Fabrication and characterization of anti-microbial and biofouling resistant nanofibers with silver nanoparticles and immobilized enzymes for application in water filtration

by
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Declaration

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Summary

Due to a global lack of access to potable water, a problem particularly affecting people in developing countries and the poor, improvement on existing water purification methods are necessary to provide more cost effective, accessible and efficient methods of water purification. In drinking water systems, biofilms are a potential source of contamination, which can affect the biological stability and hygienic safety of water. In industrial water systems, biofilms can cause corrosion, resistance in flow systems and a decrease in efficiency of membranes. Nanotechnology has been identified as a technology to utilize in water purification problem solving. Alternatives to the use of chemical biocides and antibiotics need to be investigated therefore; the focus of this study was the fabrication and characterization of polymer nanofibers containing silver nanoparticles as biocide and anti-biofouling nanofibers with hydrolytic enzymes immobilized on the surface.

The aim of this study was to synthesize and compare poly (vinyl alcohol) (PVA) nanofibers and poly (acrylonitrile) (PAN) nanofibers with silver nanoparticles to determine which type of fiber will be the most appropriate for application in water sanitation. The two types of fibers were to be compared based on morphology, silver nanoparticle content, physical distribution of silver nanoparticles, levels of silver leaching from the fibers in water, which could imply toxicity, and most importantly, anti-microbial efficacy. Back scattering electron images revealed that silver nanoparticles in PVA nanofibers were more evenly dispersed than in PAN nanofibers, but that PAN nanofibers had higher silver nanoparticle content. This was confirmed by energy dispersive X-ray (EDX) analysis. Both PVA and PAN nanofibers containing silver nanoparticles had excellent anti-microbial activity, with PVA nanofibers killing between 91% and 99% of bacteria in a contaminated water sample and PAN nanofibers killed 100%. When investigated by SEM, the biocidal effect of PAN nanofibers containing silver nanoparticles can be observed as morphological changes in the cell walls. Neither PVA nor PAN nanofibers leached silver into water. PVA is a non-toxic and biodegradable synthetic polymer, and PVA-silver nanofibers have excellent anti-microbial activity,

making it applicable in water sanitation in an environmental conscious milieu. PAN nanofibers are more conductive to the formation of silver nanoparticles, have higher silver nanoparticle content, allowing the complete sanitation of pathogenically contaminated water samples. PAN nanofibers also have better longevity and strength in water, making it ideal for water filtration and sanitation in higher throughput systems.

Furthermore, immobilized enzymes are being investigated as possible alternatives to inefficient conventional methods of controlling and removing biofilms from filtration systems. This study demonstrates the covalent immobilization of two industrial proteases and an amylase enzyme onto polymer nanofibers widely used in filtration membranes. Confirmed by FTIR, these nanofibers were successfully activated by amidination, allowing the covalent immobilization of respectively two serine proteases and an α-amylase onto the fibers. When inspected visually, fibers largely retained their original morphology after activation and enzyme immobilization. Immobilized enzymes were, however visible as aggregated particles on the nanofiber surfaces. The large surface area to volume ratio provided by the nanofibers as immobilization surface, allowed sufficient amounts of enzymes to be immobilized onto the fibers so that all enzymes retained above 80% of the specific activity of the free enzymes. For each of the immobilized enzymes, just below 30% of initial activity was retained after 10 repeated cycles of use.

Fibers with immobilized enzymes on their surface did not support the growth of biofilms, as opposed to plain nanofibers, which did support the growth of biofilms. When considering the combined advantages of this effective immobilization process, the robustness of the enzymes used in this study, and their effectiveness against biofilms in their immobilized state, a valuable addition has been made to technology available for the control of biofilm formation on filtration membranes, and could potentially be employed to control biofilm formation in water filtration systems.

A combination of anti-microbial and anti-biofouling nanofibers into a single nanofiltration product may prove to be highly applicable in water sanitation systems.

Opsomming

As gevolg van 'n wêreldwye gebrek aan toegang tot drinkbare water, 'n probleem wat veral mense in ontwikkelende lande en armes raak, is dit van belang dat bestaande metodes van watersuiwering verbeter word om voorsiening te maak vir meer koste-effektiewe, toeganklike en doeltreffende metodes van watersuiwering. In drinkwater stelsels is biofilms 'n potensiële bron van besoedeling, wat die biologiese stabiliteit en die higiëniese veiligheid van water beïnvloed. In industriële waterstelsels kan biofilms tot die verwering van pyplyne lei, weerstand in die stroomstelsels veroorsaak en 'n afname in die doeltreffendheid van membrane veroorsaak. Nanotegnologie is geïdentifiseer as 'n tegnologie wat aangewend kan word in watersuiwerings probleemoplossing. Alternatiewe vir die gebruik van chemiese antimikrobiese middels moet dus ondersoek word. Hierdie studie fokus dus op die vervaardiging en karakterisering van polimeer nanovesels met silwer nanopartikels wat ingesluit is as antimikrobiese middel en anti-biofilm vesels met hidrolitiese ensieme geïmmobiliseer op die oppervlak.

Die doel van hierdie studie was om poli (viniel alkohol) (PVA) nanovesels en poli (akrielonitriel) (PAN) nanovesels te sintetiseer waarby silwer nanopartikels ingesluit is, en te bepaal watter tipe vesel die mees geskikte sal wees vir die gebruik in water sanitasie. Die twee tipes vesels is met mekaar vergelyk gebaseer op morfologie, silwer nanopartikel inhoud, fisiese verspreiding van silwer nanopartikels, vlakke van silwer uitloging vanuit die vesels in water, wat toksisiteit tot gevolg kan hê, en die belangrikste, antimikrobiese effektiwiteit. Terug verstrooiing elektron beelde het aan die lig gebring dat die silwer nanopartikels in PVA nanovesels meer eweredig versprei was as in PAN nanovesels, maar dat PAN nanovesels 'n hoër silwer nanopartikel inhoud gehad het. Dit is bevestig deur “energy dispersive X-ray” (EDX) analise. Beide PVA en PAN nanovesels met silwer nanopartikels het uitstekende antimikrobiese aktiwiteit getoon, met PVA vesels wat tussen 91% en 99% bakterieë in besoedelde water monsters kon doodmaak en PAN vesels wat 100% bakterieë kon uitwis. Wanneer vesels ondersoek is met ’n skandeer elektronmikroskoop (SEM), kon die antimikrobiese effek van PAN vesels met silwer nanopartikels as morfologiese veranderinge in die selwande waargeneem word. Nie PVA

of PAN nanovesels loog silwer uit in water nie. PVA is 'n nie-toksiese en bioafbreekbare sintetiese polimeer, en PVA-silwer nanovesels het uitstekende antimikrobiese aktiwiteit, wat dit van toepassing maak op water sanitasie in ŉ omgewings bewuste milieu. PAN vesels is meer gunstig tot die vorming van silwer nanopartikels, en het 'n hoër silwer nanopartikel inhoud, dus word patogeen besoedelde water volledig gesteriliseer. PAN vesels het ook 'n beter langslewendheid en weerstandige sterkte in water, wat dit ideaal vir water filtrasie en sanitasie in hoër deursettings stelsels maak.

Geïmmobiliseerde ensieme word ook ondersoek as moontlike alternatiewe tot ondoeltreffende konvensionele metodes van beheer en die verwydering van biofilms uit water stelsels. Hierdie studie toon die kovalente immobilisasie van twee industriële proteases en 'n amilase ensiem op polimeer vesels wat gebruik word in filtrasie membrane.

Bevestig deur FTIR, is PAN vesels suksesvol geaktiveer deur amidinasie, sodat die kovalente immobilisasie van onderskeidelik twee serien proteases en 'n α-amilase op die vesels moontlik is. Met visuele ondersoek kan gesien word die vesels behou grootliks hul oorspronklike morfologie na aktivering en ensiem immobilisasie. Geïmmobiliseerde ensieme is egter sigbaar as saamgevoegde deeltjies op die nanovesel oppervlaktes. Die groot oppervlakarea: volume-ratio van die vesels wat dien as immobilisasie oppervlak, laat toe dat voldoende hoeveelhede van ensieme geïmmobiliseer word sodat alle ensieme meer as 80% van die spesifieke aktiwiteit van die vrye ensieme behou. Vir elk van die geïmmobiliseer ensieme, is net minder as 30% van die aanvanklike aktiwiteit behou na 10 siklusse van hergebruik.

Vesels met geïmmobiliseerde ensieme op hul oppervlaktes het nie die groei van biofilms ondersteun nie, in teenstelling met gewone vesels, sonder ensieme, wat die groei van biofilms ondersteun. As die gesamentlike voordele van hierdie doeltreffende immobilisasie proses, die robuustheid van die ensieme en hulle doeltreffendheid teen biofilms in hul geïmmobiliseerde toestand in ag geneem word, is ŉ waardevolle toevoeging gemaak tot tegnologie wat beskikbaar is vir die beheer van biofilm vorming

op filtrasie membrane, en dit kan potensieel gebruik word om biofilm vorming filter stelsels te beheer.

Die kombinasie van anti-mikrobiese en anti-biofilm vesels in ’n enkele nanofiltrasie produk moet nagestreef word, omdat dit hoogs van toepassing sal wees in water sterilisasie stelsels.

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Contents

CHAPTER 1

Introduction

Globally, water scarcity is one of the foremost health and environmental challenges faced. Climate change and drastically increasing population is threatening the availability of potable water, with detrimental environmental, social and economic impacts (Mara 2003; Montgomery and Elimelech 2007; Johnson et al. 2007; Moore et al. 2003). According to the World health organization (2004), 1 billion people lack access to safe drinking water and 2.6 billion lack adequate sanitation. Improved water supply and sanitation can drastically reduce water-borne illness related morbidities. In 2000, the United Nations adopted the "*Millennium Development Goals 2015*" part of which has set the goal of reducing the number of people without sustainable access to safe drinking water by half. Current methods of water treatment are not meeting increasing water demands (Weber 2002), thus, research into new water treatment technologies are of utmost importance. Water sanitation, reclamation and decontamination methods that are lower in cost and are more efficient than current water treatment options need to be developed and expanded to a level where it can alleviate water stress, especially in 3rd world countries, where access to potable water is often a luxury (Theron et al., 2008).

The control of pathogenic contamination and biofouling are major problems in water sanitation systems. In drinking water systems, biofilms are a potential source of contamination (Momba et al. 2000), which can affect the biological stability, hygienic safety (Emtiazi et al.,2004) and the general quality of water (Khiari and Watson, 2007); Ludwig et al. (2007)). Biofilms are structures of accumulated bacterial biomass, consisting of bacterial cells, proteins, nucleic acids, polysaccharides (Characklis, 1990)) and humic substances embedded in extra cellular polymeric substances (EPS) (Wagner et al., 2009). Biofilms often form on surfaces in an aqueous environment (Cloete et al., 1992), making water filtration membranes, water distribution systems and industrial water systems particularly vulnerable to biofouling.

Current methods of water decontamination and biofilm control are not without challenges and drawbacks. Chemical oxidants used to disinfect water such as chlorine, chloramines

and ozone can form complexes with the constituents of natural water, producing harmful disinfection by products (DBP's), many of which are carcinogens (Krasner et al., 2006). Furthermore, the eradication of anti-microbial resistant pathogens and biofilm forming bacteria in water treatment and supply systems require high dosages of disinfectants, leading to higher DBP formation and an increased cost.

Nanotechnology is the discipline of manipulating matter at the nanoscale (1-100 nm), yielding nanoparticles or materials that often possess novel biological, physical or chemical properties (Theron et al., 2008), and has been identified as a technology that can be useful in resolving current problems in water treatment (Bottero et al., 2006; Savage and Diallo, 2005).

Various forms of nanotechnology such as nanobiocides, nanofibers and nanofiltration are employed in water treatment. Examples of applications include chemical decontamination, desalination, filtration and sanitation. Nanofibers have excellent filtration properties, and due to the variety of polymers that can be used to fabricate nanofibers, and the versatility of being able to add functional molecules and chemical groups to the nanofibers, make nanofibers applicable to sanitation and purification of water.

Nanofibers are produced from a range of electrospinnable polymers by the process of needle-electrospinning. A simple and very effective variation of conventional needle-based electrospinning, known as bubble electrospinning allows much more rapid production of nanofibers for research purposes.

Anti-microbial nanofibers can be synthesized by incorporating nanobiocides such as silver nanoparticles into the nanofibers. The synthesis of nanofibers containing metal nanoparticles is well researched greatly because of the advantages involved with combining the functional properties of metal nanoparticles (Niu and Crooks, 2003) with the widely applicable properties of nanofibers.

Silver nanoparticles are considered as an alternative to conventional antimicrobial agents, and silver as a nanobiocide is under investigation in this work. Silver is considered the most toxic element to microorganisms, and the antimicrobial activity of silver ions is a well researched area.

Enzymes are highly selective biocatalysts and can be used to prevent and control biofilm formation without the production of toxic by-products. Some drawbacks concerning the use of enzymes 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)).

In the present study, these potential drawbacks were overcome by using industrial enzymes produced on a large scale which are tolerant towards working environments over large pH and temperature ranges (Table 1). Furthermore, these enzymes were covalently immobilized onto a nanofibrous support, stabilizing them and enabling re-use of the enzymes without the need for recovery from the medium.

In this work, pathogenic contamination and biofouling in water filtration was addressed. Firstly, the sanitation of water with nanotechnology was addressed by fabricating and characterizing anti-microbial polymer nanofibers with a nanobiocide. This is presented as a research article in chapter 3. Secondly, the problem of biofouling on filtration membranes was addressed by fabricating and testing polymer nanofibers with immobilized hydrolytic enzymes on the surface, also presented as a research article in chapter 4.

To address water sanitation, two types of polymer nanofibers, namely poly(vinyl alcohol) (PVA) and poly(acrylonitrile) (PAN) were to be synthesized by bubble-electrospinning, incorporating $AgNO_3$ into the polymer solutions, with subsequent in situ reduction of silver ions in $AgNO_3$ to silver nanoparticles by exposing the nanofibers to ultra violet (UV) irradiation. The aim of this study was to synthesize and compare PVA nanofibers with $AgNO_3$ to PAN nanofibers with $AgNO_3$ to determine which type of fiber will be the

most appropriate for application in water sanitation. The two types of fibers were to be compared based on morphology, silver nanoparticle content, physical distribution of silver nanoparticles, levels of silver leaching from the fibers in water, which could imply toxicity, and most importantly, anti-microbial efficacy.

Furthermore, to address the problem of biofouling, the objective was to exploit the protein and polysaccharide hydrolyzing actions of two industrial proteases and an alpha-amylase for breaking down the EPS in a biofilm, preventing biofilm formation. The hydrolytic enzymes were to be immobilized onto the surface of PAN nanofibers in an attempt to render the nanofibers resistant to biofilm formation when applied in water filtration technology. Furthermore, the effects of the immobilization process on the activity as well as the enzyme kinetics such as the maximum velocity of the enzyme reaction (V_{max}) and the substrate affinities (K_m) of these enzymes were to be investigated.

Various polymers, with or without chemical modification have been used for the immobilization of enzymes. Many of these polymers are, however heat sensitive and have poor chemical, physical and microbiological resistance, for example acrylic and vinylic supports such as polyacrylamide and PVA (Di San Filippo et al., 1990). PAN is an organic polymer with good chemical and physical stability, and can be electrospun into nanofibers with a diameter range of between 150 and 300 nm. PAN nanofibers have excellent mechanical properties without the need for any reinforcing treatment after fabrication and are used widely in the manufacture of water and air filters. PAN requires chemical activation of highly polar CN groups on the surface to make protein immobilization possible. Imidoesterification is the process of changing amide groups to imidoester groups on the surface of PAN in the presence of anhydrous hydrogen chloride. This renders the polymer modifiable (Handa et al., 1982; Handa et al.,1983; Hunter and Ludwig, 1972).

The importance and potential applications of anti-microbial and anti-biofouling technologies such as these are discussed in chapter 2.

