusion effects into the permeable structure of the electrospun nanofibres. AChE molecules diffusing from the bulk incubation solution into the nanofibres initially reacted with the exterior aldehyde chains as they were the first chains available to the enzyme. This would have made other GA chains that had less exposure to the bulk of the incubation solution less accessible for linkage (Adriano *et al.,* 2005). It was also observed that for initial concentrations of 0.5 and 0.6 mg/ml enzyme, the same immobilisation yield of about 69 % was achieved indicating that the nanofibre surface was saturated. Zuza *et al,* (2007) reported that an increase in the initial concentration of penicillin acylase led to an almost linear increase of the enzyme loading on the support followed by levelling off of the graph when initial enzyme concentration was above 9 µg/ml.

Comparative FT-IR spectra of pristine nylon-6:chitosan nanofibres, GA cross-linked nanofibres and AChE loaded GA cross-linked nanofibres were shown in Figure 17. FT-IR analysis of the nanofibres after cross-linking and enzyme loading (Figure 17c) had a characteristic peak at 1536 cm^{-1} indicating the presence of amine groups associated with the enzymes. Other studies have reported an increase in C–H stretch at 2936 cm^{-1} and a decrease at 1100 cm^{-1} due to the presence of aliphatic amino group. These results have led to the conclusion that GA cross linking resulted in more hydrophobic nanofibre surface since all the chitosan amino groups are blocked with aliphatic chains (Beppu *et al.,* 2007). Another method that could have been used to quantitatively characterise the degree of cross linking is that of acid titration (Knaul *et al.,* 1999). After GA cross linking, the nanofibres would be characterised by the absence of protonable amino groups, thus the only colour change observed would be due to the consumption of NaOH in neutralizing the added acid.

The results in this chapter indicate the optimised conditions for immobilising AChE onto nanofibres obtained from the electrospinning of a nylon-6:chitosan blend. Since it had the least adverse effect on the activity of soluble AChE as well as the best immobilising effect, it was concluded that 5% GA solution was the best concentration for the immobilisation of AChE. Increasing the initial AChE concentration in solution also increased the amount of protein retained on the support. Immobilisation on GA pre-activated supports normally occurs via the reaction between the primary amino groups of the enzyme the aldehyde groups of GA (Monsan, 1978). This reaction is normally carried out in the pH range between 7 and 8.5 due to the instability of GA outside of this pH range. The disadvantage with this method is that the reactivity of the ε-amino of Lysine residues is low and thus the multi-point covalent attachment may be weak. The results obtained in this study showed that preactivating the nanofibres resulted in higher AChE loading. However a comparative study was not carried out to determine whether using either immobilisation method yielded AChE with higher stability. Studies by Lopez-Gallego *et al.* (2005) showed that the GA treatment of adsorbed glucose oxidase on aminated supports yielded stabilization of over 400-fold over that of free glucose oxidase. Subsequent characterisation of immobilised AChE was thus carried out with AChE immobilised under the following conditions: nanofibres pre-activated with 5% GA solution, 0.5 mg/ml AChE solution, and 30 hours incubation for immobilisation. Under these conditions, 66 % AChE was immobilised onto the electrospun nanofibres. The results also showed that, after immobilisation, the enzyme retained catalytic activity. Chapter 5 outlines the comparison between free and immobilised AChE in terms of the conditions required for optimum activity as well as their operational and storage stability.

Chapter 5

Chapter 5: ENZYME CHARACTERISATION

5.1 INTRODUCTION

Enzymes have several advantages over conventional inorganic catalysts; these include selectivity, reduced side reactions and mild reaction conditions. However, since enzymes are proteins, any changes in reaction conditions may lead to the deformation of their structure, resulting in the loss of activity. Some drawbacks concerning the use of enzymes therefore include high production costs, enzyme instability towards certain pH and temperature environments, and the difficulty of recovering soluble enzymes from an aqueous medium (Brady and Jordaan 2009). The idea of immobilising an enzyme on an insoluble support provides the possibility of using the enzymatic reaction on an industrial scale. Immobilisation is typically achieved through a site-specific reaction between reactive groups of the chosen material and an amino acid residue on the enzyme. The immobilised enzyme can be reused throughout several reactions and easily separated from the product, which decreases the cost involved and the contamination of product, respectively. Moreover, the stability of enzymes is also increased after immobilisation.

Due to its pivotal role in the cholinergic nervous system, AChE has been the subject of many studies. In ecotoxicology, AChE is widely used as a biomarker of effect to evaluate the health of the aquatic environment. Biomarkers provide biological responses that are useful tools informing about pollutant exposure and their possible toxic effects in living organisms (Durieux *et al.,* 2010). OPs and CPs are extremely potent inhibitors of AChE, found in cholinergic neurons. When AChE is blocked, ACh accumulates at the cholinergic receptor sites thereby causing the excessive stimulation of the receptor ultimately leading to death. Long term exposure to low levels of OPs and CPs can lead to persistent and addictive inhibition of AChE resulting in delayed neuropathy (Williams *et al.,* 1997). Rapid, sensitive, selective and reliable determination of OPs and CPs is therefore necessary in order to take immediate necessary action. Current analytical techniques such as gas chromatography (GC) and liquid chromatography (LC) are very sensitive and reliable, but cannot be carried out in the field. In addition, these techniques are time consuming, expensive and have to be performed by highly trained technicians.

In order to address these problems, biosensors based on inhibition of the cholinesterases AChE and BChE have been developed (Singh *et al.,* 1999). Both AChE and BChE have been immobilised on different supports for constructing cholinesterase biosensors, used for detecting inhibitors (Amine *et al.,* 2006; Singh *et al.,* 1999). However, the majority of OP and CP biosensors to date have used AChE as the recognition component. The presence of these pesticides blocks AChE activity leading to decreased device response. In an AChE-based sensor, the signal is inversely proportional to pesticide concentration. The determination of pesticides has become increasingly important in recent years because of the widespread use of these compounds, which is due to their large range of biological activity and a relatively low persistence. The development of an efficient biosensor requires the immobilisation of adequate amount of recognition elements (enzyme) while maintaining its biological activity. Storage stability, or shelf life, refers to an enzyme's ability to maintain its catalytic abilities in the period between manufacture and eventual use. Operational stability describes the persistence of enzyme activity during a process, i.e. under conditions of use. Both storage and operational stabilities affect the usefulness of enzyme-based products.

Ellman's spectrophotometric method is based on the fact that thiocholine (one of the products of enzymatic hydrolysis of ATChI) reacts immediately, quantitatively and irreversibly with 5, 5-dithiobis-2-nitrobenzoic acid (Ellman's reagent, DTNB) forming the yellow product 5-mercapto-2-nitrobenzoic acid and its dissociated forms (Ellman *et al.,* 1961). The assay for AChE activity may be carried out with analogues of acetylcholine e.g. acetylthiocholine; or radiolabelled acetylcholine (Johnson and Russel, 1975), or by titrating the acid formed by hydrolysis (Jacobson *et al.,* 1957). Chemiluminescence procedures for measuring acetylcholine have also been described (Birman, 1985) where the choline formed by hydrolysis of acetylcholine is converted into betaine and hydrogen peroxide (H_20_2) by choline oxidase and the H_20_2 generated is detected with a luminescence reaction. Ellmans' method (Figure 18) is the most extensively used spectrophotometric assay for AChE activity due to its simplicity.

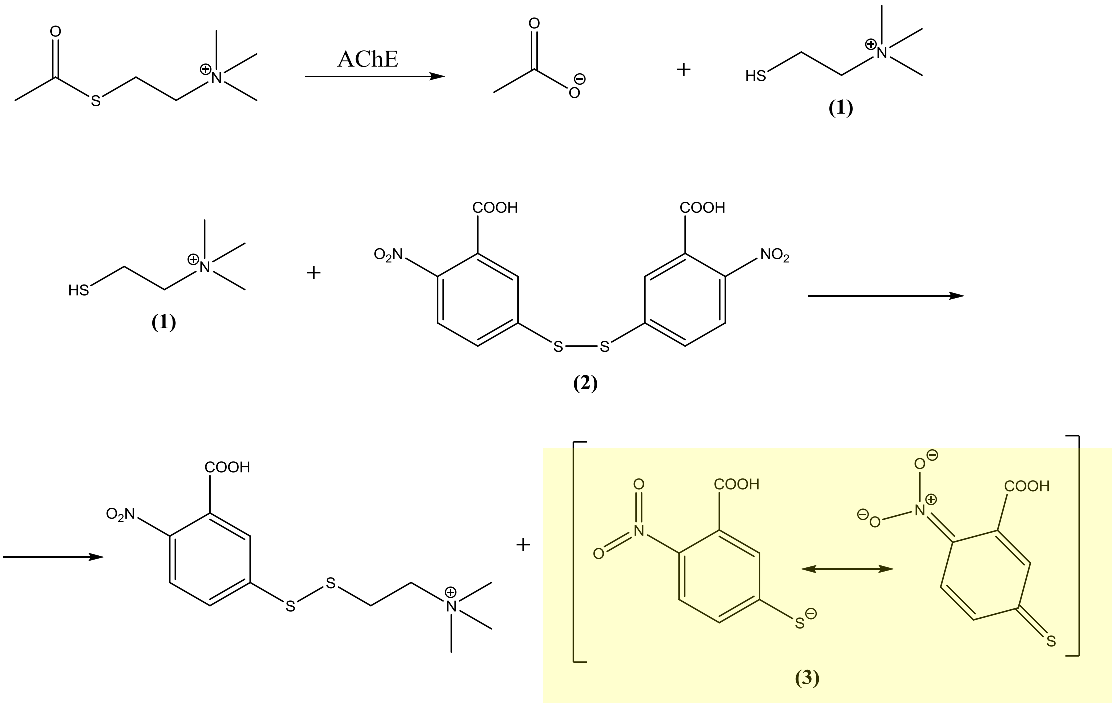


Figure 18: Reactions involved in the determination of AChE activity. Thiocholine (1) reacts with Ellman reagent (2) producing a yellow anion (3) (Marinov *et al.*, 2009).