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CHAPTER 2

Literature Review

2.1 Introduction

Water scarcity is one of the foremost health and environmental challenges faced globally. Due to climate change and drastically increasing population, the availability of potable water is both limited and threatened. This has a detrimental environmental, social and economic impact (Johnson et al., 2008; Mara, 2003; Montgomery and Elimelech, 2007; Moore et al., 2003). According to the World health organization (2004), 1 billion people lack access to safe drinking water and 2.6 billion lack adequate sanitation. Annually, 1.8 million people die as a result of water borne disease, over 90% of which are children. An improved water supply can reduce these morbidities by up to 25% and improved sanitation, up to 32%. In 2000, the United Nations adopted the "*Millennium Development Goals 2015*" part of which has set the goal of reducing the number of people without sustainable access to safe drinking water by half. Concerns regarding current methods of water treatment not meeting increasing water demands (Weber, 2002), means research into new water treatment technologies is thus of utmost importance. Water sanitation, reclamation and decontamination methods that are lower in cost and are more efficient than current water treatment options need to be developed and expanded to a level where it can alleviate water stress, especially in 3rd world countries, where access to potable water is often a luxury (Theron et al., 2008).

Evidence of water purification exists from ancient times. Sanskrit writings described methods of water purification by sand and charcoal filters. The first example of ion-exchange is recorded in the Holy Bible. Louis Pasteur studied diseases caused by micro-organisms and John Snow linked the spread of cholera in London to water. Perhaps the most important early advance made in water treatment was the introduction of chlorine as a disinfectant in municipal water supply in Belgium in 1902 (Pradeep and Anshup, 2009). Important milestones in the history of water treatment are listed in table 1.

Table 1. Milestones in water treatment history. Modified from Pradeep and Anshup (2009)

Year	Milestone
1804	World's first municipal city water treatment plant (Scotland, sand filtering technology)
1810	Discovery of chlorine as a disinfectant (H. Davy)
1852	Formulation of the metropolis water act (London)
1879	Formulation of the germ theory (L. Pasteur)
1902	Use of chlorine (calcium hypochlorite) as a disinfectant in water (Belgium)
1906	Use of ozone as disinfectant (France)
1908	Use of chlorine (calcium hypochlorite) as a disinfectant in municipal water supply (New Jersey, USA)
1914	Federal regulation of drinking water supply
1916	Use of UV in municipal water supply
1935	Discovery of synthetic ion exchange resin (B.A. Adams, E.L. Holmes)
1965	World's first reverse osmosis plant launched
1974	First reports on role of carcinogenic by-products of water disinfection with chlorine
	Formulation of the Safe Drinking Water Act (United States Environmental Protection Agency)
1975	Development of carbon block for drinking water purification
1998	Drinking water directive applied in EU
2000	Adoption of the Millennium Declaration during the UN millennium summit
2007	Launch of noble metal nanoparticle-based domestic water purifier. (T. Pradeep, A.S. Nair, Eureka Forbes Limited)

Although effective, current methods of water decontamination and treatment are not without challenges. Chemical oxidants used to disinfect water such as chlorine, chloramines and ozone can form complexes with the constituents of natural water, producing harmful disinfection by products (DBP's), many of which are carcinogens (Krasner, 2006). Furthermore, anti-microbial resistant pathogens in water and biofilm forming bacteria in water treatment and supply systems, serve as a source of microbial and chemical contamination. Eradication of such pathogens requires high dosages of disinfectants, leading to higher DBP formation and an increased cost.

Another point of concern is water loss and deterioration of water quality associated with aging water distribution networks. Furthermore, increasing costs of transporting water, alternative water sources and waste water re-use in water scarce areas also need to be addressed. Decentralized point of use water treatment provides a solution for most of these problems. Anti-microbial nanomaterials are suitable for application in highly effective, small scale point of use water treatment systems.

This review discusses the problem of biofouling in water treatment systems and how nanotechnology such as noble metal nanoparticles, hydrolytic enzymes and electrospun nanofibers with modified surface properties can be applied in water treatment and disinfection.

2.2 Biofilms in water treatment and distribution systems

Biofilms are defined as three dimensional structures of biomass, consisting of bacterial cells, proteins, nucleic acids, polysaccharides and humic substances embedded in amphiphilic extra cellular polymeric substances (EPS) (Characklis, 1990; Wagner et al., 2009). Biofilms often form on surfaces in an aqueous environment, making water filtration membranes, water distribution systems and industrial water systems particularly vulnerable to biofouling (Cloete et al., 1992). Biofilms pose resistance against anti-microbial agents and 65-80% of all pathogenic infections are estimated to be biofilm-related (Costerton et al., 1999; Hall Stoodley et al., 2004; Parsek and Singh, 2003)

In drinking water systems, biofilms are a potential source of contamination (Momba et al., 2000), which can affect the biological stability and hygienic safety of water (Emtiazi et al., 2004). Additionally, metabolites generated by organisms in the biofilm may add flavours and odours to the water, further reducing the quality (Khiari and Watson 2007; Ludwig et al., 2007). In industrial systems, biofilms can cause corrosion, resistance in flow velocity and a decrease in efficiency of filtration membranes due to physical blockage.

Two major influences on the formation of a biofilm is the roughness and composition of the surface to which the biofilm is attaching, as well as the hydrodynamic shear stress that is present. Rough surfaces of filtration membranes combined with the shear stress present within a water filtration system, may lead to the formation of biofilms with higher bacterial count and higher EPS production (Percival et al., 1999).

Biofilm formation occurs in a sequence of events (Nagant et al., 2010). Firstly, planktonic cells adhere to the substrate through electrostatic and hydrophobic interactions, during which cells also stick to one another and form aggregates on the substrate (Kumar and Prasad, 2006). When planktonic cells become stably adhered to the substrate, micro colonies of bacterial cells form. Cells multiply and chemical signalling takes place between cells, initiating the production of EPS.

Cells embedded within the EPS demonstrate group behaviour, mediated by communication via quorum sensing and are more resistant to anti-microbial agents than planktonic cells (Zhang and Dong, 2004). The EPS offers resistance to embedded cells by reacting with anti-microbial agents, inactivating them (de Beer et al., 1994), and by physically resisting access of anti-microbial agents into the biofilm (Cloete 2003a; Davies 2003; Donlan and Costerton, 2002; Gilbert et al., 2002; Lewis 2001; Mah and O'Toole, 2001). The charge of both the EPS and the anti-microbial agent, size exclusion (Cloete, 2003b), and the viscosity of the EPS (Kostenko et al., 2007) also influences anti-microbial resistance.

The EPS acts as a barrier against the penetration if anti-microbials (Anderl et al., 2000; Lewis, 2001), also, it can adsorb the anti-microbial into the EPS (Kumon et al., 1994), neutralize and inactivate the anti-microbial (Bagge et al., 2004; Sanderson et al 1997; Xu et al., 1996) and degrade the anti-microbial with enzymes produced by the EPS. Furthermore, the EPS contains extremely resistant cells known as persisters, which neither grow nor die in the presence of anti-microbial agents, and which remain viable, even after treatment with high dosages of anti-microbials (Keren et al., 2004). Carbohydrates and proteins are the major components of the EPS (Wingender et al.,

1999) with the addition of humic substances, lipids, nucleic acids and inorganic complexes (D'Azbac et al., 2010; Dignac et al., 1998; Frolund et al., 1996; Nielsen et al., 1992). Carbohydrates occur either as exopolysaccharides, which are attached to the bacterial cell, or occur freely in the EPS, and can have a linear, branched or cyclic structure. Polysaccharides are mostly in the β configuration with 1,3 or 1,4 linkages in the polymer backbone (Allison et al., 2000). Polysaccharides also form complexes with proteins and lipids (Sutherland, 2001). Proteins are responsible for the hydrophobic properties of the EPS (Allison et al., 2000) and are obtained from both living and dead cells. Proteins assist in the attachment of the biofilm to hydrophobic and negatively charged surfaces (Characklis, 1990). The most common proteins in EPS are lecitins, which adhere the pathogenic cell to its host and other cells, and polysaccharases which are responsible for the degradation of EPS and components in the surrounding environment, which supplies the biofilm with nutrients.

Current methods of biofilm disinfection in water distribution systems include chlorination, chloramination and UV irradiation (Momba et al., 2008). Micro-organisms do, however develop resistance against these treatments and become difficult to eradicate (Kieriek-Pearson and Karatan, 2005). Proposed mechanisms of anti-microbial resistance in biofilms include (i) limited diffusion of the anti-microbial into the biofilm matrix; (ii) interaction of the anti-microbial agent with the biofilm matrix; (iii) enzyme mediated resistance; (iv) level of metabolic activity within the biofilm; (v) genetic adaptation; (vi) efflux pumps and (vii) outer membrane structure (Cloete, 2003a). Furthermore, the use of oxidative disinfectants may cause the release of organic substances, encouraging biofilm formation, and when used in high concentrations may lead to the formation of harmful DBP's, which introduce toxins into the water and can damage surfaces (Momba et al., 2000). Due to these limitations, there is a need to consider alternative methods of controlling biofouling in water distribution and treatment systems.

2.3 Nanotechnology in water treatment

Nanotechnology is currently at the forefront of the latest research in water treatment and has been identified as a useful tool in resolving current problems in water treatment (Bottero et al., 2006; Cloete et al., 2010; Savage and Diallo, 2005). Nanotechnology comprises the fabrication and functionality of materials with dimensions within the nano-scale (1-100nm). Because of the larger surface area to volume ratio and smaller size, chemical and physical properties of the material are altered, giving it novel qualities. There has hence been an increase in publications in the field of nanotechnology with applications in water treatment (Fig.1).

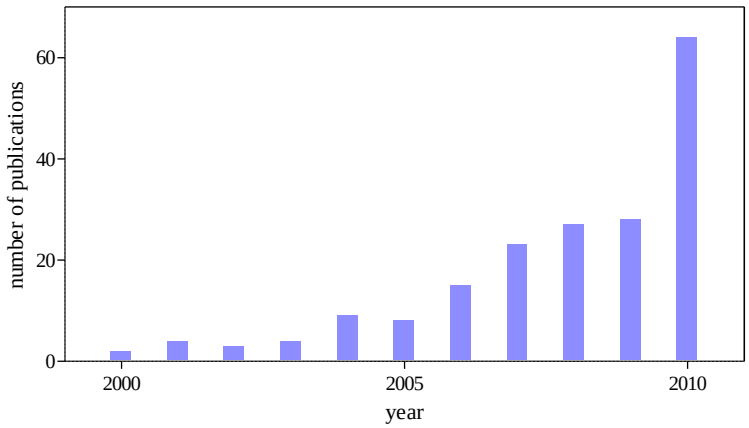


Figure 1. The number of nanotechnology-related publications for each year (2000-2010) in the journal *Water Research.*

Various forms of nanotechnology such as nanobiocides, nanofibers and nanofiltration are currently being developed and in some cases used in water treatment for chemical decontamination, desalination, filtration and sanitation. Nanofibers have enormous potential for application in water filtration and sanitation (Botes and Cloete, 2010). Due to the small pores in a non woven mat of electrospun nanofibers, nanofibrous mats have excellent filtration properties, and due to the variety of polymers that can be used to fabricate nanofibers, and the versatility of being able to add functional molecules and chemical groups to the nanofibers, make nanofibers applicable to sanitation and purification of water (Coete et al., 2010).

2.4 Nanofibers in water purification

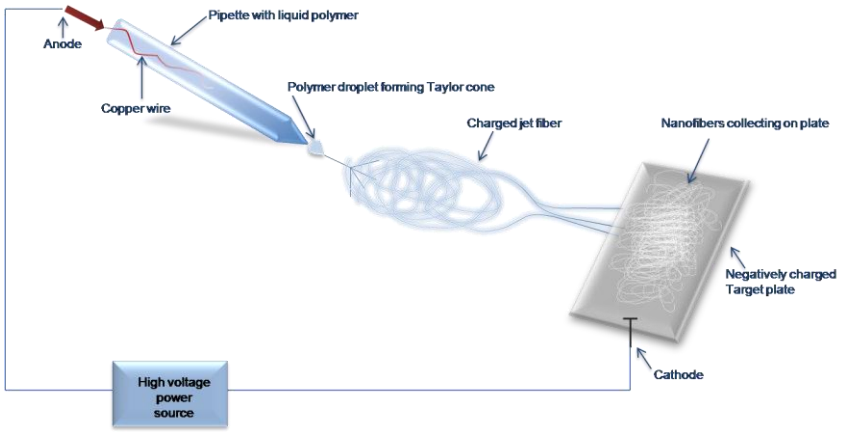
Nanofibers are solid fibers with diameters within the nanoscale with a large surface area to volume ratio, and when assembled in a non-woven mat, have a small pore size. Due to the small diameter, polymer nanofibers often possess far superior qualities to that of the polymer in any other form. When compared to micro fibers, nanofibers can have a surface area of up to 10^3 times larger; they are more flexible and have superior tensile strength. Furthermore, surface activity is determined by the polymer and additional non-soluble particles that are added (Frenot and Cheronakis, 2003). These qualities make nanofibers extremely versatile and more effective than conventional polymer membranes used in liquid filtration (Theron et al., 2008; Yoon et al., 2006). Due to the small fiber diameter and small pore sizes, a non woven nanofibrous mat has high filtration efficiency, easily trapping particles smaller than 0.5 μm without providing much flow resistance.

Nanofibers can be produced by various processes including drawing which produces singular nanofibers from viscoelastic materials only (Ondarchuhu and Joachim, 1998); template synthesis where a nanoporous membrane is used as a template to form nanofibers (Martin, 1994); phase separation which produces a nanoporous foam (Ma and Zhang, 1999); self assembly in which pre-existing components arrange themselves into fibers, which are all time consuming processes (Grzybowski and Whitesides , 2002), and finally electrospinning (Doshi and Reneker, 1995).

Electrospinning can produce nanofibers from a range of electrospinnable polymers. In the process of needle-electrospinning, a high voltage electric field is generated between a charged source of polymer solution and a grounded metal collector plate. An electrostatically driven jet of polymer solution gives rise to nanofibers, which are collected on the plate (Fig. 2(a)).

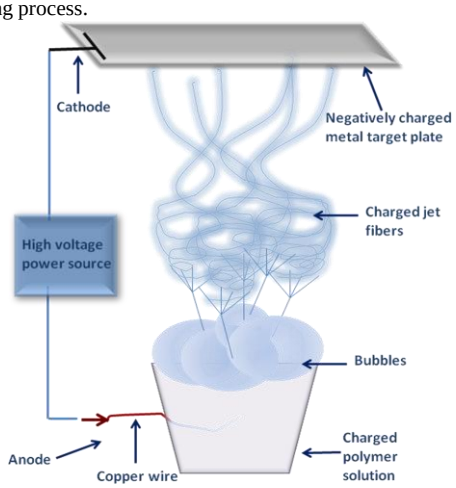
A simple variation of conventional needle-based electrospinning allows much more rapid production of nanofibers for research purposes. The process, known as bubble

electrospinning, involves the formation of multiple electrostatically driven jets of polymer solution from a charged bubble of polymer solution (Yang et al. (2009)). The electric field is of a much higher voltage than used in conventional needle spinning, and fibers generated are collected on a negatively charged metallic collector plate positioned above the bubble (Fig. 2(b)).



(a)

Figure.2 (a) Needle electrospinning process.



(b)

Figure.2 (b) Bubble-electrospinning process.

Nanofibers from a range of polymers have been widely used, specifically in water filtration and treatment, and are often modified to have antimicrobial properties (Chun et al., 2010; Lala et al., 2007; Yoon et al., 2006). The inclusion of nanobiocides into nanofibers is a common method of producing anti-microbial nanofibers (Botes and Cloete, 2010).

Table 2. Electrospun nanofibers from different polymers for application in liquid filtration.