An enzyme's reaction rate is one of the parameters used to determine the success of an immobilisation method. The rate of reaction should be proportional to the enzyme concentration or loading capacity, as mentioned earlier, and also to the enzyme activity. Increased reaction rates would imply that either the system has a high loading capacity for the immobilised enzymes or that immobilisation has increased enzymatic activity, both of which are desirable. Another parameter would be the enzyme's reusability. This would involve the measurement of enzyme activity in repeated batch experiments. Immobilisation systems that are able to maintain activity over many batches have greater economic value than those in which activity is lost. In addition to the the reaction rate, the enzyme system's activity must be characterised over varying pH and temperature. Immobilised enzymes typically show maximum activity at a particular pH and temperature, however, these maxima do not automatically correspond to the maxima of the free enzymes. The immobilisation of enzymes can have slight or drastic effects on enzyme conformation, shifting the activity maxima.

The objectives of the current study were to compare the characteristics of free AChE to those of immobilised AChE using the modified Ellman's assay for microtitre plates. The effect of immobilisation on the pH and temperature optima as well as on the thermal and pH stabilities of AChE were determined. Furthermore, the effects of the immobilisation process on the

AChE kinetic parameters (V_{max}), (k_{cat}), (K_m) and catalytic efficiency (k_{cat}/K_m) as well as the reusability and storage stability were investigated.

5.2 MATERIALS AND METHODS

The activity of the free and immobilised AChE was determined according to the micro-Ellman method (Ellman, *et al.,* 1961) using ATChI as substrate and 5,5-dithiobis- (2-nitrobenzoic acid).

5.2.1 Free AChE assay

Experiments were carried out with a volume of free enzyme solution with a protein concentration equivalent to the amount of protein immobilised onto a 1 cm^2 nylon/chitosan nanofibre sample. For each experiment, 240 µl of sodium phosphate buffer (0.1 M, pH 7.5), 30 µl DTNB, and 15 µl AChE solution were added to a microtitre plate well. The reaction was initiated by the addition of 15 µl ATChI and the enzyme reaction product reacted with DTNB and the absorbance of the yellow solution was followed spectrophotometrically at 412 nm every 30 seconds. One unit of enzymatic activity was defined as the amount of enzyme that catalysed 1 µmol of substrate to product per minute.

5.2.2 Immobilised AChE

For the immobilised enzyme assay, soluble enzyme was substituted by a 1 cm^2 section of AChE loaded electrospun nanofibre. Each reaction mixture total volume of 300 µl consisted of 255 µl of sodium phosphate buffer (0.1 M, pH 7.5) and 30 µl DTNB. To allow spectrophotometric readings of the reaction, the assay procedure was modified for the immobilised AChE assays (Figure 19). In a microcentrifuge tube, the reaction was initiated by the addition of 15 µl ATChI, and allowed to continue for 5 minutes, after which the reaction was terminated at the appropriate time by placing the reaction tube on ice. The supernatant from the reaction was then transferred to a 96 well microtitre plate and the absorbance determined at 412 nm.

Figure 19: A modified AChE assay procedure for immobilised enzyme (discontinuous assay). For a 5 minute assay, a microcentrifuge tube was prepared for each 15 minute interval read (t0 = initial read; t1 = 30s, t2 = 60s etc). After the addition of ATChI, each reaction was incubated for the designated time, after which the reaction was terminated by the removal of the AChE loaded nanofiber and the reaction tube was placed on ice.

5.2.3 Free and immobilised AChE assay optimisation

AChE assay conditions for both the free enzyme and its immobilised counterpart were optimised by assessing the following parameters: pH (4, 5, 6, 7, 7.5, 8, and 9), temperature (4, 10, 20, 25, 30 and 37 °C), substrate concentration and DTNB concentration. All experiments were conducted in triplicate with the appropriate enzyme and substrate controls. Results were analyzed at 412 nm with the Powerwave$_x$ spectrophotometer using KC Junior software.

5.2.4 Kinetic parameters

Values of K_m and V_{max} for the free and immobilised AChE were determined at 25°C, and at pH 7.5 and pH 7.0, respectively. Different concentrations of ATChI ranging from 0.1 – 0.5 mM were used.

5.2.5 Temperature and pH stability

Thermal stability of the free and immobilised AChE was studied at 25°C and 60°C. The reaction mixture was incubated at 25°C and 60°C in the absence of a substrate. An appropriate amount of free AChE or 1 cm^2 of AChE loaded nanofibres were then withdrawn from the incubated solutions at the same time intervals (15 minutes) and the residual activities were determined.

The pH stability of free and immobilised AChE was determined according to the following procedure. Free and immobilised AChE were stored in 0.1 M potassium acetate buffer of pH 5 and 0.1 M sodium phosphate buffer of pH 9 at 25°C, respectively. The residual activity of the enzymes was determined at 15 minute intervals as described above for thermal stability.

5.2.6 AChE inhibition studies

The inhibitory effects of a CP, carbofuran, and an OP, demeton-S-methyl sulfone on the activities of the free and immobilised AChE were determined by measuring the reduction of the activities with a fixed concentration of ATChI and in the presence of different concentrations of the pesticides. Stock solutions of 1-10 µg/L and 1-5 µg/L carbofuran and demeton-S-methyl sulfone were prepared by dissolving these pesticides in ethanol. The reaction mixture, consisting of 15 µl (free enzyme) or 1 cm^2 immobilised AChE, sodium phosphate buffer and 30 µl DTNB was pre-incubated within defined concentrations of either carbofuran or demeton-S-methyl sulfone ranging from 0.1 µg/L to 10 µg/L for 15 minutes at 25°C before initiating the enzymatic reaction with 15 µl ATChI to give a total volume of 300 µl. The effect of pre-incubation time was also determined. A control experiment was carried out to determine the effect of ethanol in AChE activity. Enzyme inhibition was determined according to the following equation:

$$\text{Inhibition percentage (\%)} \quad = \quad [(Ao - Ai)/\, Ao\,] \times 100$$

Where A*o* represents the AChE activity in the absence of the inhibitor and A*i* the enzyme activity after exposure to the inhibitor

5.2.7 Reusability of Immobilised AChE

Reusability of bound AChE was examined by re-using the same functionalised nanofibres to conduct the activity measurement of the bound AChE at 15 minutes time intervals. After each reaction, the nanofibres on which AChE was immobilised were washed several times with double-distilled water to remove any residual substrate. The washed membranes were then reintroduced into a fresh reaction medium and enzyme activity determined under optimum conditions. The percentage of specific activity retained relative to the specific activity after one use was calculated.

5.2.8 Storage Stability

The activities of the free and immobilised AChE, after storage in 0.1 M sodium phosphate buffer of appropriate pH at 4°C, were determined at 7 day intervals over a period of 49 days.

5.3 RESULTS

5.3.1 pH and temperature optimum

Two of the major parameters that contribute to the limitation of enzyme applications are the operating pH and temperature range. The effect of pH on the relative activity of the free and immobilised AChE was studied; a pH range of 4 – 9 was used (Figure 20), whilst the effect of temperature was studied in the temperature range of 4–37°C (Figure 21).

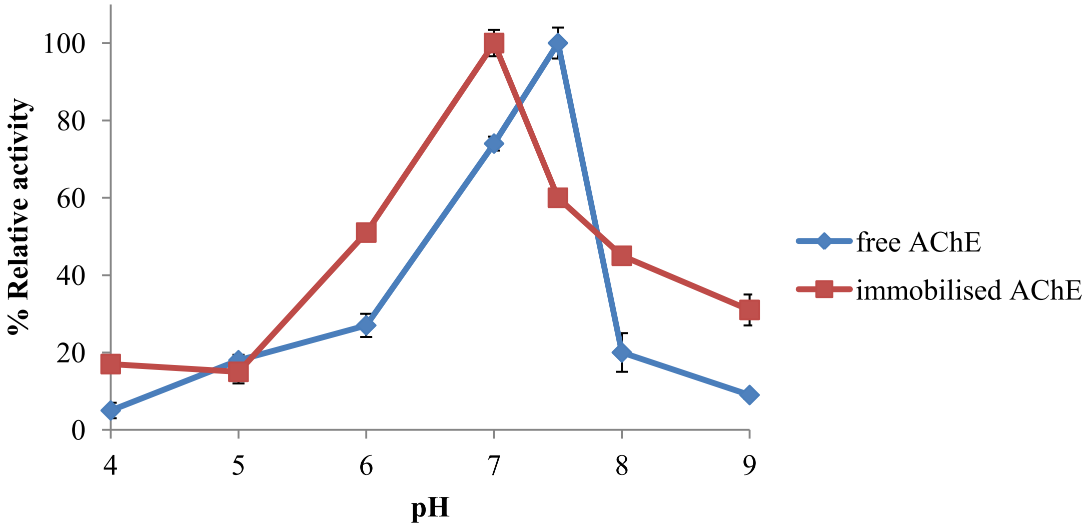


Figure 20: Effect of pH on the activity of free and immobilised AChE. The reactions were carried out at ambient temperature in 0.1 M potassium acetate buffer (pH 4.0 and pH 5.0) and 0.1 M phosphate buffer with pH ranging from 6.0 to 9.0. Data points represent the means ±SD (n=3).

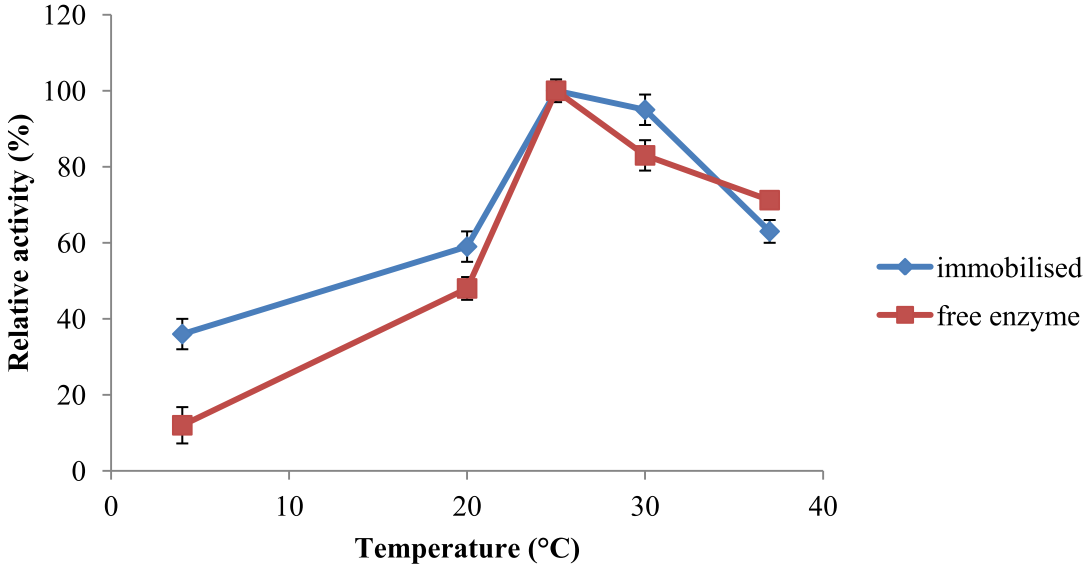


Figure 21: Effect of temperature on the activity of AChE immobilised on nylon-6:chitosan electrospun nanofibres. Data points represent the means ±SD (n=3).