Polymer	Solvent	Concentration	Reference
Poly (acrylonitrile)	Dimethylformamide	8 wt%	Lala et al., 2007
		4-12 wt%	Yoon et al., 2006
Cellulose acetate	Acetone: TFE: DMF	16 wt%	Lala et al., 2007
Poly (vinyl chloride)	THF	10 wt%	Lala et al., 2007
Poly (vinyl alcohol)	Distilled water	8-16 wt%	Chun et al., 2010

2.5 Nanobiocides

Nanobiocides are anti-microbial nanoparticles and generally fall into one of three categories, namely metals and metal oxides, of which silver and gold nanoparticles, copper, zinc and titanium oxides are most widely used; fabricated nanoparticles such as fullerines and naturally occurring anti-microbial materials such as chitosan. Currently, the most commoly used nanobiocides are noble metal nanoparticles, and in particular, silver nanoparticles (Botes and Cloete, 2010; Maynard and Michaelson et al., 2006). The chemistry of noble metal particles started with the synthesis of colloidal gold by Faraday (1857), which was followed by many studies into the synthesis of colloidal gold (Pradeep and Anshup, 2009). The potential of nano-scaled noble metal nanoparticles was highlighted by pioneering work by Henglein (1989) in which the change in reactivity properties of metals in the nanoscale were described by the size-quantization effect. It was stated that the number of atoms in the crystal lattice of a metal has an effect on the chemical properties of that metal because of a change in electrochemical properties, for example, bulk silver has an electrochemical potential of 0.799V, but with a reduction in the number of atoms, the electrochemical potential decreases, with a single atom of Ag measured to have an electrochemical potential of -1.8V (Gu et al., 2004). This change in

electrochemical potential lends novel properties to the metal when fewer atoms are present, such as in nanoparticles.

2.6 Silver as nanobiocide

The domestic use of silver to preserve perishable items and to disinfect water dates back to the ancient civilizations of Greece, Rome, Phoenicia and Macedonia (Lansdown 2004). Alexander the Great (335 BC) stored his water in silver vessels and boiled it prior to use (Russell 1994). The first research on the anti-microbial properties of silver was carried out in 1869 by Ravelin and in 1893 by Nageli, who showed that extremely low concentrations of silver salt were toxic to *Spirogyra* and *Aspergillus niger* spores (Lansdown 2006). A silver colloid was first prepared in the late 19th century (Lea, 1889) by reduction of silver nitrate. In the early 20th century, a porous metallic mesh of silver, known as *Katadyn silver* was produced and used in water sanitation (Lansdown 2006).

Noble metals are toxic to microorganisms in the following order of effectivity: Ag >Hg >Cu >Cd >Cr >Pb >Co >Au >Zn >Fe >Mn >Mo >Sn (Berger et al., 1976; Golubovich and Rabotnava, 1974). The broad spectrum anti-microbial activity of silver against Gram-positive and Gram-negative bacteria, including drug resistant strains, fungi, protozoa and viruses has been well studied and proven (Balazs et al., 2004 ; Melaiye et al., 2005; Stobie et al., 2008). As the size of a silver particle decreases, the anti-microbial efficacy increases because of the larger surface area per unit volume (Qian et al., 2001). Therefore, silver nanoparticles are being considered as an alternative to conventional antimicrobial agents.

Currently, the proposed mechanism for the inhibitory and bactericidal activity of silver is the adherence of silver nanoparticles to the microbial cell membrane where it interacts with thiol (sulfhydryl) group-containing proteins. Thiol group-containing amino acids, such as cysteine neutralized the activity of silver against bacteria, as opposed to amino acids without thiol groups, which had no effect on the anti-microbial activity of silver (Liau et al., 1997), therefore implying the interaction of silver with thiol groups. Silver

ions cause the release of K^+ ions from bacteria, meaning that the cytoplasmic membrane is a target site for silver ions (Schreurs and Rosenberg, 1982). The nanoparticles penetrate the cell where it interacts with the phosphorous-containing DNA and attack thiol group compounds of respiratory chain enzymes, inhibiting respiration and cell division, finally leading to cell death (Klasen, 2000). Furthermore, silver ions are believed to interact with ribosomes, inhibiting the expression of ATP producing enzymes, also inhibiting respiration. Large concentrations of ionic silver catalyze the complete destructive oxidation of microorganisms in an oxygen rich aqueous environment (Davies et al., 1997). In a study on the antibacterial mechanism of silver ions in *Escherichia coli* and *Staphylococcus aureus* it was demonstrated that upon exposure to silver ions, free DNA condensed and lost its replication abilities (Feng et al., 2000). *E. coli* cells showed cell wall damage, and in *S. aureus* cells, the cytoplasm membrane shrank and became detached from the cell wall. Gram-positive organisms are more resistant towards silver ions due to extra protection offered by the peptidoglycan layer of the cell wall (Feng et al., 2000). Sondi and Salopek-Sondi (2004) reported the anti-microbial activity of silver nanoparticles towards *E. coli*. The biocidal properties of silver nanoparticles are suggested to be mediated by silver ions, which are chemisorbed onto the partially oxidized nanoparticles (Lok et al., 2006) Trace amounts of silver have been found to be effective against biofilm formation (Sreekumari et al., 2001).

The advantageous characteristics of silver nanoparticles as biocides can be expanded for further applications by incorporating it into other materials, especially polymer nanofibers.

2.7 Incorporation of silver nanoparticles into polymer nanofibers

Metal nanoparticles can be incorporated into polymer nanofibers by either physically blending the nanoparticles with the polymer prior to electrospinning, in situ polymerization of a monomer in the presence of metal nanoparticles, or incorporation of metal salts into the polymer with subsequent in situ reduction of metal ions to

nanoparticles (Lala et al., 2007). With advances in nanofabrication techniques, silver has been incorporated into a range of nanostructures to exploit its chemical and biological properties (Sharma et al., 2009). Silver nanoparticles can be included into polymer nanofibers with an even distribution, making it useful in various applications such as water filtration (Li et al., 2004). The antimicrobial properties make it applicable for water sanitation and prevention of biofouling on filtration membranes.

Silver is the most commonly used biocide in electrospun nanofibers (Teo et al., 2009). Recently, electrospun nanofibers containing silver nanoparticles have successfully been fabricated for antimicrobial applications using polymers such as poly(vinyl alcohol) (Barakat et al., 2010; Chun et al., 2010; Hong et al., 2006; Nguyen 2010) polyamide (Bjorge et al., 2009); poly(ε-caprolactone) (Nirmala et al., 2010); gelatin (Rujitanaroj et al., 2008); cellulose acetate (Son et al., 2006); polyurethane (Yao et al., 2008) and poly(L-lactide) (Xu et al., 2006).

2.8 Hydrolytic enzymes as anti-biofouling agents

A potential target for the prevention of biofouling is the prevention of EPS formation and the degradation of EPS, since the EPS is central to the formation, attachment, protection and stability of the biofilm (Mahmoud, 2004). The main components of the EPS are polysaccharides and proteins, therefore hydrolytic polysaccharases and proteases can be used to prevent biofouling by preventing EPS formation and biofilm attachment. Polysaccharide lyases, and more prevalently polysaccharide hydrolases are commonly used to degrade EPS (Wingender et al., 1999). Since proteins play an important role in biofilm structure and EPS attachment, proteases are also used to prevent biofouling. Proteases, and specifically microbial proteases are one of the major industrial enzymes, especially due to the efficient production of proteases by microbes. Proteases occur in one of two major groups, depending on their target site, namely endopeptidases and exopeptidases. Endopeptidases cleave bonds between inner peptides in the polypeptide chain, leading to the denaturation of the three dimensional structure and inevitably the functionality of the protein, and are classified as serine proteases, cysteine proteases,

aspartic proteases and metalloproteases, depending on their catalysis. Exopeptidases cleave peptide bonds close to the polypeptide chain terminals, and are either carboxypeptidases, which target the C-terminal of the polypeptide chain, or aminopeptidases which cleave at the N-terminal of the polypeptide chain. Furthermore, proteases are classified according to the pH environment in which they function optimally (Rao et al 1998).

Studies have been conducted on the activity of protein and polysaccharide degrading enzymes on biofilms (Molobela and Cloete 2010). Fungal cellulose was tested against a *P. aerugunosa* biofilm, and was found to cause a decrease in CFU and EPS biomass (Loiselle and Anderson, 2003).

Proteases from the Antarctic krill shrimp, including endo- and exopeptidases removed mixed biofilms from surfaces and prevented the formation of additional biofilm growth by removing EPS proteins and preventing adhesion-receptor interactions responsible for cell to cell and cell to surface attachment (Hahn Berg et al., 2001).

Johansen et al. (1997) found that in a wide range of commercial enzymes that were tested against mixed biofilms, individual enzymes either had a bactericidal effect on cells in a biofilm or lead to biofilm detachment without eliminating cell viability, but that combinations of these commercial enzymes both removed and killed biofilms. This was ascribed to the heterogeneous nature of biofilms, comprising a complex mixture of bio molecules, and therefore explained why combinations of enzymes with different targets will be more efficient in the control of biofouling.

Similarly, Böckelmann et al. (2003) showed the efficient detachment of mixed biofilms in soil when using a combination of polysaccharidases, galactosidase, glucosidase and lipase.

This was confirmed again in a recent study by Wang et al. (2009). The anti-biofouling potential of α-amylase, protease, lysozyme and cellulase was studied. The activity of each

enzyme was measured as detachment of a mature mixed biofilm both independently and in combination of more than one enzyme at a time. The detachment ratio was the ratio of the weight of a glass surface with biofouling on it, before and after treatment with enzymes. When used independently, each enzyme caused a detachment ratio of between 12 and 25% after 20h of exposure to each enzyme. Cellulase was the most efficient, followed by protease, amylase, and lysozyme being least effective. After trying different combinations of two and three enzymes, it was concluded that the most effective was a combination of all four enzymes, with protease as the largest concentration, followed by cellulase, lysozyme and then amylase, which caused a biofilm detachment ratio of 40.24%. This was similar to detachment caused by a chemical biocide bromogeramine, but, when the enzymes were used in combination with bromogeramine, the detachment ratio increased by about 10% to 54.01%. This confirmed the potential of hydrolytic enzymes as anti-biofouling agents in water systems.

Furthermore, the pre-treatment of surfaces prone to biofilm formation with enzymes have proved to be effective in preventing the attachment, formation and maturation of biofilms (Walker et al., 2007)

Despite being effective against biofilm formation and attachment without the problem of DBP formation, the use of enzymes as anti-microbials on a large scale is not without drawbacks. Enzymes are relatively expensive to produce and purify when compared to chemical biocides. Therefore it would be preferable to re-use enzymes in reactions, but enzymes are generally difficult to recover when dispersed in the reaction medium for re-use. Furthermore, enzymes are very sensitive to conditions in the reaction environment such as temperature and pH. A solution to these challenges is the immobilization of enzymes.

2.9 Immobilization of hydrolytic enzymes

Enzyme immobilization can stabilize the three dimensional structural conformation of an enzyme by attachment to a substrate at many points in the polypeptide chain. This prevents denaturation and loss of enzyme activity when exposed to unfavourable pH or temperature reaction conditions (Cao, 2005; Lopez-Serrano et al., 2002; Mateo et al., 2007). Furthermore, the recovery and re-use of the enzymes is much easier.

Enzymes can either be immobilized onto solid supports or they can be self-immobilized by a cross-linking process (Brady et al., 2008). Self immobilization eliminates the need for a supporting medium, reducing cost and delivering immobilized enzymes with retained specific activity (Brady et al., 2008). Currently, published or patented methods of self-immobilization include cross-linked enzyme aggregates (Lopez-Serrano et al., 2002), cross-linked spray dried enzyme (Amotz, 1987), cross-linked enzyme from solution and cross-linked enzyme crystals (Khalaf et al., 1996), which involves the cross linking of purified enzyme crystals, which is an expensive process with limited range. Recently, a novel method of self-immobilization has been developed. Enzymes are cross-linked whilst in emulsion, yielding spherical catalytic macro-particles known as Spherezymes (Brady et al., 2008; Richards, 2010). This relatively inexpensive method yields immobilized enzymes with activity in both aqueous and organic solvents, with superior activity in organic solvent when compared to free enzymes.

Most commonly, enzymes are immobilized by attachment to solid supports by methods such as physical adsorption, encapsulation or covalent binding (Goldstein et al., 1976).
Physical adsorption is a simple process of reversibly binding enzymes to the supporting medium through weak interactions, and enzymes can dissociate under specific temperature, pH or ionic conditions. The drawback is the reversibility of the bonds causing enzymes to often end up in the reaction medium after use. Encapsulation is achieved by immobilizing enzymes within the structure of the supporting media, for example a polymer. Encapsulation is advantageous because the process is relatively simple and high concentrations of enzymes and mixed enzymes can be included.

Furthermore, the supporting medium provides physical protection against enzyme denaturation by unfavourable temperature, pH and solvents, enhancing the retention of enzyme activity. Conversely, encapsulation can obstruct many of the enzyme reactive sites from substrate interaction, lowering the enzyme efficiency. Covalent bonding entails irreversible chemical binding between a group on the polypeptide chain of the enzyme protein to reactive moieties on the surface of the polymer (Wang et al., 2008). The usefulness of such a covalent bond depends on the effect of this immobilization process on the function of the enzyme. Functionality of the enzyme will be compromised if binding takes place in such a manner that the reactive site of the enzyme is obstructed or altered. Covalent enzyme immobilization thus inevitably goes hand in hand with some loss of enzyme activity, as random protein-polymer covalent bonds will affect the active sites of at least a proportion of the enzymes. It is thus advantageous to use an immobilization system which allows for the immobilization of a large concentration of enzymes, as to retain a sufficient level of enzyme activity.

The most commonly used polymeric support for enzyme immobilization is polymer fibers. This can be ascribed the large specific surface area available for immobilization, the inter fiber porosity, which allows the penetration of the substrate and excellent mechanical strength (Wang et al., 2008), allowing for application in many fields of use such as filtration. Immobilized enzyme efficiency can be improved by reducing the size of the supporting media to nanoscale (Li et al., 2007). Nanomaterials such as nanofibers, nanotubes and nanoparticles have been used as supports for enzyme immobilization, creating nano-biocatalysts (Ding et al., 2005; Kim and Grate, 2003). Nanofibers are thus excellent candidates for supporting enzyme immobilization.

Enzymes have various functional groups on their surface that can be utilized in covalent immobilization. The amino groups ($–NH_2$) on lysine amino acid residues, the carboxylic groups (-COOH) on aspartic and glutamic amino acid residues and hydroxyl groups (-OH) on serine and tyrosine amino acid residues are all capable of forming covalent bonds with the substrate. Thus, to immobilize an enzyme covalently onto a polymer structure, a polymer with chemically available reactive sites on its surface is required. Inert polymers,

although very stable, do not have reactive groups on their surface and can thus only be used for immobilization if the surface can be chemically altered in such a way that surface groups become reactive and compatible with protein binding.

Many polymers such as PVA do have reactive groups on their surface, but most polymers lack reactive groups on their surface (Marinov et al., 2009; Perez et al., 2007), and thus need chemical activation. Such methods include carbonization at high temperatures or chemical alteration.

An example of one such a polymer is poly (acrylonitrile) (PAN), with nitrile groups (CN) on the surface. The process of amidination, first demonstrated in 1972 by Hunter and Ludwig, is an excellent way of converting inert nitrile groups on PAN surface to reactive imidoester moieties, to which enzymes can bind covalently by interaction with amino groups. Imidoester formation is based on the Pinner reaction, first described in 1892, and involves the anhydrous conversion of a nitrile group to an imidate salt formed by a reaction with alcohol in the presence of a halide acid, generally HCl (Figure 2) (Hunter and Ludwig 1972, Pinner 1892).

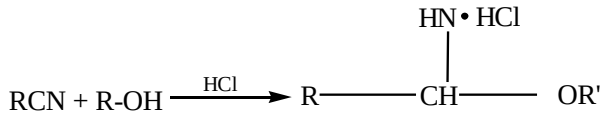


Figure 2. The Pinner reaction of imidoester formation.

Imidoesters are well known to react with both α- and ϵ-amino groups of proteins in an aqueous environment (Hunter and Ludwig 1972). The activation of nitrile groups on PAN by imidoesterification for the immobilization of enzymes was first carried out by Handa et al. (1982 and 1983). Similarly, imidoesterification was used to successfully activate the nitrile groups on PAN electrospun nanofibers for the immobilization of lipase (Li et al., 2007 and 2009) (Figure 3).

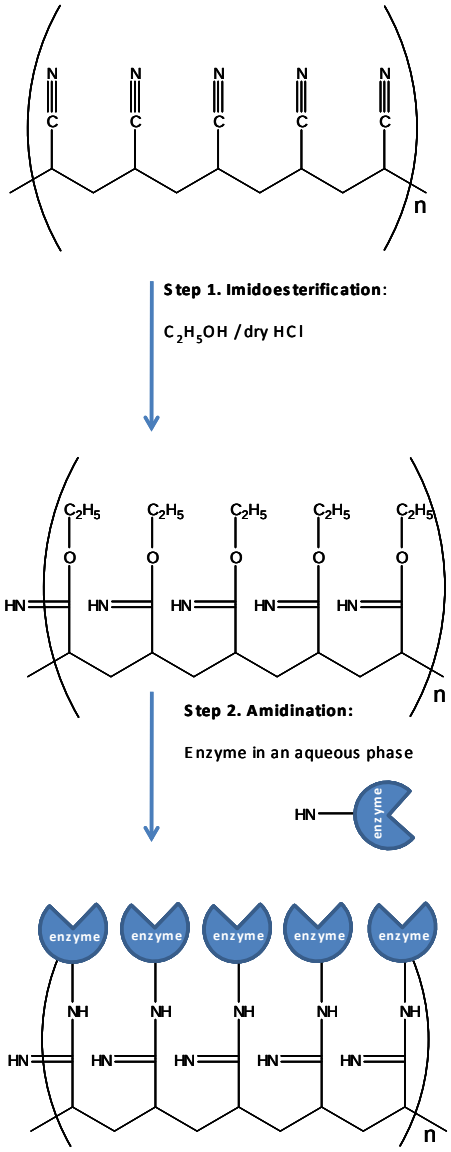


Figure 3. Enzyme immobilization onto PAN by activation through imidoesterification and subsequent amidination or protein binding modified from Li et al., (2007).

Imidoesterification is the process of changing amide groups to reactive groups on the surface of PAN in the presence of anhydrous hydrogen chloride. This renders the polymer functional and modifiable (Handa et al., 1982; Handa et al.,1983; Hunter and Ludwig 1972).

It was concluded that this method was very effective in immobilization of lipase, yielding a product with a large surface area, carrying enzymes retaining high activity with improved storage stability when compared to the free enzyme. Additionally, the immobilized lipase was found to be highly re-usable (Li et al., 2007 and 2009). Enzyme immobilization through covalent binding to chemically activated PAN nanofibers is thus an effective and feasible method of enzyme immobilization and stabilization (Li et al., 2007 and 2009). Such technology is highly applicable to the field of filtration where debris in contact with the nanofiber surface needs to be eliminated.

2.10 Conclusion

In the light of the urgent need for development of new, more efficient, accessible, economically viable and environmentally friendly techniques of water sanitation products, current research developments in the field of nanobiocides, nanofiltration, enzymatic control of biofouling and the efficient immobilization of enzymes onto nanofibers offer promising solutions. Conventional disinfection methods in water treatment often include the use of large amounts of chemical disinfectants, which produce harmful by-products. Nanobiocides, such as noble metal nanoparticles, and silver nanoparticles in particular, offer an alternative method of disinfection without reacting with the water itself, not adding harmful by-products to the water.

There is, however growing evidence that silver nanoparticles exhibit cytotoxic effects on higher organisms, raising the need for further investigation into the impact of the use of silver as a nanobiocide on the environment and human health (Marambia-Jones, 2010), especially when used in water treatment. Furthermore, the exact mechanism of silver nanoparticles as biocides has yet to be fully elucidated.

Polymer nanofibers have characteristics making it highly applicable in the field of water treatment. The high surface area and porosity, the ease of fabrication and the highly modifiable characteristics allow for the development of nanofilters with a wide range of possible applications.

Silver nanobiocide can be included into nanofibers through a simple process, yielding efficient anti-microbial nanofibers. Equally successful is the immobilization of hydrolytic enzymes onto the surface of polymer nanofibers. When immobilizing enzymes targeted specifically against the components of a biofilm, anti-biofouling nanofibers are created. Anti-microbial nanofibers will eradicate the viability of contaminant cells, but will not remove the biomass remaining from dead cells when used to filter contaminated water. The remaining biomass is likely to accumulate on the nanofibers and in the pores of the nanofiber mat, blocking filtration efficiency, and providing substrate for biofouling.

Further studies need to be done into the combination of antimicrobial nanofibers with nanofibers with immobilized enzymes into a single nanofiltration product, which will have both anti-microbial and anti-biofouling properties. Such a product will be highly applicable in water treatment systems.

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CHAPTER 3

The fabrication and characterization of anti-microbial poly (vinyl alcohol) and poly (acrylonitrile) nanofibers containing silver nanoparticles

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Abstract

Due to a global lack of access to potable water, a problem that particularly affects people in developing countries and the poor, improvement on existing water purification methods are necessary to provide more cost effective, accessible and efficient methods of water purification. Nanofiltration has been used as a technology in water purification. Electrospun nanofibers have shown potential application for filtration and a possible alternative to the use of chemical biocides. The focus of this study was to produce PVA and PAN nanofibers containing silver nanoparticles as biocide, using an electrospinning technique. The two types of fibers were to be compared based on morphology, silver nanoparticle content, physical distribution of silver nanoparticles, levels of silver leaching from the fibers in water, which could imply toxicity, and most importantly, anti-microbial efficacy. Both types of fibers had excellent anti-microbial activity, and PAN nanofibers with silver nanoparticles completely sanitized contaminated water samples.

Introduction

On a global basis, approximately 1 billion people lack access to potable water. People in developing countries and communities living in rural areas are most severely affected (WHO 2004). Therefore a demand exists to improve on existing water purification methods, providing more cost effective, accessible and efficient methods of water purification (Theron et al., 2008). Conventional methods of water treatment include the use of chemical biocides such as free chlorine, chloramines and ozone. Disinfection by-products produced by disinfectants have carcinogenic properties (Krasner et al., 2006). Furthermore, antibiotic and biocide resistance in pathogens is becoming more prevalent. Alternatives to the use of chemical biocides and antibiotics need to be investigated (Sondi and Salopek-Sondi 2004).

Nanotechnology has been identified as a tool with many applications in the water industry (Bottero et al., 2006, Cloete et al., 2010). One of these applications involves the use of nanofibers for water filtration (Cloete et al., 2010). Nanoparticles or scaffolds thereof often possess novel biological, physical or chemical properties (Theron et al., 2008).

Nanofibers are solid fibers with diameters within the nanoscale with a large surface to volume ratio, and when assembled in a non-woven mat, have a small pore size. Furthermore, the specific physical properties of nanofibers, such as strength, porosity and surface activity are determined by the polymer and additional non-soluble particles being used in the synthesis process (Frenot and Chronakis, 2004). These qualities make nanofibers extremely versatile and more effective than conventional polymer membranes used in liquid filtration (Theron et al., 2008; Yoon et al. 2006).

Electrospinning can produce nanofibers from a range of electrospinnable polymers. In the process of needle-electrospinning, a high voltage electric field is generated between a charged source of polymer solution and a grounded metal collector plate. An

electrostatically driven jet of polymer solution gives rise to nanofibers, which are collected on the plate (Fig. 1a).

A variation of conventional needle-based electrospinning, known as bubble electrospinning (Fig. 1b), allows much more rapid production of nanofibers. The process involves the formation of multiple electrostatically driven jets of polymer from a charged bubble of polymer solution (Yang et al., 2009). The electric field is of a much higher voltage than used in conventional needle spinning, and fibers generated from polymer jets are collected on a negatively charged metallic collector plate positioned above the bubble.

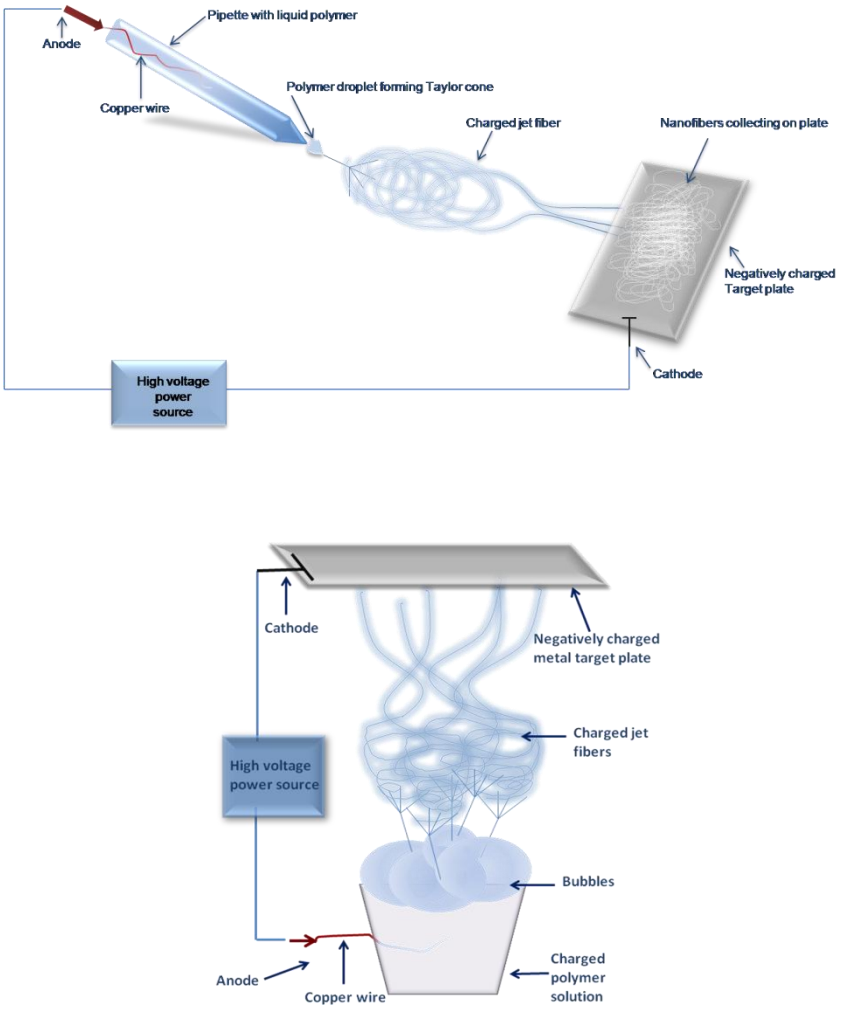


Figure.1 (a) Needle electrospinning and (b) bubble-electrospinning.

The synthesis of nanofibers containing metal nanoparticles is a well researched field (Botes and Cloete, 2010; Ochoa-Fernandez and Chen, 2004; Randall et al., 2001), mostly because of the advantages involved with combining the functional properties of metal nanoparticles with the widely applicable properties of nanofibers (Niu and Crooks, 2003). These properties include biocidal activity.

Silver is considered to be the most toxic element to microorganisms followed by Hg, Cu, Cd, Cr, Pb, Co, Au, Zn, Fe, Mn, Mo and Sn (Berger et al., 1976 ; Golubovich and Rabotnava, 1974). Although having a low toxicity toward mammalian cells (Maynard and Michaelson, 2006), silver has a broad spectrum of anti-microbial activity and inhibits

the growth of Gram-positive and Gram-negative bacteria, including drug resistant strains, fungi, protozoa and viruses (Balazs et al., 2004; Melaiye et al., 2004; Stobie et al., 2008). As the size of a silver particle decreases, the anti-microbial efficacy increases because of the larger surface area per unit volume (Qian et al., 2001). Silver nanoparticles are thus considered an alternative to conventional antimicrobial agents and are one of the most commonly used nanomaterials in consumer products.

Silver nanoparticles adhere to the microbial cell membrane where it interacts with sulfur-containing proteins. Nanoparticles can also penetrate the cell membrane, where it interacts with phosphorous-containing DNA and attack thiol groups of respiratory chain enzymes, inhibiting cell division and respiration, finally leading to cell death (Feng et al. 2000; Klasen, 2000). Furthermore, large concentrations of ionic silver catalyze the complete destructive oxidation of microorganisms in an oxygen rich aqueous environment (Davies et al., 1997). Due to the large specific surface area and high fraction of surface atoms on silver nanoparticles, it can be expected that silver nanoparticles will also have an oxidative effect on microorganisms, similar to that of large concentrations of ionic silver (Cho et al., 2005).

Metal nanoparticles can be incorporated into polymer nanofibers by either physically blending the nanoparticles with the polymer prior to electrospinning, *in situ* polymerization of a monomer in the presence of metal nanoparticles, or incorporation of metal salts into the polymer with subsequent *in situ* reduction of metal ions to nanoparticles (Lala et al., 2007).

In the present study, poly (vinyl alcohol) (PVA) and poly (acrylonitrile) (PAN) nanofibers were synthesized by bubble-electrospinning, incorporating $AgNO_3$ into the polymer solutions. *In situ* reduction of silver ions in $AgNO_3$ to silver nanoparticles was achieved by exposing the nanofibers to ultra violet (UV) irradiation.

PVA is a water soluble, non-toxic and biodegradable synthetic polymer. PVA nanofibers can be cross-linked to be water resistant. Alternatively, PAN nanofibers have better

durability and longevity in water. PAN is dissolved in dimethylformamide (DMF), a toxic solvent that evaporates during nanofiber synthesis. DMF assists in the *in situ* reduction of $AgNO_3$ silver ions to silver nanoparticles. Both PVA and PAN nanofibers are thus excellent candidates for carrying silver nanoparticles and for use in water treatment.

The aim of this study was to synthesize and compare PVA nanofibers with $AgNO_3$ to PAN nanofibers with $AgNO_3$ to determine which type of fiber would be the most appropriate for application in water sanitation. The two types of fibers were compared based on morphology, silver nanoparticle concentration, physical distribution of silver nanoparticles, levels of silver leaching from the fibers into water, which could imply toxicity, and anti-microbial efficacy.

Materials and Method

Materials

Poly (vinyl alcohol) (PVA; Mr 146 000 – 186 000 Dalton, 87 – 89% hydrolysis), glyoxal (40% aqueous solution), concentrated HCl, and silver nanoparticles (10wt% dispersion in ethylene glycol), poly(acrylonitrile) (PAN) and JSYK silicone surfactant were purchased from Sigma-Aldrich (Aston Manor, South Africa). Silver nitrate was from BDH (Poole, England).

Anti-microbial activity of silver nanoparticles in suspension

Pseudomonas aeruginosa (Xen 5), *Escherichia coli* (Xen 14), *Salmonella typhimurium* (Xen 26), *Klebsiella pneumonia* (Xen 39) and *Staphylococcus aureus* (Xen 36) (Caliper Life Sciences, Hopkinton, MA, USA), were used in this study. These strains each contain a stable copy of the *Photorhabdus luminescens lux* operon on the bacterial chromosome, rendering them bioluminescent.

Each pathogen was cultured in 10 ml Brain Heart Infusion (BHI) (Biolab Diagnostics) supplemented with tetracycline or kanamycin overnight on a rotating wheel at 37˚C. Cultures were standardized to 1 x 10^9 cells/ml using a SmartSpec Plus spectrophotometer

(Bio Rad) and 200 µl of each strain was plated into 12 wells of a 96 well culture plate (Greiner). The plate was incubated for 48 h at 30˚C. Viability of cells was confirmed by monitoring bioluminescence (Fig. 2(a)) using the IVIS *in vivo* imaging system 100 series (Caliper life sciences, Hopkinton MA, USA). Aqueous solutions of silver nanoparticles (10wt% dispersion in ethylene glycol, Sigma Aldrich) of 0.1, 1 and 5% (wt/v) were added to each of the wells in triplicate, and cell viability was recorded by determining bioluminescence after 1, 30 and 60 min (Fig. 3(b-d).