As shown in Figure 20, the optimum pH for free AChE was 7.5, whereas the pH optimum for the immobilised enzyme was 7.0. For both the free and the immobilised AChE an optimum temperature of 25°C was obtained (Figure 21).

5.3.2 pH and temperature stability

The thermal stability was investigated at 25°C and at 60°C (Figure 22). The reaction mixture in the absence of substrate was incubated at either temperature and at 15 minute intervals, and the residual activities of the enzymes was determined.

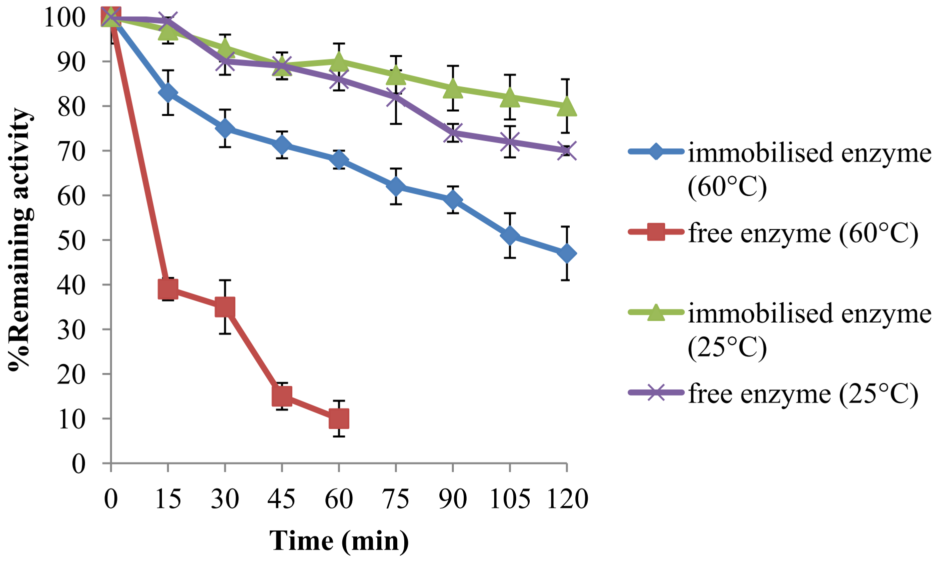


Figure 22: Thermal stability of free and immobilised electric eel AChE after pre-incubation at 25°C and 60°C over time. All other reaction conditions were kept at optimum levels, i.e. pH 7.5 and 7.0 for the free and immobilised AChE, respectively. Data points represent the means ±SD (n=3).

Immobilised AChE showed significantly higher thermal stability compared to the free enzyme (Figure 22).Incubation at 25°C for 2 hours resulted in the free AChE losing ~28% of its initial activity whilst immobilised AChE lost ~17% of its initial activity. On the other hand, after incubation at 60°C for 2 hours, immobilised AChE retained ~49% of its initial activity whilst free AChE lost >90% of its activity after 1 hour incubation.

The pH stability was investigated at pH 5 and pH 9 (Figure 23a and 23b). The reaction mixture in the absence of substrate was incubated in buffer solutions of either pH value and at 15 minute intervals and the residual activities of the enzymes was determined.

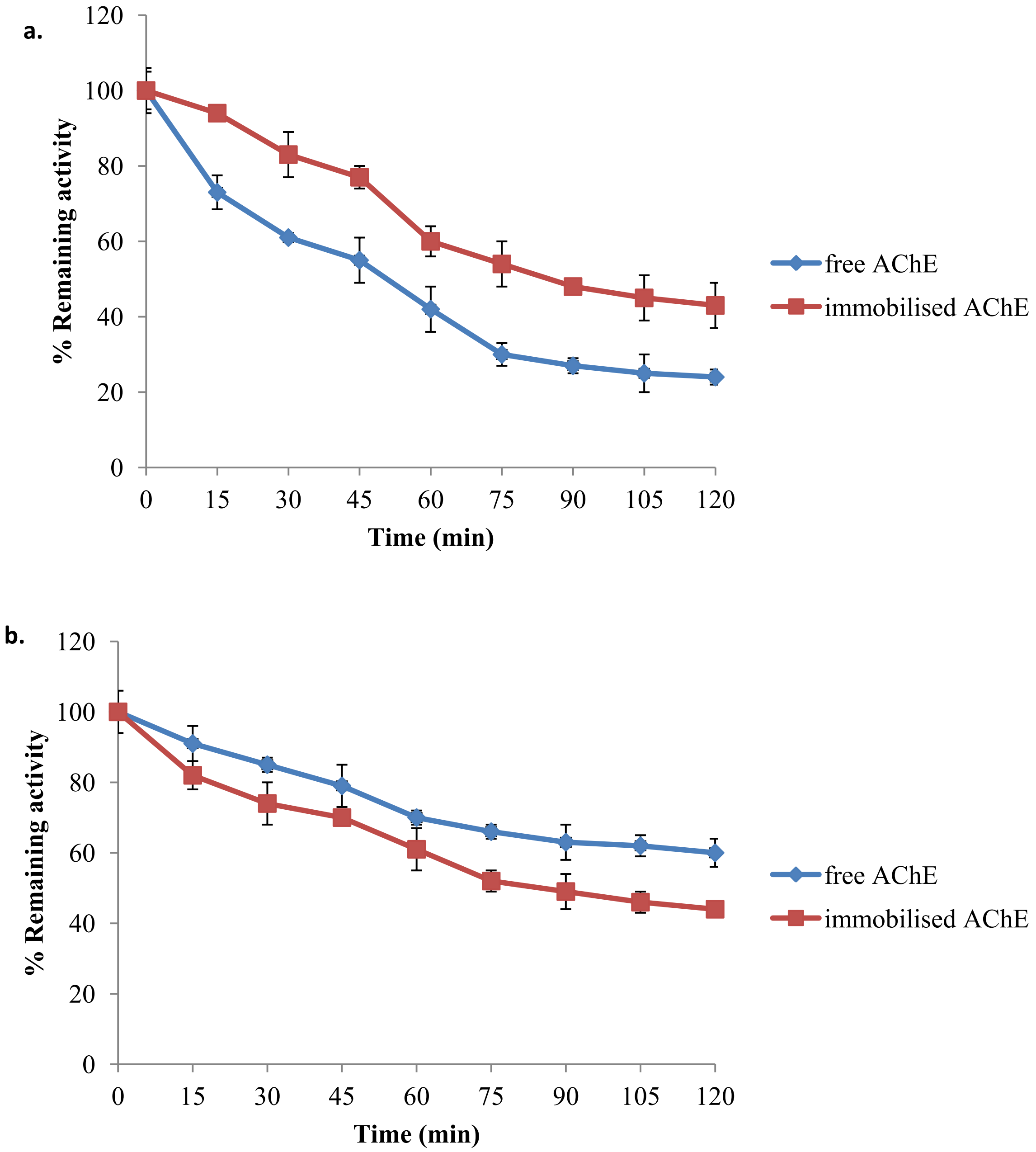


Figure 23: pH stabilities of free and immobilised AChE after pre-incubation in (a) 0.1 M potassium acetate buffer, (pH 5) and (b) 0.1 M sodium phosphate buffer (pH 9) at 25°C over time. Data points represent the means ±SD (n=3).

In 0.1 M potassium phosphate buffer (pH 5.0), both free and immobilised AChE lost a considerable amount of activity -80 % and -60 %, respectively. When incubated in 0.1 M sodium phosphate buffer (pH 9.0) for 2 hours, free AChE had a higher residual activity compared to immobilised AChE. These results showed that immobilised AChE was more stable at acidic pH whilst free AChE was more stable at alkaline pH.

5.3.3 Michaelis-Menten and Lineweaver-Burk plots

The kinetic parameters K_m and V_{max} were determined by varying the concentration of ATChI (from 0 – 0.5 mM) in the reaction mixtures. The Michaelis constant (K_m) and the maximum velocity (V_{max}) values were determined by using the relationship between the initial rate of the reaction (v_o) at various substrate concentrations (Figure 24).