For statistical analysis, one way ANOVA followed by Dunnett's Multiple Comparison test as post test was performed. *P<0.05, **P<0.01 and ***P<0.001 when compared to photons in the absence of silver nanoparticles (nAg), (solid first column of each set, Fig. 3).

Synthesizing cross-linked poly (vinyl alcohol) (PVA) nanofibers with silver nanoparticles

A polymer solution of 8wt% PVA was prepared by dissolving PVA powder in water with gentle stirring at 90°C. The polymer solution was left to cool down and 8%v/v glyoxal was added as cross-linking agent. The pH was adjusted to 5.0 with concentrated HCl to aid the cross-linking process. Finally 5 % (wt/v) $AgNO_3$ was thoroughly mixed into the polymer solution. Bubble spinning was set up as illustrated in Figure 1(b). A bubble spinning widget for lab scale nanofiber production, specially designed by Dr. Eugene Smit, was used. Five ml of the polymer solution was poured into the well of the bubble spinning widget. A copper wire attached to the positive electrode of a high voltage power supply was inserted into the polymer solution, while the negative electrode was attached to a tinfoil collector plate suspended 20 cm above the widget. A current of 50 kV was applied. PVA nanofibers containing $AgNO_3$ was collected on the plate, and was cross linked by curing at 60°C for 4 d. Subsequent to cross-linking, the nanofibers were exposed to UV irradiation for 1h to reduce silver ions in the nanofibers to silver nanoparticles.

Plain PVA nanofibers without silver nanoparticles were used as negative controls in the antimicrobial tests. These were fabricated similarly to PVA nanofibers containing silver nanoparticles, but $AgNO_3$ was omitted from the polymer solution.

Synthesizing poly (acrylonitrile) nanofibers

A polymer solution of 6% (wt/v) PAN in dimethylformamide (DMF) (Sigma Aldrich) was prepared. DMF was heated up to 90°C and stirred while PAN was added gradually. The mixture was stirred at 90°C for 5 h until a clear, dark yellow solution was obtained. Silicone surfactant, JSYK L580 was added (0.95 g/l) to stabilize bubble formation during bubble-electrospinning. Finally, 5% (wt/v) $AgNO_3$ was thoroughly mixed into the polymer solution. Five ml of the polymer solution was poured into the well of the bubble spinning widget. A copper wire attached to the positive electrode of a high voltage power supply was inserted into the polymer solution, while the negative electrode was attached to a tinfoil collector plate suspended 20 cm above the widget. A current of 50 kV was applied. PAN nanofibers containing $AgNO_3$ and already reduced silver nanoparticles were collected on the plate. Subsequently, the nanofibers were exposed to UV irradiation for 1 h to reduce any remaining silver ions in the nanofibers to silver nanoparticles.

Plain PAN nanofibers without silver nanoparticles were used as negative controls in the antimicrobial tests. These were fabricated similarly to PAN nanofibers containing silver nanoparticles, but $AgNO_3$ was omitted from the polymer solution.

***Characterization of PVA and PAN nanofibers containing silver nanoparticles with SEM and EDX*.**

The morphology of PVA and PAN nanofibers containing silver nanoparticles were studied using the Zeiss Evo MA 15 scanning electron microscope (SEM) and energy dispersive X-ray analysis (EDX). The physical distribution pattern of silver nanoparticles within the nanofibers was visualized using SEM with a back scattered electron detector

(BSE). Additional elemental analysis and quantification of silver in the fibers was done with EDX.

Anti-microbial activity of PVA and PAN nanofibers with silver nanoparticles

The antimicrobial activity of nanofibers containing silver nanoparticles was tested by immobilizing the nanofibers onto filters with 0.22 µm pores (Durapore, Millipore). Pathogenic cells were brought into contact with the nanofibers by filtering pathogen contaminated water samples through the filters with the nanofibers on the filter surface. The filter pore size of 0.22 µm excluded bacterial cells, causing the cells to remain in contact with the fibers. This allowed investigation of the effect of nanofibers containing silver nanoparticles on bacterial cells upon contact. A negative control was included in each experiment, namely nanofibers without silver nanoparticles that were immobilized onto filters and subjected to the same test conditions.

For these tests, *P. aeruginosa* (Xen 5), *E. coli* (Xen 14) and *K. pneumonia* (Xen 39) were used. Each pathogen was cultured in 10 ml brain heart infusion (BHI, Biolab Diagnostics) supplemented with antibiotics, overnight on a rotating wheel at 37˚C. Cells were harvested (10 min, 1000xg) from each culture and were washed 3 times with sterile saline to remove nutrients possibly remaining from the growth medium. For each pathogen, a test water sample was prepared by inoculating 10^6 cells/ml into 250 ml of sterile distilled H_2O. Water samples were then filtered through 0.22 µm filters, with either clean PVA nanofibers, or PVA nanofibers containing silver nanoparticles immobilized on the filter surface, using a Sartorius-Stedim polycarbonate filter holder. The same experiments were repeated with 0.22 µm filters with either clean PAN nanofibers, or PAN nanofibers containing silver nanoparticles immobilized on the filter surface.

After filtration, the viability of the cells remaining on the nanofibers with silver nanoparticles was investigated, firstly by quantification of bioluminescence as an indication of viability with IVIS visualization and secondly by determining the number of viable cells recovered from the nanofibers. This was done by repeated rinsing with sterile

PBS. Serial dilutions were plated onto selective solid BHI agar plates, and after incubation overnight at 37˚C, CFU was determined.

When bioluminescence of the cells remaining on the nanofibers with silver nanoparticles was quantified as an indication of viability, the bioluminescence of cells remaining on nanofibers without silver nanoparticles served as a control. When determining the CFU of cells remaining on nanofibers containing silver nanoparticles, the CFU of the water sample before filtration, as well as the CFU of cells remaining on nanofibers without silver nanoparticles, served as controls. All experiments were done in triplicate.

SEM imaging of anti-microbial activity of PAN nanofibers with silver nanoparticles

Water samples containing pathogenic cells were filtered through both plain PAN nanofibers and PAN nanofibers containing silver nanoparticles that were immobilized onto 0.22 µm filters. This allowed visualization of the morphological effect of nanofibers containing silver nanoparticles on bacterial cells upon contact.

A sample of 10^6 cells/ml distilled H_2O of *P. aeruginosa* (Xen 5), *E. coli* (Xen 14), *S. typhimurium* (Xen 26), *K. pneumonia* (Xen 39) and *S. aureus* (Xen 36) were filtered through plain PAN nanofibers and PAN nanofibers containing silver nanoparticles that were immobilized onto 0.22 µm filters. Immediately following filtration, the samples were fixed in 2.5% (v/v) gluteraldehyde in PBS at 4°C for 4 h. Fibers were allowed to air dry and were prepared for SEM imaging by fixing on carbon adhesive tape and sputter coating with gold. All experiments were done in triplicate.

UV analysis of silver leaching from PVA and PAN nanofibers with silver nanoparticles

UV spectroscopy with the Perkin Elmer UV/VIS spectrophotometer was used to quantify any possible leaching of silver from both PVA and PAN nanofibers containing silver nanoparticles submerged in distilled water for 1h and 18h respectively. Results were normalized for distilled water.

Results and discussion

Anti-microbial activity of silver nanoparticles in suspension

Bioluminescent images of *P. aeruginosa* (Xen 5), *E. coli* (Xen 14), *S. typhimurium* (Xen 26), *K. pneumonia* (Xen 39) and *S. aureus* (Xen 36) (Fig. 2), have shown that a solution containing 1% of silver nanoparticles (nAg) caused a significant decrease in the number of viable cells within a 1 min of exposure (Fig. 3). This is in agreement with work by Choi et al. (2008) that reported strong anti-microbial activity of a silver nanoparticles colloid solution against *E. coli*.

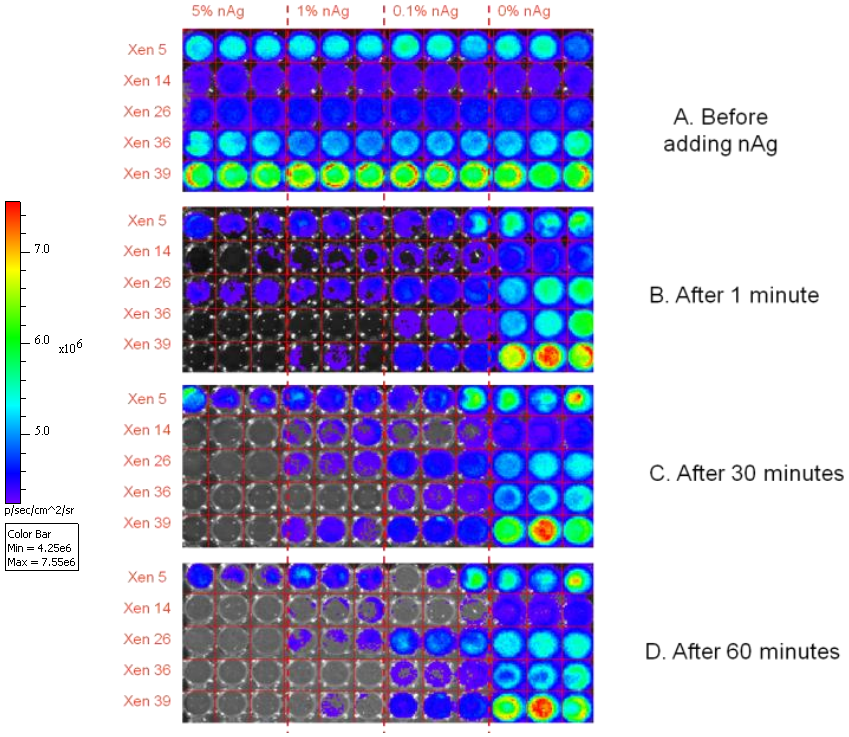


Figure.2 IVIS image of bioluminescence of *P. aeruginosa* (Xen 5), *E. coli* (Xen 14), *S. typhimurium* (Xen 26), *K. pneumonia* (Xen 39) and *S. aureus* (Xen 36) in the absence of silver nanoparticles (a), and in the presence of 0.1% (wt/v) , 1% (wt/v) and 5% (wt/v) silver nanoparticles (nAg) in aqueous suspension after (b) 1 min (c) 30 min and (d) 1h.

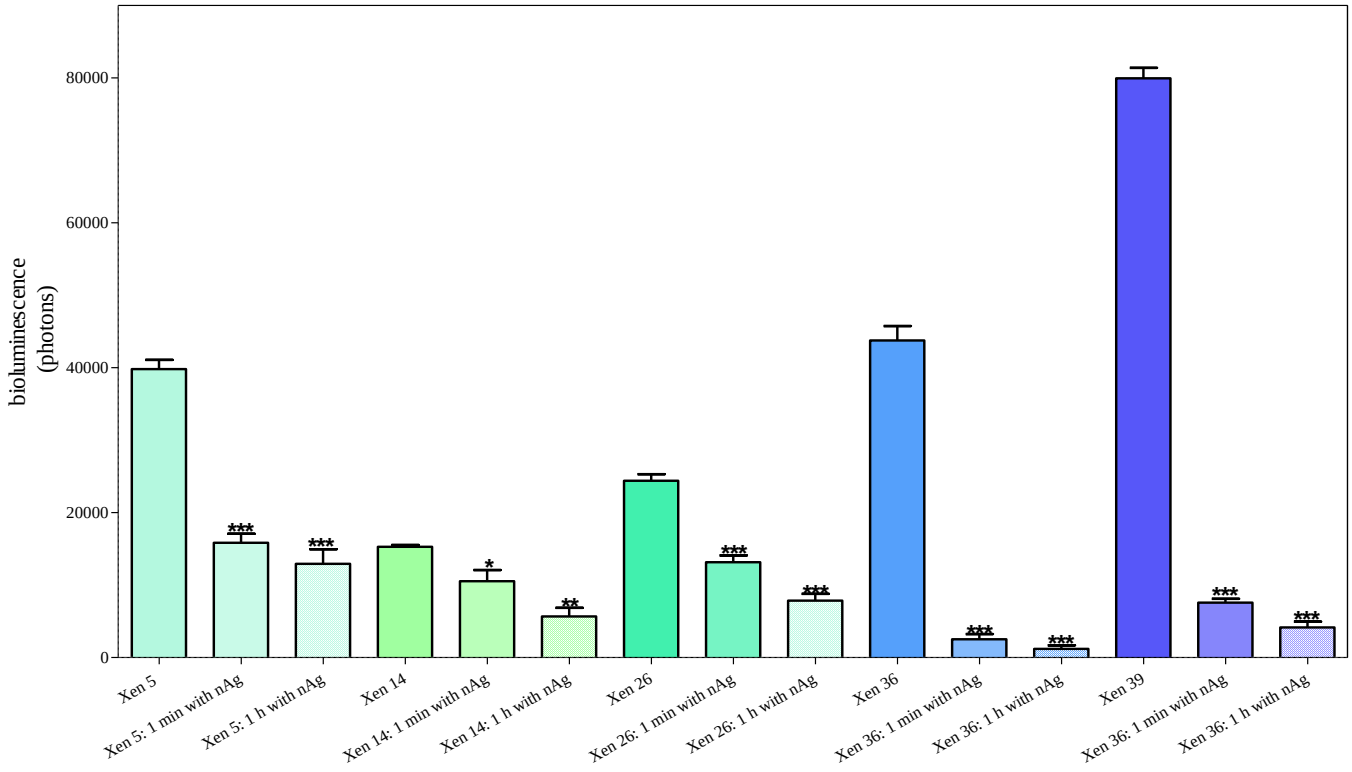


Figure.3 Bioluminescence of *P. aeruginosa* (Xen 5), *E. coli* (Xen 14), *S. typhimurium* (Xen 26), *K. pneumonia* (Xen 39) and *S. aureus* (Xen 36) in the absence of silver nanoparticles, and in the presence of 1% (wt/v) silver nanoparticle suspension after 1 min and 1h.

Characterization of PVA and PAN nanofibers containing silver nanoparticles with SEM and EDX.

No structural differences were observed between cross-linked PVA nanofibers containing 5% (wt/v) $AgNO_3$, which was exposed to 1 h of UV irradiation and PAN nanofibers that were prepared in the same way. PVA fibers were slightly smaller in diameter (Fig. 4a and 5a). Using a back scattered electron detector, contrast imaging shows the position of silver particles on the nanofibrous membranes in white, with each white speck indicating the presence of silver. When comparing Fig. 4b and 5b, a greater abundance of larger silver particles are visible in white. Fig. 4c and 5c are SEM/BSE images showing only the distribution of silver. Each tiny white dot represents flecks of silver which are visibly widely dispersed in both samples, with bigger particles of silver or areas of more concentrated distribution of silver particles visible as larger white areas. The EDX analysis of both the PVA and PAN nanofibers with silver nanoparticles shows a

distinctive energy peak at around 3 keV, characteristic of silver. The much higher x-ray intensity observed in PAN nanofibers with silver nanoparticles (Fig. 5d) indicates a higher silver content than in PVA nanofibers with silver nanoparticles (Fig. 4d). This is more clearly demonstrated in the analysis of the silver contents of the sample by EDX. The PVA-$AgNO_3$ nanofiber sample contained 8.9% (wt) of silver, whereas the PAN-$AgNO_3$ nanofiber sample contained 49.9% (wt) of silver. This was attributed to the fact that, in addition to UV irradiation, the solvent, dimethylformamide, also reduces Ag^+ ions in $AgNO_3$ to produce silver nanoparticles, thus yielding more silver nanoparticles than in PVA-$AgNO_3$ nanofibers, in which UV irradiation is the only step to reduce silver ions to silver nanoparticles.

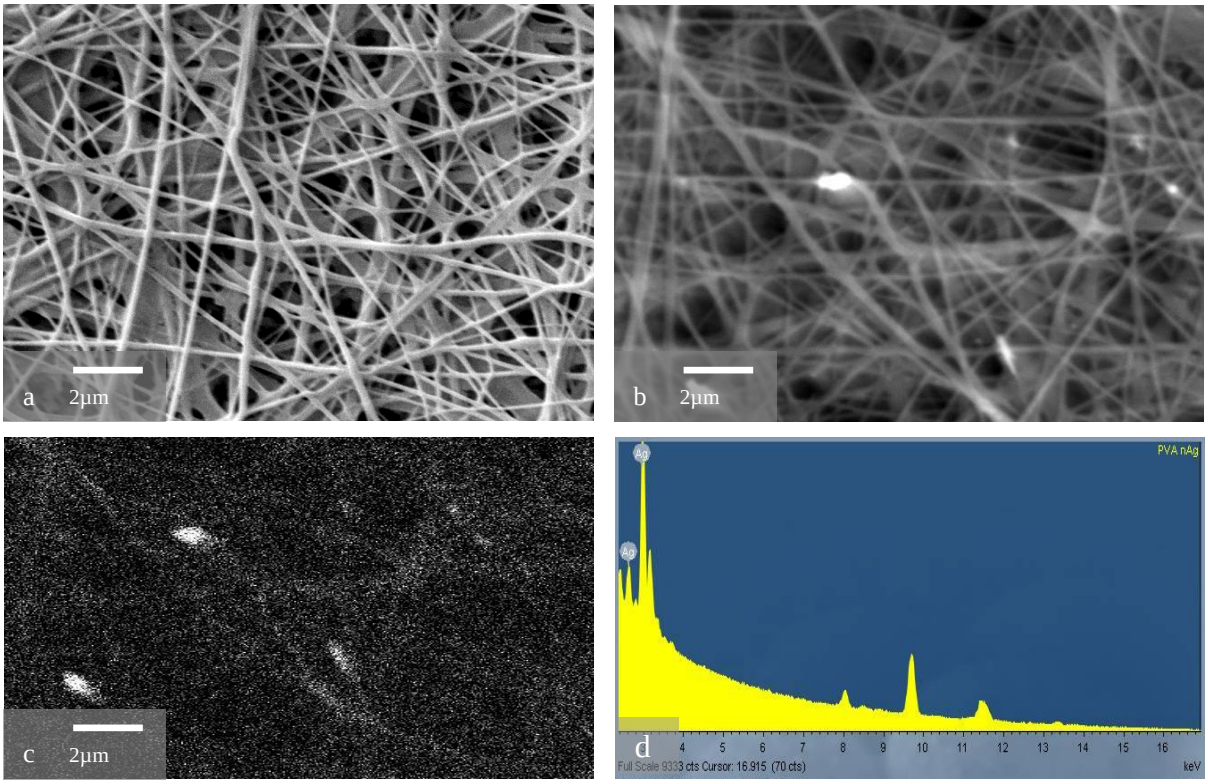


Figure.4 SEM/EDX of cross-linked PVA nanofibers with 5% (wt/v) $AgNO_3$ after exposure to UV. (a) SEM micrograph (b) SEM/back scattering image of silver particles in PVA nanofibers (c) Back scattering image of silver particle distribution in PVA nanofibers (d) EDX spectrum of silver in PVA nanofibers.

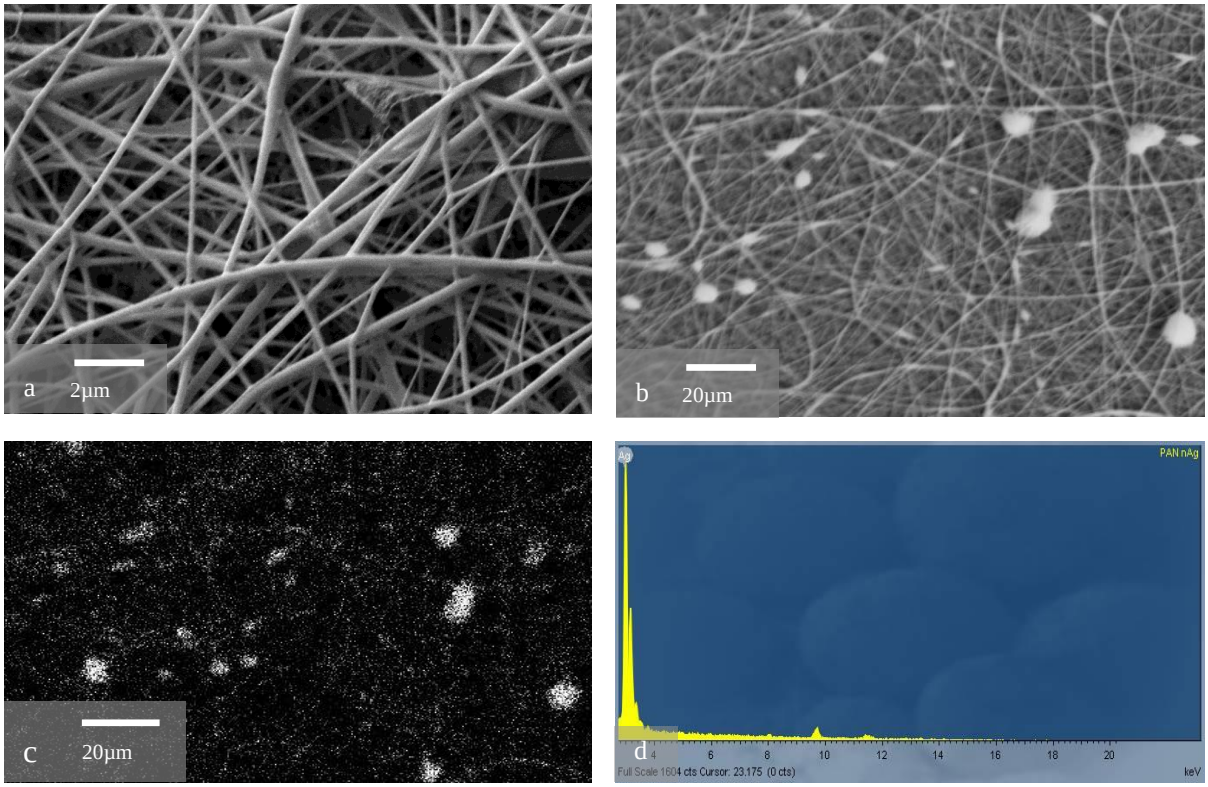


Figure.5 SEM/EDX of PAN nanofibers with 5% (wt/v) $AgNO_3$ after exposure to UV. (a) SEM micrograph (b) SEM/backscattering image of silver particles in PAN nanofibers (c) Back scattering image of silver particle distribution in PAN nanofibers (d) EDX spectrum of silver in PAN nanofibers.

Anti-microbial activity of PVA and PAN nanofibers with silver nanoparticles

Cells of each of the three pathogens in this study remain bioluminescent and thus viable when in contact with plain PVA fibers not containing silver nanoparticles. However, when these cells are in contact with PVA nanofibers containing silver nanoparticles, between 96% and 98% of bioluminescence is lost in all three pathogens which were tested (Table 1).

After filtration through membranes with plain PVA nanofibers on the surface, viable cells increased by a 100 fold as opposed to the number of CFU before filtration, indicating the potential for biofilm formation, should the fiber surface remain moist. However, when filtered through membranes with PVA nanofibers containing silver nanoparticles on their surface, there was a reduction of between 91% and 99% in CFU in all three pathogens

tested as opposed to the number of CFU before filtration. This confirmed that the observed decrease in bioluminescence correlated with a decrease in viable cells (Table 1).

Due to the larger prevalence of silver nanoparticles in PAN nanofibers (Fig. 5), a greater anti-microbial effect could be expected. In all three pathogens that were tested, there was a decrease in bioluminescence of between 98% and 100% in cells that were in contact with PAN nanofibers containing silver nanoparticles, as opposed to cells in contact with plain PAN nanofibers.

Similar to plain PVA fibers, filtration through membranes with plain PAN nanofibers on the surface led to roughly a 100 fold increase in viable cell numbers as opposed to the number of CFU before filtration. When filtered through a filter with PAN nanofibers containing silver nanoparticles on the surface, there was a 100% reduction in CFU in all three pathogens tested as opposed to the number of CFU before filtration (Table 2). This showed that PAN nanofibers containing 5% (wt/v) $AgNO_3$ not only had higher silver nanoparticle content, but also had a higher antimicrobial efficacy than PVA nanofibers containing 5% (wt/v) $AgNO_3$. These results are comparable to that found in the literature (Table 3).

Table 1. Summary of the anti-microbial effects of PVA nanofibers containing 5% (wt/v) $AgNO_3$

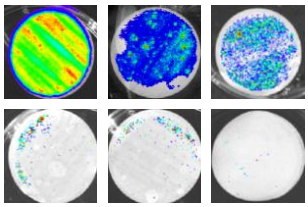
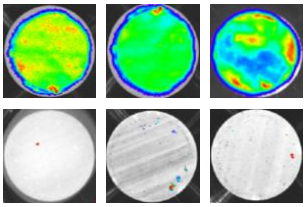
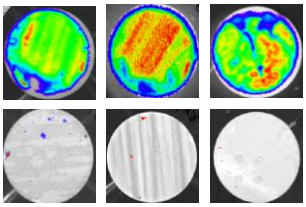
		P.aeruginosa (Xen 5)	*E.coli* (Xen 14)	*K.pneumonia* (Xen 39)
IVIS imaging of organism in contact with plain PVA fibers (A) and PVA fibers with 5% (wt/v) $AgNO_3$ (B).		A  B	A  B	A  B
% Reduction in photons SEM (n=3) R%=[(A-B)/A] x100		96.41% ± 1.7	98.59% ± 0.36	97.89% ± 1.4
CFU (average of triplicates)	Before filtration (A)	3.6×10^4	5.9×10^5	4.7×10^5
	Filtration with PVA	9.2×10^6	1.0×10^7	1.2×10^7
	Filtration with PVA+$AgNO_3$ (B)	3.0×10^3	1.0×10^4	2.0×10^3
% Reduction in CFU R%=[(A-B)/A] x100		91.67 %	98.39%	99.57%

Table 2. Summary of the anti-microbial effects of PAN nanofibers containing 5% (wt/v) $AgNO_3$

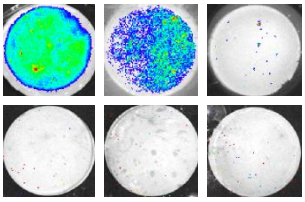
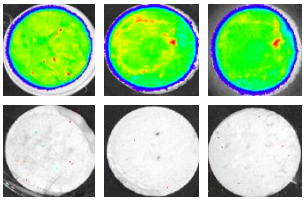
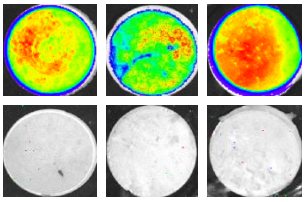
		P.aeruginosa (Xen 5)	*E.coli* (Xen 14)	*K.pneumonia* (Xen 39)
IVIS imaging of organism in contact with plain PAN fibers (A) and PAN fibers with 5% (wt/v) $AgNO_3$ (B).		A  B	A  B	A  B
% Reduction in photons (average of triplicates) SEM (n=3) R%=[(A-B)/A] x100		98.49% ± 1.3	99.96% ± 0.03	99.99% ± 0.01
CFU (average of triplicates)	Before filtration (A)	3.0×10^2	6.4×10^5	1.1×10^6
	Filtration with PAN	2.0×10^4	1.4×10^7	4.2×10^7
	Filtration with PAN+$AgNO_3$ (B)	0	0	0
% Reduction in CFU R%=[(A-B)/A] x100		100 %	100%	100%

Table 3. Summary of anti-microbial nanofibers containing silver nanoparticles in the literature.

Polymer Nanofibers	Concentration of silver	Antimicrobial activity	Reference
PVA/Chitosan blend	1%wt AgNO3	Good activity against *E. coli*	Hang et al., 2010
Gelatin	2.5%wt AgNO3	Active against *P. aeruginosa*, *S. aureus*, *E. coli*and methicillin-resistant *S. aureus.*	Rujitanaroj et al., 2008
N-Carboxyethylchitosan / poly(ethylene oxide)	5%wt AgNO3	*S.aureus*	Penchev et al., 2010
Cellulose Acetate	5%wt AgNO3	*E. coli* and *P.aeruginosa*	Lala et al., 2007
Poly (vinyl chloride)	5%wt AgNO3	*E. coli* and *P.aeruginosa*	Lala et al., 2007
Poly (acrylonitrile)	5%wt AgNO3	*E. coli* and *P.aeruginosa*	Lala et al., 2007
Poly(L-lactide)	8-32%wt AgNO3	very strong activity against *E. coli* and *S. aureus*	Xu et al., 2006

SEM imaging of anti-microbial activity of PAN nanofibers with silver nanoparticles

Of the 5 pathogens visualized (results not shown) only Gram-positive *S. aureus* cells showed a noticeable morphological change when in contact with PAN nanofibers with silver nanoparticles. When in contact with plain PAN nanofibers, *S. aureus* cells appeared spherical and intact (Fig. 6a). In a SEM micrograph where silver nanoparticles on the surface of PAN nanofibers were clearly visible, it was observed that *S. aureus* cells which were in contact with the nanofibers appeared pitted and lysed (Fig. 6b). This matches results obtained by Feng et al. (2000), which confirmed that silver nanoparticles physically compromises the integrity of the cell membranes of *S. aureus.*

As only the Gram-positive organism showed a morphological change in this study, it can be suggested that peptidoglycan, which is present in Gram-positive organisms at the exterior of the cell membrane may indicate that targeting the integrity of peptidoglycan could be a mechanism for anti-microbial action of silver nanoparticles.

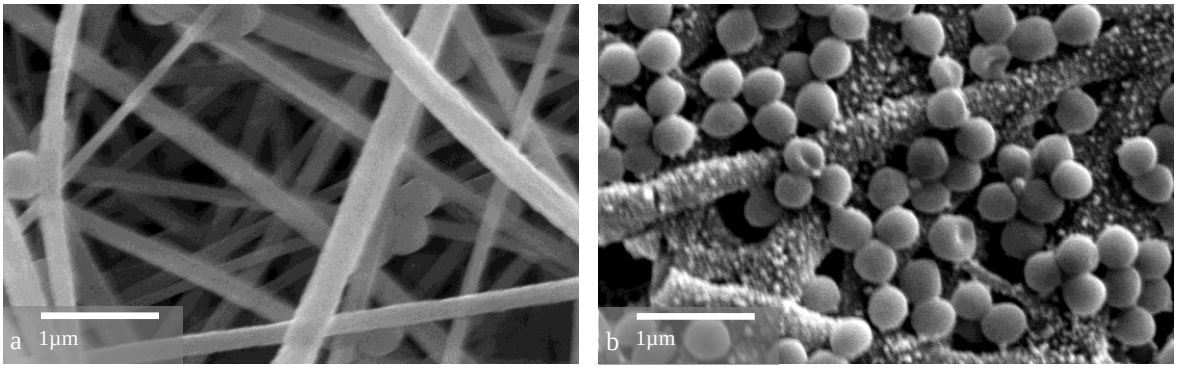


Figure 6. SEM micrographs of *S. aureus* cells in contact with (a) plain PAN nanofibers and (b) PAN nanofibers with silver nanoparticles.

UV analysis of silver leaching from PAN nanofibers with silver nanoparticles

It may be expected that cross linked PVA nanofibers containing 5% (wt/v) $AgNO_3$ are prone to leaching silver upon exposure to water, as PVA is not as resistant to water when compared to PAN, and usually swells a small amount when exposed to water. Interestingly, samples taken from water which PAN nanofibers were soaked in, had a higher UV absorbance than samples taken from water which PVA nanofibers containing silver were soaked in, indicating the presence of slightly more silver. For both PVA and PAN fibers containing silver, there was only slightly more silver present in the water samples of fibers that soaked for 18 h as opposed to only 1 h. This suggests that silver present in the samples did not originate from leaching, but was more likely residual surface silver nanoparticles that washed off upon contact with water.