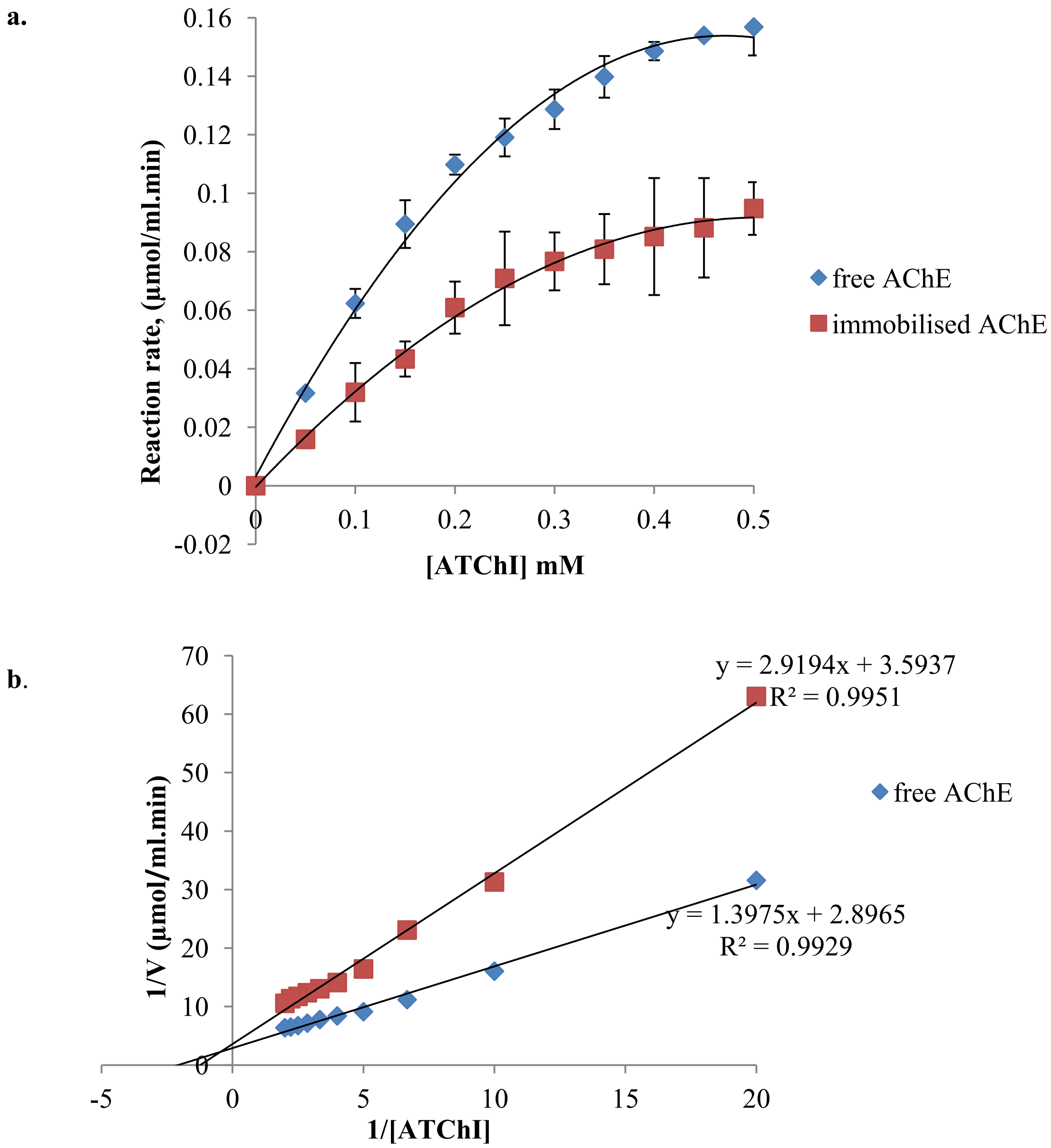


Figure 24: Michaelis Menten (a) and (b) Lineweaver-Burk plots for free and immobilised AChE under optimum conditions.

When initial velocity (v_o) was plotted against substrate concentration hyperbolic curves were obtained, indicating typical Michaelis-Menten kinetic behaviour (Figure 24 a) and the dependence of the reaction rate on substrate concentration. A double reciprocal (Lineweaver-Burk) plot is shown in Figure (24 b).

5.3.4 Reusability and storage stability

The influence of the number of reuses on the activity of immobilised AChE with repeated cycles was determined (Figure 25). Storage stability of free and immobilised AChE at 4 °C was also determined (Figure 26).

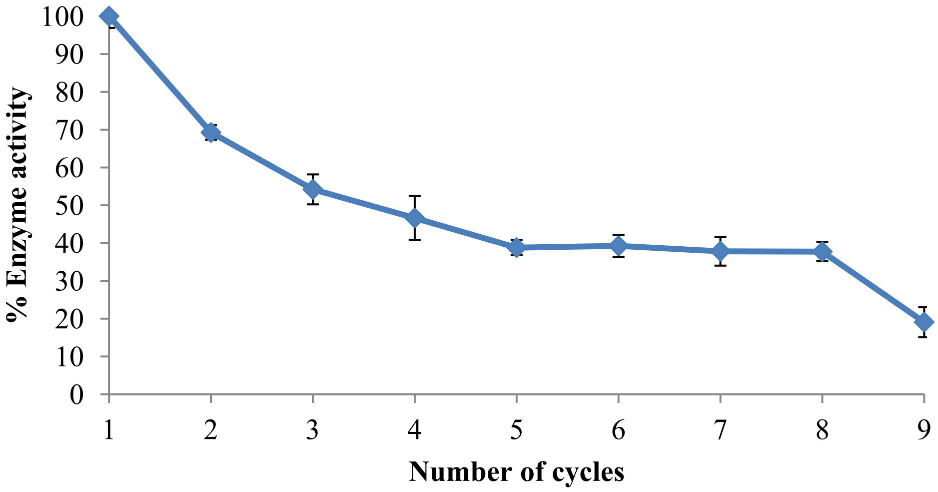


Figure 25: The influence of the number of re-uses on the activity of immobilised AChE with repeated cycles. All cycles were carried out at ambient temperature (± 22 °C). Reusability of bound AChE was examined by conducting activity assays at time intervals of 15 minutes. Data points represent means ± SD (n=3).

It is clear that the specific activity of AChE decreased by roughly 45% after the first 3 cycles (Figure 25). After that, a constant specific activity of just above 40% was retained until after 8 repeated usages when activity dropped below 30 %.

5.3.5 Storage stability

Storage stability of free and immobilised AChE was determined. The enzyme preparations were stored in phosphate buffer solution at 4 °C separately and activities were measured periodically over duration of 49 days (Figure 26).

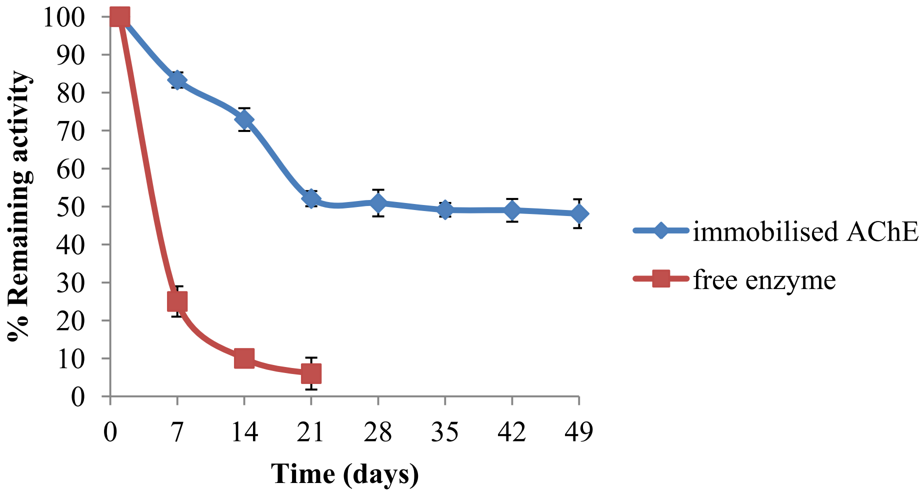


Figure 26: Storage stability of immobilised and free AChE: Both immobilised and free enzymes were stored in 0.1 M phosphate buffer (pH 7.0 and pH 7.5, respectively) at 4°C and the enzyme activity was tested at 7 day intervals. Data points represent means ± SD (n=3).

The activity of the free AChE decreased rapidly in the first 7 days (35%) and had lost most of its activity (>90 %) on day 21 (Figure26). In contrast, the activity of the immobilised AChE for the first 7 days was >80% and after 49 days, the immobilised enzyme still retained 50 % of activity.

5.3.6 Effect of a CP and an OP on AChE activity

In order to demonstrate the usefulness of the immobilised acetylcholinesterase in the analysis of pesticides, its response to different concentrations of a CP (carbofuran) and an OP (demeton-S-methyl sulfone) were determined (see Figures 27 and 28).

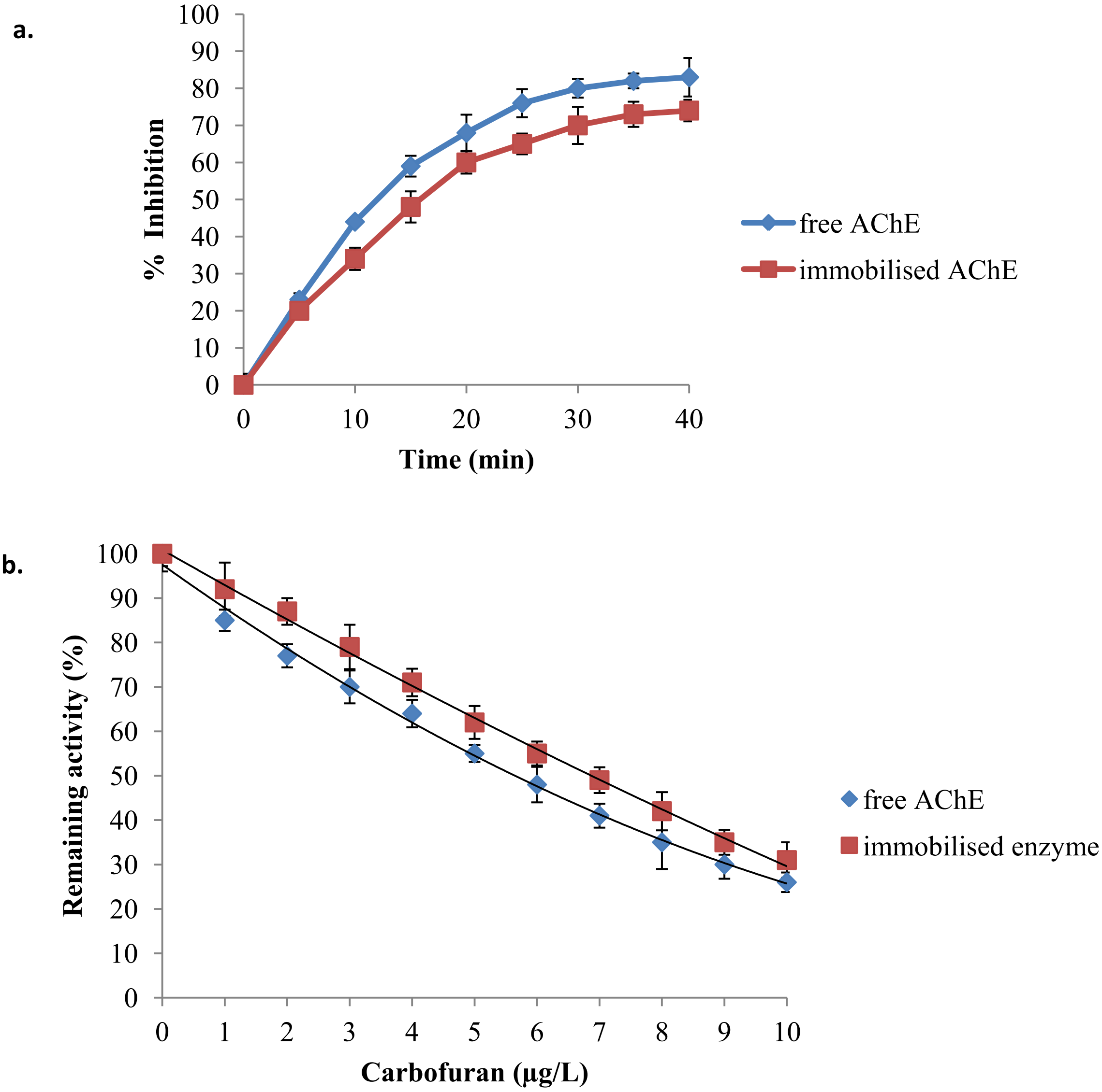


Figure 27: Effect of carbofuran on the activity of AChE (a) effect of incubation time at a fixed concentration (1 µg/L) of carbofuran and (b), the effect of different concentrations of the CP on AChE activity. Data points represent means ± SD (n=3).