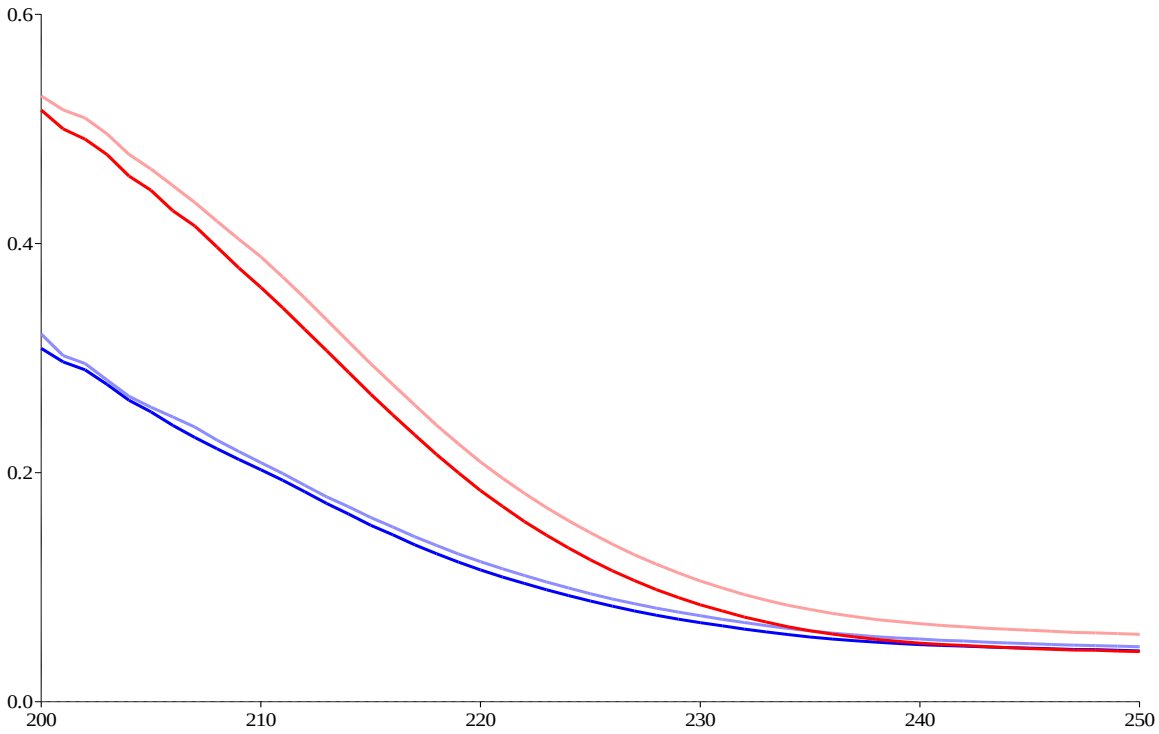


Figure 7. UV/VIS spectrum of PVA and PAN nanofibers containing silver nanoparticles that were soaked for 1h and 18h respectively.

Conclusion

PVA and PAN nanofibers containing silver nanoparticles were successfully fabricated by bubble-electrospinning. This was confirmed by EDX/SEM analysis. When inspected visually by SEM, PVA and PAN nanofibers containing silver nanoparticles appeared morphologically similar. Back scattering electron images revealed that silver nanoparticles in PVA nanofibers were more evenly dispersed than in PAN nanofibers, but that PAN nanofibers had higher silver nanoparticle content. This was confirmed by EDX analysis. Both PVA and PAN nanofibers had excellent anti-microbial activity, with PVA nanofibers with silver nanoparticles killing between 91% and 99% of bacteria in a contaminated water sample. PAN nanofibers with silver nanoparticles had an even higher anti-microbial activity, killing 100% of bacteria in water samples contaminated by 3 different pathogens. When investigated by SEM, the biocidal effect of PAN nanofibers containing silver nanoparticles on *S. aureus* can be observed as morphological changes in the cell walls. Neither PVA nor PAN nanofibers showed signs of leaching silver into water, even after 18h of soaking. When considering that PVA nanofibers with silver

nanoparticles are fabricated from a water soluble, non-toxic and biodegradable synthetic polymer, and has excellent anti-microbial activity, it can be highly applicable in water sanitation, especially in an environmental conscious milieu. On the other hand, the fabrication process of PAN nanofibers is more conductive to the formation of silver nanoparticles, and thus delivers fibers with a higher silver nanoparticle content, allowing the complete sanitation of pathogenically contaminated water samples. PAN nanofibers also have better longevity and strength in water, making it ideal for water filtration and sanitation in higher throughput systems.

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CHAPTER 4

Immobilization of commercial hydrolytic enzymes on electrospun poly(acrylonitrile) nanofibers and possible anti-biofouling application

*The following research paper will be submitted for publication in *Water Research.*

Abstract

In drinking water systems, biofilms are a potential source of contamination, which can affect the biological stability and hygienic safety of water. In industrial water systems, biofilms can result in conditions that will induce microbially influenced corrosion, reduced flow rates by increasing the fluid frictional resistance and a decrease in efficiency of membranes through biofouling. Immobilized enzymes were investigated as a possible alternative to inefficient conventional methods for controlling and removing biofilms from filtration systems. This study demonstrated the covalent immobilization of two industrial proteases and an amylase enzyme onto polymer nanofibers widely used in filtration membranes. Enzymes retained activity after immobilization and could be re-used. Furthermore, fibers with immobilized enzymes on their surface did not support the growth of biofilms, as opposed to plain nanofibers, which did support the growth of biofilms.

Introduction

Biofilms are three dimensional structures of accumulated bacterial biomass, consisting of bacterial cells, proteins, nucleic acids, polysaccharides (Characklis, 1990) and humic substances embedded in amphiphilic extracellular polymeric substances (EPS) (Wagner et al., 2009). Biofilms often form on surfaces in an aqueous environment (Cloete et al., 1992), making water filtration membranes, water distribution systems and industrial water systems particularly vulnerable to biofouling. In drinking water systems, biofilms are a potential source of contamination (Momba et al., 2000), which can affect the biological stability and hygienic safety of water (Emtiazi et al., 2004). Additionally, metabolites generated by organisms in the biofilm may add flavours and odours to the water, further reducing the quality (Khiari and Watson 2007; Ludwig et al., 2007). In industrial systems, biofilms can cause corrosion, resistance in flow systems and a decrease in efficiency of filtration membranes due to physical blockage.

Conventional methods of disinfection in drinking water and industrial water systems include the use of chemical biocides such as free chlorine, chloramines and ozone. Potential reactions between these biocides and the components of natural water can produce harmful disinfection by products (DBPs), many of which are carcinogens (Krasner et al., 2006). Although very effective against planktonic cells, bacteria in biofilms are resistant to chemical biocides (Cloete 2003; Xu et al., 2000) Consequently, alternatives to the use of chemical biocides against biofilms need to be investigated (Cloete 2003). One possible alternative is the use of hydrolytic enzymes as biocides and anti-biofouling agents (Molobela et al., 2010).

Enzymes are highly selective biocatalysts that can be employed to break down specific components of a biofilm under certain conditions without the production of toxic by-products. Some drawbacks concerning the use of enzymes 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).

In the present study, these potential drawbacks were overcome by using industrial enzymes produced on a large scale which are tolerant towards working environments over large pH and temperature ranges (Table 1). Furthermore, these enzymes were covalently immobilized onto a nanofibrous support, enabling re-use of the enzymes without the need for recovery from the medium.

Nanofibers are solid fibers with diameters within the nanoscale. They have a large surface to volume ratio and when assembled in a non-woven mat, create a permeable macrostructure with small pores. Furthermore, the specific physical properties of nanofibers, such as strength, porosity and surface activity are determined by the polymer and additional non-soluble particles being used in the synthesis process (Frenot and Chronakis 2003). Nanofibers can also be functionalized by adding molecules or chemical groups to reactive groups on the polymer surface. These qualities make nanofibers extremely versatile and more effective than conventional polymer membranes used in liquid filtration (Theron et al., 2008; Yoon et al., 2006).

Electrospinning can efficiently produce nanofibers from a range of electrospinnable polymers. A high voltage electric field is generated between a charged source of polymer solution and a grounded metal collector plate. An electrostatically driven jet of polymer solution gives rise to nanofibers, which are collected on the plate (Figure 1a).

A simple and very effective variation of conventional needle-based electrospinning allows much more rapid production of nanofibers for research purposes. The process, known as bubble electrospinning, involves the formation of multiple electrostatically driven jets of polymer solution from a charged bubble of polymer solution (Yang et al., 2009). The electric field is of a much higher voltage than used in conventional needle spinning, and fibers generated are collected on a negatively charged metallic collector plate positioned above the bubble (Figure 1b).

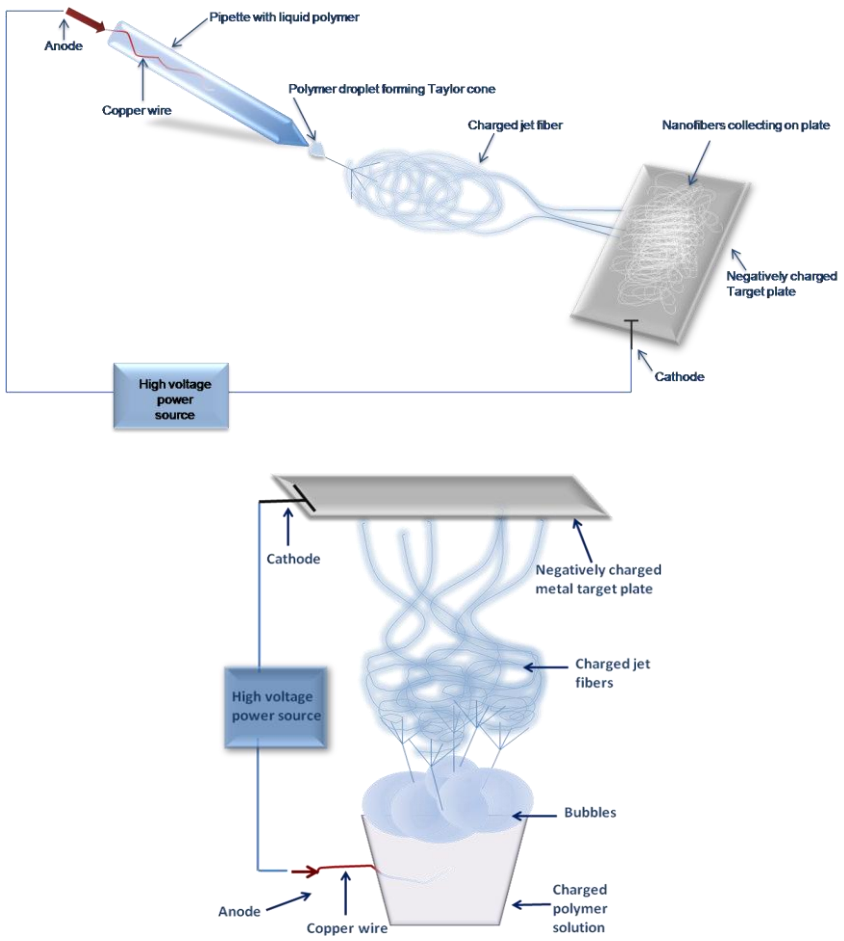


Figure.1 (a) Needle electrospinning and (b) bubble-electrospinning process.

Various polymers, with or without chemical modification have been used for the immobilization of enzymes. Many of these polymers are, however heat sensitive and have poor chemical, physical and microbiological resistance, for example acrylic and vinylic supports such as polyacrylamide and poly (vinyl alcohol) (Di San Filippo et al., 1990). Poly (acrylonitrile) (PAN) is an inert organic polymer with good chemical and physical stability, and can be electrospun into nanofibers with a diameter range of between 150 and 300 nm. PAN nanofibers have excellent mechanical properties without the need for any reinforcing treatment after fabrication and are used widely in the manufacture of water and air filters. PAN has no reactive groups on the surface;

therefore, PAN requires chemical activation to convert highly polar CN groups on the surface to chemically reactive groups. Imidoesterification is the process of changing amide groups to reactive groups on the surface of PAN in the presence of anhydrous hydrogen chloride. This renders the polymer functional and modifiable (Handa et al., 1983; Handa et al., 1982; Hunter and Ludwig 1972).

The objectives of the current study were to exploit the protein and polysaccharide hydrolyzing actions of two industrial proteases and an alpha-amylase for breaking down the EPS in a biofilm, preventing biofilm formation. The hydrolytic enzymes were immobilized onto the surface of PAN nanofibers in an attempt to render the nanofibers resistant to biofilm formation when applied in water filtration technology. Furthermore, the effects of the immobilization process on the activity as well as the enzyme kinetics such as the maximum velocity of the enzyme reaction (V_{max}) and the substrate affinities (K_m) of these enzymes were investigated.

Materials and Methods

Materials

Poly (acrylonitrile) (PAN), JSYK silicone surfactant and HCl gas were form Sigma-Aldrich. The BCA protein determination kit was from Pierce Scientific, and the enzymes Alcalase 2.4L FG, Savinase 16L type EX and BAN 480L were kindly supplied by Novozymes^{TM}, Sandton, South Africa, and were used in their commercial state without further treatment. Other reagents used in these experiments were all of analytical grade.

Synthesizing poly (acrylonitrile) nanofibers

A polymer solution of 6% (wt/v) PAN (Sigma Aldrich) in dimethylformamide (DMF) was prepared. DMF was heated to 90°C and stirred while PAN was added gradually. The mixture was stirred at 90°C for 5 h until a clear, dark yellow solution was obtained. Silicone surfactant, (0.95 g/l) JSYK L580 was added to stabilize bubble formation during bubble-electrospinning. Bubble spinning was set up as illustrated in Fig.1b). A bubble spinning widget for lab scale nanofiber production, designed by Dr. Eugene Smit, was

used. 5ml of the polymer solution was poured into the well of the bubble spinning widget. A copper wire attached to the positive electrode of a high voltage power supply was inserted into the polymer solution, while the negative electrode was attached to a tinfoil collector plate suspended 20 cm above the widget. A current of 100kV was applied.

Surface activation and functionalization of poly (acrylonitrile) nanofibers

Nitrile groups on the surface of PAN nanofibers were activated by imidoesterification in the presence of ethanol and anhydrous HCl. Imidoesters were then covalently replaced by enzymes through amidination in an aqueous environment. This process has successfully been used to immobilize lipase onto PAN nanofibers (Li and Wu 2009).

Imidoesterification of PAN nanofibers

PAN fiber sheets were cut into squares of 2cm x 2cm each with an approximate weight of 3mg. These fibers were submerged in absolute ethanol and HCl gas (Sigma) was bubbled through at a constant rate for 5 min at 30°C. Fibers were removed from the ethanol and rinsed thoroughly with copious amounts of distilled water to remove any residual HCl. The fibers were air dried.

Enzyme immobilization

Three enzymes from Novozymes^{TM} were used: Savinase and Alcalase, both microbial serine proteases, and BAN (Bacterial Amylo Novo) an α-amylase. Activated PAN nanofibers were submerged in undiluted enzyme solutions for 2h. In an aqueous environment, enzymes chemically replace the imidodiester groups that were formed during activation on the –NH groups on the PAN nanofiber surface (Figure 2.). After immobilization, the fibers were washed thoroughly with PBS and allowed to air dry.