When carbofuran concentration was fixed at 1 µg/L, the degree of inhibition for both free and immobilised AChE increased over time and reached a plateau (maximum inhibition of 80 and 70%, respectively) (See Figure 27a). An increase in carbofuran caused a decrease in activity for both the free and immobilised AChE (Figure 27b). Free AChE lost 50% of its initial activity after exposure to 5 µg/L carbofuran whilst immobilised AChE lost 50% of its activity after exposure to 7 µg/L carbofuran.

5.3.7 Effect of Demeton-S-methyl Sulfon

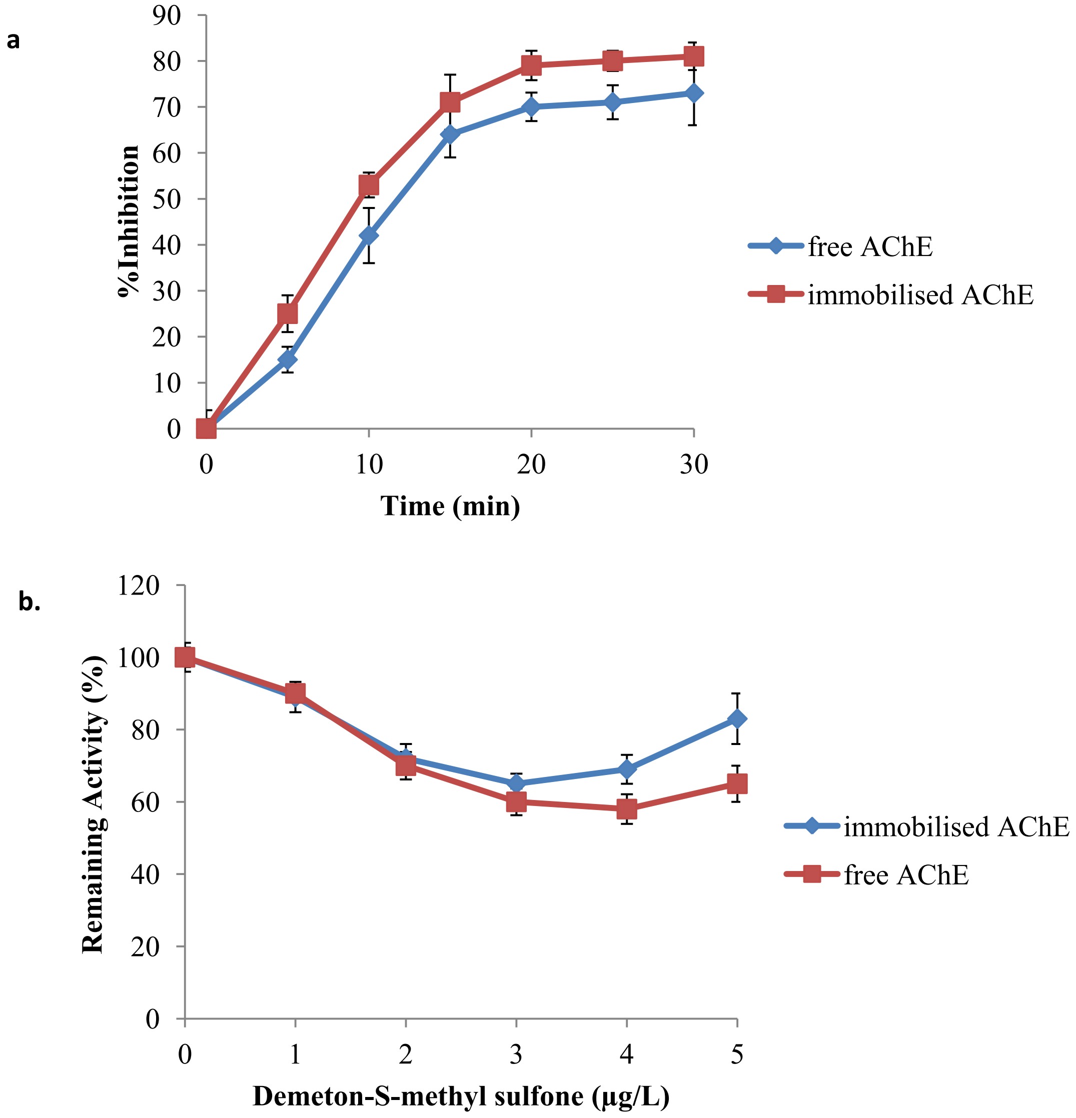


Figure 28: Effect of demeton-S-methyl sulfone on the activity of AChE, (a) effect of incubation time at a fixed concentration (1 µg/L) of OP and (b) the effect of different concentrations of demeton-S-methyl sulfone on AChE activity. Data points represent means ± SD (n=3).

As observed for carbofuran, at a fixed concentration of demeton-S-methyl sulfone, both free or immobilised AChE lost activity gradually over time (Figure 28 a). There was a decrease in AChE activity after the enzyme was incubated in demeton-S-methyl sulfone concentrations ranging from 1 µg/L – 3 µg/L. However, above 3 µg/L demeton-S-methyl sulfon, there was a slight increase in activity for both enzyme preparations (Figure 28 b).

AChE was successfully immobilised on nylon:chitosan electrospun nanofibres via GA cross-linking. Subsequent characterisation of immobilised AChE showed that the enzyme retained activity and was susceptible to pesticide inhibition. The results from the comparative study carried out between free and immobilised AChE are summarised in Table 7.

Table 7: Summary of results from the characterisation of free and immobilised electric eel AChE

	Free AChE		**Immobilised AChE**	
	Literature	**This study**	Literature	**This study**
Optimum pH	7.3 – 8.0	**7.5**		**7.0**
Optimum T°C	30 - 37	**25**	30 or 37	**25**
T°C stability (25°C)	Not done	**>70% activity retained after 2 hours**	Not done	**>80% activity retained after 2 hours**
T°C stability (60°C)	>25% activity retained after 1 hour	>10% activity retained after 1 hour	40% to 50% sctivity retained after 2 hours	~40% activity retained after 2 hours
V_{max} (µmol/cm²/min)	23.1*	**15.8**	8.1*	**9.41**
K_m (mM)	0.90*	**0.491**	0.96*	**0.812**
k_{cat} (s^{-1})	Not done	**526.7**	Not done	**313.7**
Catalytic efficiency (s^{-1} M^{-1})	Not done	**1.075×10^6**	Not done	**3.863×10^5**
Reusability	Not done	**Not done**	10-12 cycles**	**9 cycles**
Susceptible to inhibitors	yes	**yes**	yes	**yes**

***Stoilova *et al.* (2010), ** Moradzadegan *et al.* (2010).**

The successful immobilisation of commercial electric eel acetylcholinesterase is reported in this study. An investigation into the optimum pH and temperature conditions for the immobilised AChE showed a shift from values reported in literature for the free enzyme. Immobilised AChE also showed improved pH, thermal and storage stability and the enzyme could be reused in 9 consecutive cycles. The kinetic parameters of the free and immobilised AChE were determined using acetylthiocholine iodide as substrate (0.5 M) at constant temperature and pH. The V_{max}, K_m, k_{cat} and k_{cat}/K_m values of the immobilised enzyme were calculated from Lineweaver–Burk plots and the results were compared with those obtained for the free AChE.

5.4 DISCUSSION

The development of biosensors based on immobilised enzymes arose from the need to solve several problems such as loss of enzyme activity and stability as well to offer disposable devices which can be easily used in stationary or in flow systems (Amine *et al.,* 2006). Enzymes are complex proteins made up of charged hydrophilic and neutral hydrophobic amino acids. The tertiary structure of an enzyme is dictated by the formation of intra-molecular interactions that include hydrogen bonding, pi-pi stacking, disulfide bridging and electrostatic interactions. These interactions are sensitive to the pH of the immediate environment and pH changes in this environment can result in changes in the pattern of charges on the enzyme and ultimately its tertiary structure. A change in the tertiary structure of the enzyme can alter the active site, resulting in either an increase or most commonly a decrease in enzymatic activity (Voet *et al.,* 2006). pH is therefore one of the important parameters capable of altering enzymatic activities in aqueous solution.

The effect of pH on the activity of the free and immobilised AChE was investigated within pH 4.0 and pH 9.0 ranges at ambient temperature. The relative activity as a function of pH (Figure 20) showed that the pH optimum for free AChE and immobilised AChE on the hybrid nylon/chitosan nanofibres was pH 7.5 and 7.0, respectively. The optimum pH for free AChE in literature ranges from pH 7.5 to pH 9.0 depending on the type of buffer used (Monsan, 1978). In this study, the optimum pH observed for immobilised AChE was similar to that reported for AChE immobilised within Ca-alginate and alginate/ carrageenan beads (Sahin *et al.,* 2005). In addition to the shift in pH optimum (Figure 20), the immobilised AChE bell shaped curve also broadened. This was similar to the observations made in a previous study by Sahin *et al,*(2005). Several studies in which AChE has been immobilised reported different values for the enzyme's pH optimum (Marinov *et al.,* 2009; Stoilova *et al.,* 2010). This can be attributed to the different methods of immobilisation utilised, the type of working buffer and the nature of the solid matrix on which the enzyme was immobilised.

The nature of the immobilisation matrix has been shown to have a significant impact on the modification of pH in the enzyme's microenvironment. Since ionic charges influence an enzyme's activity profile, binding enzymes to polyelectrolyte supports, such as chitosan, often shift the pH optimum (Vaillant *et al.,* 2000). This effect has been demonstrated through the use of charged supports which resulted in a shift in the enzyme's pH optimum (Zhang *et al.,* 2010). Marinov *et al.* (2009) reported a shift in the pH optimum from pH 8 to pH 7.5 after the immobilisation of AChE on positively charged membranes. It is generally observed that positively charged supports such as chitosan displace pH activity curves of the enzymes attached to them towards acidic pH values. The isoelectric point (pI) for chitosan is 5.4. At high H^+ concentrations (low pH) the amino groups of chitosan would be protonated, thus attracting OH^- ions which maintain a higher microenvironmental pH than the bulk solution, thereby stabilising the enzyme (El-Tanash *et al.*, 2011). Contradicting observations were reported by Cetinus and Oztop (2000) who reported that, if the immobilised enzymes are prepared from neutral chitosan supports using either GA or epoxy as the cross-linking agent, the pH optimum does not shift. Nevertheless, the shift in the pH optimum for immobilised AChE towards the more acidic pH relative to free AChE was also reported by Stoilova *et al.* (2010) for AChE immobilised on chitosan modified PAN nanofibrous membranes (PANNF/CHI).

The effect of temperature on the relative activity of the free and immobilised AChE was studied in the temperature range 4 – 40°C (Figure 21). Both the immobilised and soluble AChE had an optimum temperature of 25°C. Different results have been obtained for AChE immobilised on other supports including electrospun nanofibrous material (Moradzadegan *et al.,* 2010; Stoilova *et al.,* 2010) where the immobilised enzyme had a higher temperature optimum (30°C). However, it can be noted that, at 30°C, immobilised AChE had above 90% relative activity, which was considerably higher than that observed for soluble AChE at the same temperature. This indicated that the immobilised AChE had improved thermal stability and resisted denaturation due to a rise in temperature. This improved thermal stability could be advantageous as it may allow the development of 'field ready' devices which can withstand relatively high environmental temperatures.

The pH and temperature stabilities of aqueous and immobilised AChE were investigated further. Although immobilised AChE appeared to be more stable at 25°C relative to the free enzyme, (Figure 22), both the free and the immobilised AChE exhibited considerably high residual activities after incubation at 25°C for 2 hours.The thermal stability of immobilised AChE is higher in comparison to its free counterpart at 60ºC (Figure 22). This may have been due to the conformational limitation of the immobilised enzyme as a result of being covalently bonded to the support. Because immobilisation and cross-linking provided a more rigid external backbone for AChE molecules, the effect of higher temperatures in breaking the interactions that were responsible for the proper globular, catalytic active structure, became less pronounced, thus increasing the thermal stability of the immobilised enzyme (Kilinc *et al.,* 2002; Kilinc *et al.,* 2006).

In order to study the kinetic effect of immobilisation, the rates of the ATChI hydrolysis reaction by the free and immobilised AChE were measured at various substrate concentrations (0.1 – 0.5 mM). Both the free and immobilised AChE showed typical Michaelis-Menten behaviour (Figure 24). The Michaelis constant (K_m) and the maximum reaction rate (V_{max}) calculated from Lineweaver-Burk plots (Figure 24 b) are reported in Table 7 together with the values reported in literature (Stoilova *et al.,* 2010). The comparison between the K_m values for free and immobilised AChE provides some information about the interaction between the enzyme and the support. The V_{max} value for free AChE was 1.68 times higher than the V_{max} determined for immobilised AChE, whilst the K_m for the free enzyme was lower than that for the immobilised enzyme. These observations are widely reported in literature and described as inevitable when enzyme immobilisation is concerned.

The Michaelis constant, (K_m) can be interpreted as the degree of affinity between the enzyme and the substrate when the rate-limiting step in reaction is the dissociation of enzyme-substrate complex to form the product (Voet *et al.,* 2006). An increase in K_m following enzyme immobilisation indicates that the immobilised enzyme has a lower affinity for its substrate relative to the free enzyme (Gabrovska *et al.,* 2008). For immobilised enzymes, K_m is strongly dependant on the diffusional resistance; this is most important where the enzyme has been entrapped within porous matrices, (Singh *et al.,* 1999). In our study however, the enzyme was immobilised on the surface of the nanofibers thus the mass transfer resistance was limited to the resistance at the boundary layer on the enzyme surface, (Singh *et al.,* 1999). Studies by Singh and colleagues (1999) reported a 2-fold increase in the K_m of AChE immobilised on the surface on non-porous silica beads using GA as a cross-linking agent. The values of K_m for immobilised lipases in all cases were higher than that of free lipase. K_m can also be influenced by the charges on the surface of the immobilisation matrix; this is because, during immobilisation, surface functionality affects the orientation and conformational changes in enzymes thus determining substrate accessibility (Wilson *et al.,* 2004).

Loss of substrate affinity (increase in K_m value) may be due to steric hindrance towards the catalytic site due to the solid support, loss of enzyme flexibility required for sufficient substrate binding and substrate or product diffussional resistance to or from the catalytic site (Thavasi *et al.,* 2008). Moreover, the decrease in substrate affinity may be due to solution partitioning effects where the substrate concentration in the vicinity of the enzyme may be significantly different from that in the bulk solution (Tumturk *et al.,* 2007). In this study, V_{max} values for free and immobilised AChE were found as 0.345 and 0.287 μmol/min/ml, respectively and the Michaelis constants, K_m, of the free and immobilised AChE were determined to be 0.482 and 0.812 mM, respectively. The K_m value of immobilised AChE was approximately 1.7-fold higher than that of free enzyme. The difference in K_m values between the free and immobilised enzyme can be attributed to the limited accessibility of ATChI molecules to the active sites of the immobilised AChE as a result of the spatial distribution of the enzyme molecules on the polymer layer as well as the conformational changes of the enzyme caused by the immobilisation. In some studies, the decrease in V_{max} value as a result of immobilisation has been associated with diffusional resistances with regards to the transport of the substrate and products to and from the surface of the membrane (Danisman *et al.,* 2004). Another possible reason for the lower activity yields with the immobilised enzyme may be close packing of the enzyme on the support surface, which could limit the access of substrates needed in the hydrolysis reaction. However, it must also be added that the intrinsic decrease of enzyme activity also contributes to decrease in overall reaction rate (Sousa *et al.,* 2001). Nevertheless, the immobilised enzyme generally showed good performance considering that GA cross-linking leads to significant inactivation of enzymes.

The kinetics of immobilised enzymes has been extensively studied and results have shown that the charge of the carrier is of great importance for enzyme kinetics (Godjevargova and Gabrovska, 2003). According to the hypothesis of Kobayashi and Laidler (1974), immobilisation of an enzyme does not bring any change in pH optimum and K_m values when the charge on the matrix used for immobilisation is zero and diffusional effects are negligible. In the study, the diffusional effects were considered negligible for two reasons (i) AChE was immobilised on the surface on nanofibres and (ii) electrospun nanofibres have reduced mass transfer resistance (Jia *et al.,* 2007). However, chitosan is a cationic polymer thus it was reasonable to expect a change in the pH optimum and K_m values.

The turnover number, (k_{cat}) represents the number of reactions catalysed per unit time by each active site. When hydrolysing acetylthiocholine, the turnover number for free electric eel AChE is 1.4×10^4 sec^{-1}. As with K_m, k_{cat} for immobilised enzymes is usually lower than that of free enzymes. These observations were also reported by Kim, (thesis, 2011) where higher turnover rates were observed for free lipase compared to those observed for immobilised lipase. Turnover rates were also affected by the presence of a spacer arm between the alumina surface and the enzymes. The catalytic efficiency ratio (k_{cat}/K_m) allows direct comparison of the effectiveness of an enzyme towards different substrates (Voet *et al.,* 2006). The catalytic efficiency, k_{cat}/K_m of free AChE was higher than that of immobilised AChE.

The determination of the inhibitory effect of inhibitors includes the following steps: the determination of initial enzymatic activity, the incubation of a biosensor in a solution that contains an inhibitor, and finally the measurement of the residual activity (activity after exposure of the biosensor to the inhibitor). In this study, the effect of exposing free and immobilised AChE to two pesticides, carbofuran (CP) and demeton-S-methyl sulfon (OP) was determined. Ethanol was shown not to have a significant effect on the activity of AChE (data not shown). However, iochemical studies of the actions of ethanol on the activity of soluble electric eel (AChE) were performed to determine ethanol-enzyme-solvent interactions (Shin *et al.,* 1991). The results from this study showed that at low concentrations, ethanol enhanced AChE activity. This observation was consistent with the result from enzyme-kinetic studies that ethanol might noncompetitively activate AChE activity at this lower concentration range. This was attributed to the alteration of hydrophobic interactions in the enzyme which ultimately leads to a favourable conformation in the enzyme's active site. Similar results were observed with sarcoplasmic reticulum membrane bound AChE (Cabezas-Herrera *et al.,* 1992).

Results showed that, at a fixed pesticide concentration, the inhibitory effect of both pesticides increased with increasing incubation time. The inhibition started to level off when the incubation time was longer than 15 minutes (Figures 27 and 28). Demeton-S-methyl sulfone was more potent in its inhibition of both free and immobilised AChE, since maximum inhibition was attained within half the time it took for carbofuran. The observations made in this study were in agreement with literature where it is reported that, unlike carbamylated enzymes which can be spontaneously regenerated to their active form, phosphorylated enzymes are highly stable and in some cases may cause irreversible inhibition (Fukuto, 1990). The degree of inhibition has been shown to be dependent on incubation time at a fixed inhibitor concentration, with inhibition increasing over time and often reaching a plateau (Kok *et al.,* 2002). For the rest of the inhibition studies, a pre-incubation time of 15 minutes was used; this is because longer incubation periods would have increased the analysis time and also exaggerated the effects of low pesticide concentration. Reasonably short incubation times with OPs are also important to avoid a permanent inhibition which occurs when the phoshorylated (inhibited) enzyme is left for a period of time. This phenomenon called "ageing" has been shown to set in after 15 minutes, when the inhibited enzyme is left without reactivation and increases with time (Sun *et al.,* 1979).

Carbofuran is a hydrophobic molecule in the super family of carbamates which inhibit AChE by carbamylation of a serine residue in the active site of the AChE. In this study, increasing carbofuran concentration from 1 µg/L to 10 µg/L resulted in a decrease in the activity of both immobilised and free AChE (Figure 27). For AChE exposed to varying concentrations of demeton-S-methly sulfon mixed results were observed (Figure 28). At OP concentrations below 3 µg/L, both AChE preparations showed a decrease in activity. However, above 3 µg/L, the relative activity of the enzymes incubated in the pesticide increased. OPs inhibited AChE activity via the phosphorylation of the catalytic serine residue often resulting in a highly stable phosphorylated enzyme that is not easily regenerated (Fukuto, 1990). The results obtained at higher concentrations of demeton-S-methyl sulfon were therefore unexpected and no explanation could be found in the literature. Although several studies have shown that the chemical reactivity of the phosphate atom is of paramount importance for AChE inhibition, steric properties have also been shown to play a major role (Fukuto 1990). This may have significant effects in altering the response of immobilised AChE to different OPs. Immobilisation may result in conformational changes that may either reduce the effect of steric hindrance thus allowing larger OP to access the active site or it could enhance steric hindrance, thereby only allowing OPs with smaller side groups.

The degree of enzyme inhibition is affected by the pH of the working solution. Moreover, the instability of inhibitors may also contribute to the deviation of the working pH from optimal conditions, resulting in reduced enzyme activity. Some OPs have been shown to be spontaneously hydrolysed in alkaline media resulting in a significant decrease in the reproducibility of the inhibition of the free and immobilised AChE. Immobilisation of an enzyme can also result in a significant change of the pH which will correspond to the optimal enzyme activity. This can be attributed to changes in the ionic equilibrium constants caused by the difference in dielectric conductivity, as well as the ionic strength of the microenvironment of the enzyme catalytic site (Evtugyn *et al.,* 1999). OPs and CPs are characterised by their low solubility in water and high solubility in organic solvents. Enzyme activity is strongly affected by the presence of organic solvents that interact with the aqueous layer around the enzyme molecule and, usually a decrease in activity is reported (Ebrahimi *et al.,* 2010). Hence, the inhibition measured depends on both the pesticide and the organic solvent.

Reusability of immobilised enzymes is important for their practical application, and this was investigated by measuring the activity of the immobilised enzyme consecutively. The stability of the immobilised enzyme after repeated use was investigated in a batch assay and the effect of repeated use on the activity of the immobilised AChE was shown in Figure 25. After 9 reuse cycles, the relative activity of the immobilised AChE decreased to below 20% of the initial activity. This observation was similar to that reported by Stoilova *et al.* (2010), where the facile immobilisation of AChE was reported on PANNF/CHI. However, studies by Moradzadegan *et al.* (2010) reported that immobilised AChE retained 70% of its initial activity after 10 consecutive reuses. This high retention of activity can be attributed to the immobilisation method used in this method where AChE solution and bovine serum albumin (BSA) were added to the PVA solution and subsequently electrospun, thus reducing the chances for enzyme leaching from the support. In addition, whereas electric eel AChE was used in our study, genetically engineered AChE from *Drosophila melanogaster* was used by Moradzadegan *et al.* (2010). AChE purified *D. melanogaster* has much higher activity if compared to that purified from *E. electricus* and bovine erythrocytes. In spite of this, AChE from *E. electricus* is widely used due to its availability.

For industrial applications, the enhanced storage stability and reusability of immobilised enzymes is of great importance because of its potential to effectively reduce costs. To determine the storage stability, the immobilised and free AChE was stored in phosphate buffer solution at 4°C and the enzyme activity was measured at regular intervals during 49 days (Figure 26). The activity of the free AChE decreased rapidly in the first 7 days (25%) and had lost most of its activity (>90 %) on day 21 (Figure 26). In contrast, the activity of immobilised AChE for the first 7 days was >80% and after 49 days, the enzyme had retained

50 % of its activity. The storage stability of immobilised AChE can be attributed to the covalent immobilisation which could have held the enzyme in a more stable conformation in comparison to soluble enzyme (Hopwood, 1975) and to the biocompatibility of the support due to the presence of chitosan (Cremisini *et al.,* 1995; Jia *et al.,* 2002). Compared to reports in literature, the storage stability of immobilised AChE in this study was significantly low. The rapid loss in activity during both the reusability and storage stability studies can be attributed to several reasons, including leaching from the support matrix and conformational changes due to immobilisation. However, leaching from the support matrix did not contribute significantly to loss of activity since negligible amounts of protein were observed in the wash solutions after each reuse cycle as well as in the storage buffer.

Chapter 6

Chapter 6: GENERAL DISCUSSION AND FUTURE RECOMMENDATIONS

The concept of immobilising proteins and enzymes to insoluble supports has been the subject of considerable research for over 30 years and consequently, many different methodologies and a vast range of applications have been suggested. Aims often include such factors as the (i) reuse or better use of enzymes, especially if they are scarce or expensive, (ii) the generation of products with greater quality as there should be little enzyme in the product requiring inactivation or downstream purification and (iii) the production of biosensors, flow-through analytical devices or the development of continuous manufacturing processes. Biosensors based on the inhibition to AChE have been widely used for the detection of OPs and CPs. During the fabrication of biosensors, immobilisation of enzymes to solid surface is a crucial step (Corcia and Marchetti, 1991; Wissiack *et al.,* 2000; Amine *et al.,* 2006).

The first objective of this study was the preparation of nanofibres via the electrospinning of a nylon-6 and chitosan blend. The electrospinning method of nanofibres is widely documented and was chosen because of its reproducibility and cost efficiency. Even though the electrospinning of polyamides is widely documented, pure chitosan is rarely electrospun due to its tendency for gelation. A solvent system consisting of formic acid and acetic acid (50:50) was prepared to allow the dissolution of both polymers to produce a homogenous solution. The SEM analysis showed that nanofibres were successfully formed from nylon-6 concentrations of 13 and 16%. However, the addition of 1% chitosan to low concentrations of nylon-6 resulted in the formation of beaded nanofibres. The 16% nylon-6: 3% chitosan polymer blends produced smooth nanofibres with minimal beading. The results also showed that the electrospinning of polymer blends produced nanofibres with a suitable surface for the immobilisation of AChE. This electrospinning protocol was a less tedious process relative to that of electrospinning followed by surface functionalisation to overcome the chemical inertness and hydrophobicity presented by nylon-polymer chains.

A key requirement of enzyme immobilisation is attachment without sacrifice of bioactivity. In this study, multi-point AChE immobilisation through GA cross-linking onto electrospun nanofibres was achieved. Of the two protocols reported for the immobilisation of enzymes via GA cross-linking, the GA preactivation of the aminated nylon-6:chitosan nanofibres produced the best results. It has been shown by Monsan (1978) that it is relatively simple to activate aminated supports with GA, resulting in between one and two molecules of GA per primary amino group, thus allowing multipoint attachment of proteins. As low ionic strength buffer was used in this study, the protein immobilisation on GA activated supports proceeded initially via ionic interactions between the protein and the amino groups on the support, followed by the covalent reaction. Khan and Ghani (2012) suggested the addition of Nafion, a tetrafluoroethyle to help reduce AChE deactivation during the cross-linking process. In addition, due to its biocompatibility, Nafion may improve enzyme stability.This study also showed that the concentration of GA used for cross-linking had an effect on both the immobilisation procedure as well as the AChE activity.

The knowledge on thermal stability of an immobilised enzyme is important for the exploration of its potential applications. The effect of the immobilisation procedure on AChE optimum temperature and pH were determined and it was shown that both soluble and immobilised AChE displayed an optimum temperature of 25 °C while the pH optimum shifted from pH 7.5 to pH 7.0. Immobilised AChE also showed thermal stability by retaining over 50% of its activity after incubation at 60 °C for 2 hours. Bearing in mind that immobilised AChE is most commonly used for the construction of biosensors that operate at standard conditions; loss of activity at temperatures above 50°C will be of no consequence to the biosensor functionality. Immobilised AChE, however, was slightly more stable after incubation at 25°C compared to free AChE. Coupled with the observed reusability and storage stability, the use of immobilised AChE for rapidly detecting CPs and OPs may be be more economical than using the free enzyme.

AChE is often used as the biocomponent for biosensors for pesticide detection. In this study, the free and immobilised AChE showed similar susceptibility to carbofuran as well as demeton-S-methyl sulfon. These results showed that the immobilisation process and the new microenvironment introduced by the solid support did not have an effect on the enzyme's sensitivity to its inhibitors. However, despite the clear advantages of utilising AChE as the biocomponent, the major disadvantage is the susceptibility of the enzyme to interfering signals arising from other compounds such as heavy metals present in real samples (Binning and Baird, 2001). The use of immobilised AChE can thus only serve as an early detection methods for determining the presence of pollutants with potential AChE inhibitory activity.

Although there was a change in the V_{max} and K_m values observed for immobilised AChE relative to the free enzyme in this study, the values were still in the same order of magnitude, therefore it was concluded that immobilisation did not alter AChE substrate kinetics greatly. Although the immobilised AChE was characterised in terms of its rate of reaction, it must be noted that when an enzyme is immobilised, its rate of activity is not only determined by substrate concentration, but also on immobilisation effects. These are a result of the following alterations on the enzyme due to immobilisation:

(i) Conformational changes which usually reduce substrate affinity (i.e. increase K_m)
(ii) Microenvironment effects between enzyme and support material
(iii) Non-uniform distribution of substrate and or product between the enzyme and bulk solution

The present study successfully demonstrated that the immobilisation of AChE resulted in an improvement in the enzyme's storage stability (Table 7). Moreover, the enzyme could be reused in nine consecutive recycles with retention of activity. Stoilova *et al.* (2010) and Moradzadegan *et al.* (2010) also reported the immobilisation of AChE on electrospun nanofibres. Considering that different support matrices as well as immobilisation procedures were used, there was no significant difference in the number of reuses that were achieved between the two studies mentioned above and this study. The stability observed may be due to cross linking since cross-linking with agents such as glutaraldehyde to produce stabilized enzymes has been well documented (Mendes *et al.,* 2011), and has been shown to improve the stability of enzymes under wide ranges of pH, ionic strength, and temperature (Richards and Knowles, 1968).

6.1 FUTURE RECOMMENDATIONS

To reduce enzyme loss via leaching from the solid support, enzyme solutions may be mixed with polymer solutions and then electrospun. However, this method is limited to the use of water soluble polymers such as dextran since using organic solvents e.g. formic acid may result in enzyme denaturation. In addition, the immobilised enzyme molecules may be buried within the nanofibres thus substrate accessibility to the active site may be reduced. The problem associated with using water soluble polymers is their potential to dissolve in aqueous environments thus enzyme leaching. To avoid dissolution, vaporised GA may be used to cross-link the solid supports. In addition, it may be useful to add another protein such as BSA to enhance the stability of the immobilised enzyme. In a study carried out by Moradzadegan *et al.* (2010), the presence of BSA resulted in a significant increase in the activity retention of immobilised AChE. This was attributed to the presence of large number of amino acid groups on the surface of BSA which were potential reaction sites for the covalent attachment of the enzyme. When immobilisation is used, the observed loss in enzyme activity and substrate affinity may be due to the various reasons stated above either individually or simultaneously. Regarding the change in enzyme conformation after immobilisation, several techniques such as circular dichroism and electronic spin resonance can be used to investigate whether any conformational changes have occurred.

The strong inhibition of the enzymes can present a serious problem for practical applications by limiting the reuse of biosensors. Since most of the pesticides with inhibitory effects towards AChE are soluble in organic solvents, it would be important to carry out studies to determine the stability of immobilised AChE when exposed to organic solvents. Improved stability of the immobilised AChE under such conditions would be an advantage to its application for pesticide detection. The strong inhibition of the enzymes can present a serious problem for practical applications by limiting the reuse of biosensors. To overcome the problem of irreversible enzyme inhibition in the application of AChE based biosensors, reactivation by oximes was investigated (Gulla *et al.,* 2002). Reactivation of inhibited AChE was investigated using pyridine-2-aldoxime methyliodide (2-PAM) and 4- formylpyridinium bromide dioxime (TMB-4). TMB-4 was found to be a more efficient reactivator with repeated use, retaining more than 60% of initial activity after 11 cycles of reuse, whereas in the case of 2-PAM, the activity retention dropped to less than 50% after only six cycles of reuse. Reactivation studies could also be useful for determining the reusability of immobilised AChE for pesticide detection in real environmental samples.

Enzyme electrodes are becoming popular for the determination of specific substrates in clinical analysis. In the brain, AChE is involved in learning and memory and research has shown the involvement of AChE in Alzheimer's disease where there is a shortage of ACh. AChE is thus an important drug target and its inhibitors have proven useful in the symptomatic treatment of Alzheimer's disease (Chiou *et al.,* 2005). In the brain, AChE is often associated with membranes via a phospholipid tail, thus an understanding of the substrate and inhibition kinetics of immobilised AChE may contribute to the development of drugs for Alzheimer's disease. AChE activity can be used as a biomarker of neurotoxic metal elements, such as lead, cadmium and copper.

Although immobilised AChE showed susceptibility to inhibitors similar to that displayed by free AChE in this study, the limit of detection for the immobilised enzyme was not determined. With regards to determining the presence of OPs and or CPs in real samples, it is important to have a highly sensitive detection method with a very low limit of detection (LOD). LOD is defined as the concentration of the species being measured which gives a minimum detectable difference signal (reduction in activity) that is equal to 2 or 3 standard deviations (S.D.) of the mean response of the blank samples (zero concentration of the inhibitor). According to Van Dyk and Pletschke (2011), where there are large samples, enzymatic detection methods can serve as screening tools which can then be followed up my more traditional analytic methods.

Despite being widely documented, the application of AChE as either a biomarker for pollution or a biocomponent in biosensors is strongly debated due to the non-specificity of the the enzyme. Several studies have shown that in addition to inhibition by OPs and CPs, AChE activity is also subject to interference by heavy metal ions such as lead (Pb) and cadmiuim (Cd). A study by Muzzarelli and colleagues (1976), however, showed that the inhibitory effect of ions was less pronounced with the immobilised enzyme. Two possible reasons were given for this observation (i) protection of the immobilised enzyme by the carrier due to structural changes in the enzyme molecules introduced by the immobilisation procedure and consequently, lower accessibility of inhibiting ions to the active site of the enzyme; and (ii) the chelating effect of chitosan which is known to be a very powerful chelating agent. Immobilising AChE on chitosan modified surfaces may therefore contribute immensely to the application of AChE based biosensors in the environment. The increase in K_m value and decrease in V_{max} value after enzyme immobilisation have also been reported by other investigators (Moradzadegan *et al*., 2010; Stoilova *et al*., 2010).

In general, the objectives of this study were met; AChE was successfully immobilised on nanofibres electrospun from a blend of nylon-6 and chitosan. The method for the preparation of the support matrix was cost effective and most importantly, did not involve the use cytotoxic agents. Although the enzyme lost its activity, it showed remarkable reusability and storage stability providing a promising tool for the rapid detection of AChE inhibitors. The most challenging aspects, however, are: (i) improving the selectivity of AChE for pesticide detection (ii) improving reusability and (iii) reducing loss of enzyme activity during immobilisation.

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APPENDICES

APPENDIX 1: *Bradford standard curve*

Protein standard curves were generated using a modified Bradford protein assay (Bradford, 1976). Bovine serum albumin (BSA) was used as a standard with commercial Bradford reagent (Sigma). Concentrations of BSA ranging from 0.01 to 1.0 mg/ml were prepared by dissolving BSA in distilled water. A standard curve was generated using 20 µl sample and 235 µl Bradford reagent. The protein samples were mixed with Bradford reagent in a 96 well plate and left to stand at room temperature (± 22°C) for 5 minutes after which the absorbance was read at 595 nm.

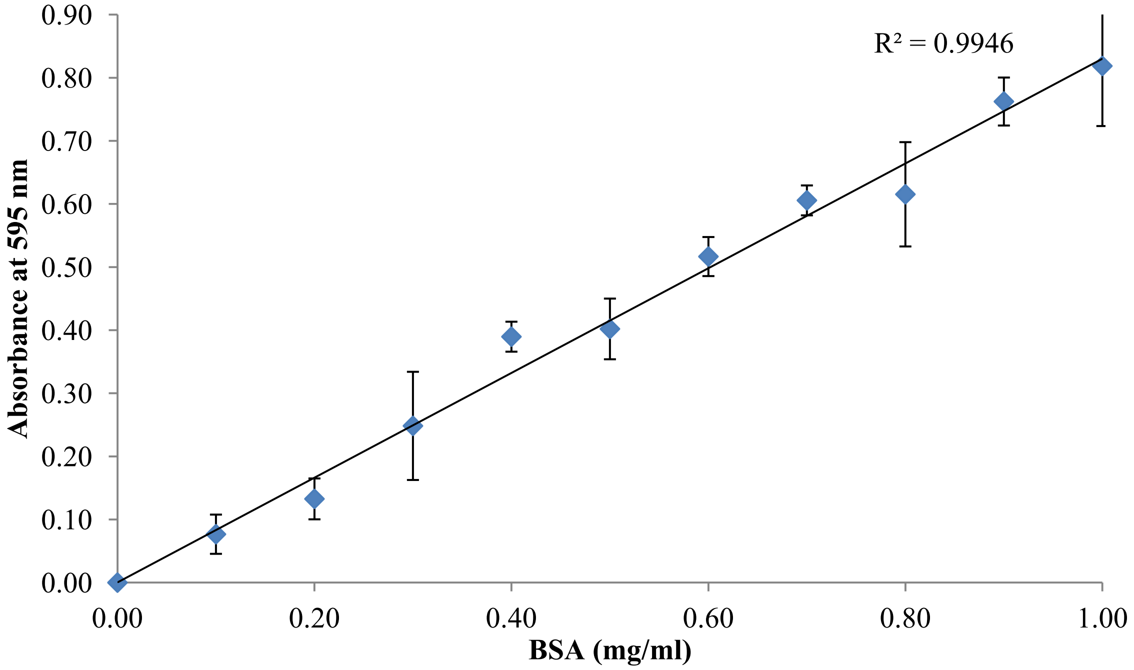

Figure A.1: Bradford standard curve

APPENDIX 2: LIST OF REAGENTS

5’5’-dithio-bis-2-nitrobenzoic acid (DNTB)	Sigma Aldrich (Cat. No. D21820-0)
Acetic acid (glacial)	Merck (CAS-No. 64-19-7)
Acetone for spectroscopy	Merck (CAS-No. 67-64-1)
Acetylcholinesterase (Electric eel)	Sigma Aldrich(Cat.No.C2888-1KU)
Acetylthiocholine iodide (ATChI)	Sigma Aldrich (Cat. No. A5751-59)
Bovine Serum Albumin (BSA)	Sigma Aldrich (Cat. No. A4503)
Bradford reagent	Sigma (Cat. No. B6916)
Carbofuran	Sigma Aldrich (Cat. No.32056)
Chitosan from crab shell (85% DD)	Sigma Aldrich, (Cat. No. 0792)
Demeton-S-methyl sulfon	Sigma Aldrich (Cat. No.45424)
Ethanol (absolute)	Merck (Cas-No 64-17-5)
Formic acid (98-100%)	Merck (CAS-No. 64-18-6)
Glutaraldehyde (25% solution in water)for synthesis	Merck (Cat. No. 8.20603.1000)
Nylon 6	CAS Number 25038-54-4
Potassium acetate	Merck (CAS-No. 127-08-2)
Di-sodium hydrogen orthophosphate anhydrous	Merck, (CAS-No. 10028-24-7)
Sodium di-hydrogen orthophosphate dehydrate	Merck(CAS-No. 7558-80-7)
Methanol	Merck (CAS. No. 67-56-1)