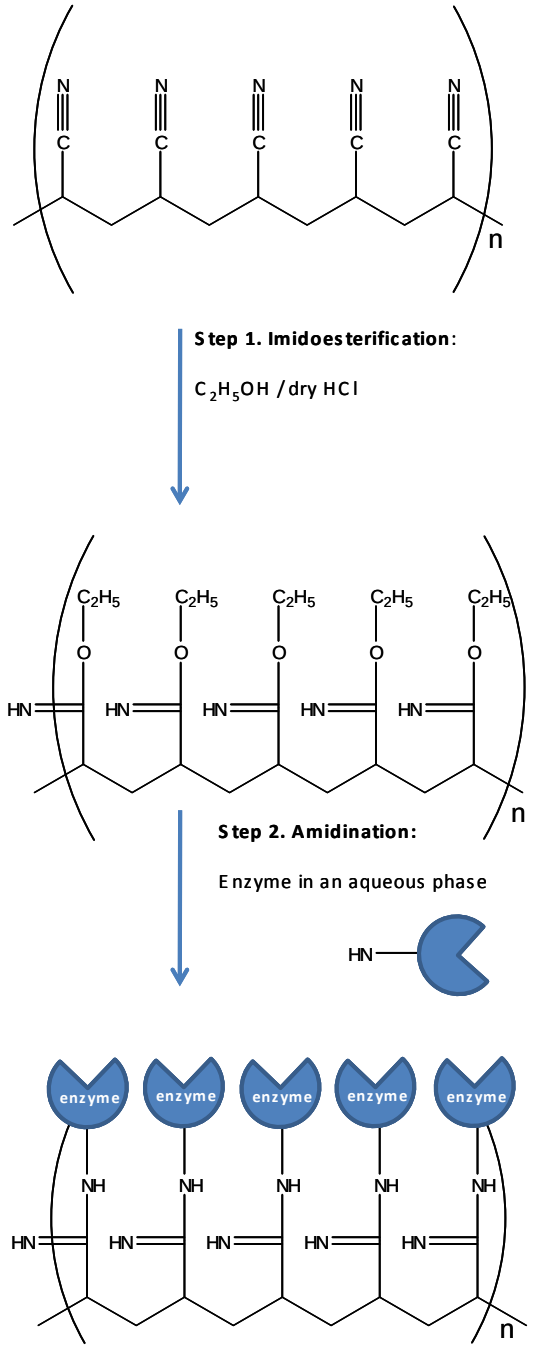


Figure 2. Schematic illustration of the use of the imidoesterification and amidination reactions to immobilize lipase on the PAN nanofibrous membrane.

Characterization of enzyme-functionalized poly (acrylonitrile) nanofibers

Non-activated, activated and functionalized fibers with immobilized enzymes were analyzed by scanning electron microscopy (SEM) and Fourier transfer infrared spectroscopy (FTIR). The infrared spectra of PAN fibers, both activated and native as well as PAN fibers with immobilized enzymes Alcalase, Savinase and BAN were recorded on a Nicolet Avatar 330 FTIR over the wave number range of 600-4000 cm^{-1}. Subsequently, functionalized fibers were subjected to protein determination to confirm the presence of proteins. Immobilized protein on washed and dried fibers with immobilized enzymes were determined by using a bicinchoninic acid (BCA) protein assay test kit^{TM} (Pierce Scientific) with bovine serum albumin as standard. Activated PAN fibers without immobilized enzymes were included as a negative control. Enzyme activity assays were carried out to determine if immobilized enzymes retained activity.

Determining the activity of free enzymes and enzyme-functionalized poly(acrylonitrile) nanofibers

Azocasein assay

Azocasein substrate stock was prepared by dissolving 2,5% (wt/v) azocasein (Sigma) into 50mM Borax NaOH buffer (pH 9.5) with gentle heating and stirring. Experiments were carried out with a volume of free enzyme solution with protein content equivalent to the amount of protein immobilized onto a 3mg functionalized fiber sample, as well as a functionalized fiber sample with immobilized enzymes. For each experiment, 500µl of azocasein solution was added to a tube. Either free enzyme or a 3mg functionalized fiber sample with immobilized enzymes were added, and after gentle mixing, was allowed to react at 30°C for 20 min. After incubation, 1 ml of 5% (wt/v) trichloroacetic acid solution was added to interrupt proteolysis and precipitate any residual azocasein. Samples were centrifuged (19 000 x *g*) for 15 min, and the absorbance of the supernatant was measured at 340nm. Azocasein consists of casein conjugated to an azo-dye. Absorbance at 340nm is a measurement of free azo-dye liberated from hydrolysed azocasein. This is directly

proportionate to the amount of azocasein hydrolysed, and thus it is an indicator of enzyme activity. Protease activity (V_1) was calculated by using the following formula:

$V_1 = \Delta A \times (V/\varepsilon tv)$ (Li and Wu, 2009).

Where (ΔA) represents the change in absorbance at 340nm, (V) is the reaction volume, (ε) is the extinction coefficient of the product of azocasein hydrolysis at 340nm and has a value of 38, (t) represents the reaction time of 2 min and (v) is the volume of the sample used to measure the absorbance.

Starch-iodine assay

The method of Xiao et al. (2006) was used to measure α-amylase activity. Experiments were carried out with a volume of free enzyme solution with protein content equivalent to the amount of protein immobilized onto a 3mg functionalized fiber sample, as well as a functionalized fiber sample with immobilized enzymes. A substrate stock of 2mg/ml soluble starch (Sigma S-2630) was prepared by dissolving the starch in boiling dH_2O. The assay was carried out by reacting 500µl of starch substrate with either the appropriate volume of free enzyme or 3mg of a functionalized fiber sample with BAN immobilized on its surface at room temperature for 30 min. Subsequently, 100µl of the reaction mixture was plated out into a 96 well titre plate. 100µl of iodine reagent (5mM I_2 and 5mM KI) was added to each well, and the absorbance was measured at 580nm. Starch undergoes a colorimetric reaction with iodine reagent, forming a starch iodine complex that can be measured at 580nm. A decrease in iodine binding starch after hydrolysis with BAN was measured spectrophotometrically at 580nm as an indication of α-amylase activity. The formula used to calculate the activity of BAN was:

$V_1 = [(\Delta A_{580})/(A_{580}\ 1mg\ starch)]/[(t)/(v)]$ (Xiao, Storms and Tsang, 2006)

Where (ΔA_{580}) represents the change in absorbance at 580nm that took place during the reaction time. (A_{580} 1mg starch) represents the absorbance of 1mg/ml of starch substrate that was reacted with iodine reagent at 580nm. (t) Is the reaction time of 30 min and (v) represents the reaction volume.

Re-usability of immobilized enzymes

The re-usability of immobilized Alcalase and Savinase was tested by re-using the same functionalized fibers 10 times for azocasein hydrolysis. For BAN, the same functionalized fibers were re-used 10 times for starch hydrolysis. The percentage of specific activity retained relative to the specific activity after one use was calculated.

Comparing the activity of free enzymes versus immobilized enzymes

The specific activity of all the enzymes in their free form and their immobilized forms were determined (Table 4). Enzyme activity was also studied as a function of substrate concentration, time and enzyme concentration. The Michaelis Menten constant (K_m) and the maximum velocity (V_{max}) were determined by using the relationship between the initial rate of the reaction (V_o) at various substrate concentrations. When initial velocity (V_o) was plotted against substrate concentration (Figure 5 (a-c)), hyperbolic curves were obtained, indicating Michaelis-Menten kinetics and the dependence of the reaction rate on substrate concentration.

Data manipulation and statistical analysis

GraphPad Prism® version 5.00 for Windows (GraphPad Software) was used for graphical representations and statistical analyses. All the error bars represent the standard error of the mean (SEM) of three independent experiments, where each point was determined in triplicate.

Determining the anti-biofouling activity of functionalized nanofibers

A mixed culture of biofilm forming bacterial pathogens was prepared. Three pathogens, each possessing a stable copy of a *Photorhabdus luminescens lux* operon on the bacterial chromosome, rendering them bioluminescent, were used. They were Gram negative

Pseudomonas aeruginosa (Xen 5), *Escherichia coli* (Xen 14) and Gram positive *Staphylococcus aureus* (Xen 36) from Caliper life sciences, Hopkinton,MA, USA.

The mixed culture was inoculated into 10ml of BHI medium and incubated overnight at 37°C without agitation. Subsequently, 1 ml of the overnight culture was added to 50ml BHI medium in an Erlenmeyer flask, and was incubated on a shaker for 6h to obtain a culture in log phase. The cells were harvested by centrifugation at 1000 x *g* for 10 min. The supernatant was decanted, and cells were washed by suspending in physiological saline solution, followed by another centrifugation step. This was repeated three times to ensure that all nutrients were removed from the culture. The cells were kept in suspension after the final wash step.

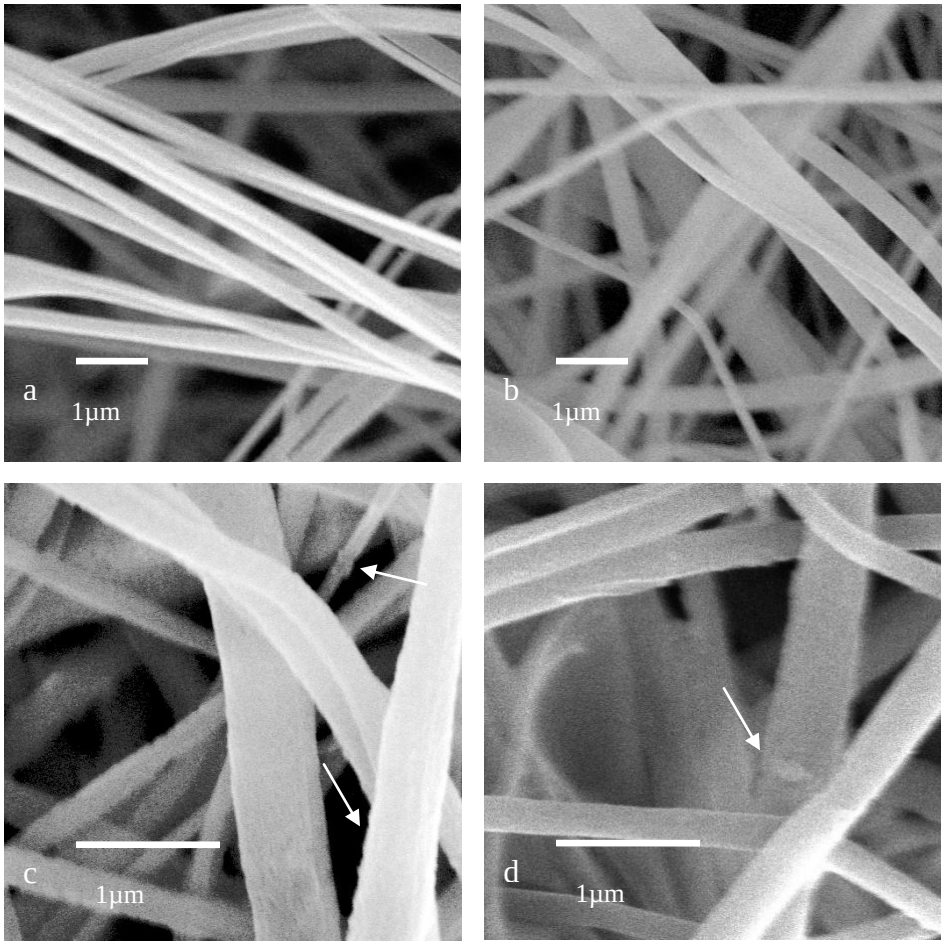
Flasks with test cultures were prepared by diluting the prepared bacteria to approximately $1x10^7$ cells per ml in 150 ml physiological saline. Fiber squares of 2 x 2cm (0.003g) of functionalized with Alcalase, Savinase and BAN respectively, as well as a cocktail of Alcalase, Savinase and BAN were submerged into separate flasks with test cultures. Two negative controls were included, namely non-activated neat PAN fibers, and activated PAN fibers that did not have enzymes immobilized on their surface. Flasks were sealed and placed on a shaker, 25 rpm at 37°C for 20 h. After the incubation period, viability of cultures was confirmed by inspecting their bioluminescence using the IVIS *in vivo* imaging system 100 series (Caliper life sciences, Hopkinton MA). The cultures in all the flasks still had bioluminescence and thus viability after 20 h.

The fibers were removed from the test cultures and were rinsed with copious amounts of sterile distilled water to remove any loose cells possibly remaining on the fibers. Any growth that may have adhered to the fibers was fixed by submerging the fibers in 2.5% (v/v) gluteraldehyde in PBS at 4°C for 4 h. This was followed by a stepwise dehydration with 25, 50, 75 and 100% ethanol. Fibers were allowed to air dry and were prepared for SEM imaging by fixing on carbon adhesive tape and sputter coating with gold.

Results and discussion

SEM

The amidination process did not have a noticeable effect on the structure of PAN nanofibers (Figure 3). Non-activated PAN (a) and activated PAN nanofibers (b) showed a uniform visual appearance with similar fiber diameter and surface morphology. After enzyme immobilization of Alcalase (c), BAN (d) and Savinase (e), a rougher surface morphology and small particles attached to the nanofiber surface were visible, indicating the presence of enzymes. The particles were likely to be aggregated enzymes, adsorbing to immobilized enzymes by molecular interactions (Li and Wu, 2009).



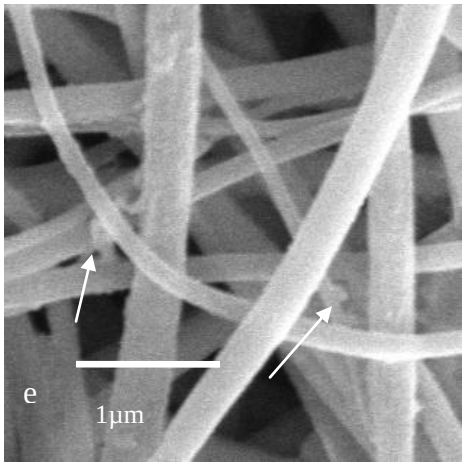


Figure 3. SEM micrographs of PAN (a) and activated PAN (b) (5000x) and activated PAN with immobilized Alcalase (c), BAN (d) and Savinase (e) (10 000x).

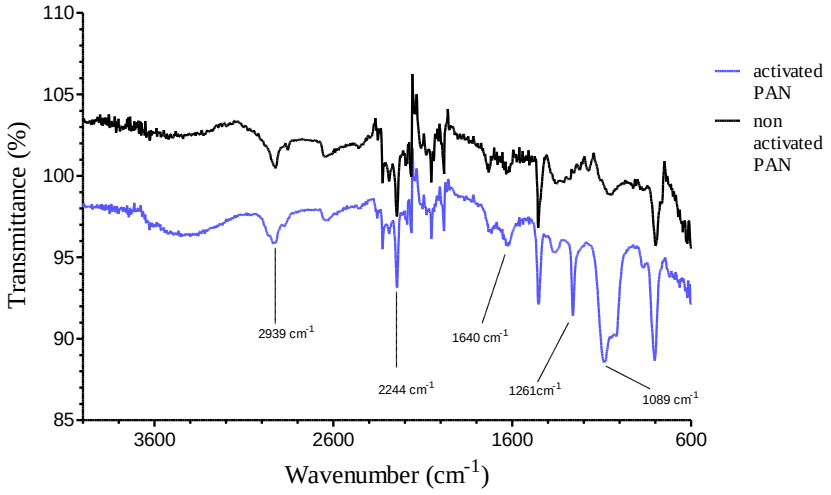
FTIR

To determine the successful activation of nitrile groups on the surface of PAN, as well as the subsequent immobilization of enzymes on the surface of PAN, the infrared (IR) spectra of native PAN fibers were measured and compared to that of activated PAN fibers (Fig 4a) and fibers with immobilized enzymes Alcalase, Savinase and BAN (Fig 4b-d). Present in all the spectra were the characteristic peaks of PAN at 2244 cm^{-1}, representing stretch vibration of the nitrile groups (C≡N) and another at 2939cm^{-1} due to the stretch vibration of methylene groups (CH_2). The presence of a C≡N stretch vibration in activated PAN indicated incomplete activation of all the C≡N groups. When comparing the spectra of non-activated PAN and activated PAN (Fig 4a) it was observed that two peaks were present at 1261 cm^{-1} and 1089cm^{-1} in activated PAN that were not present in non-activated PAN. These can be attributed to the stretch of the C-O bonds in the imidoesterified PAN. Activated PAN showed stronger absorbance at around 1640 cm^{-1}, indicating the presence of imines (C=N). When comparing the spectra of fibers with immobilized enzymes (Fig. 4b-d) to that of activated PAN fibers, they all showed similar general strength of absorbance. A decrease in the peak at 1261 cm^{-1} and at 1089 cm^{-1} was observed in all the immobilized enzyme fibers due to the replacement of the C-O-CH_5 imidoester groups with the amino bonds of the enzymes. Fibers with Savinase and BAN

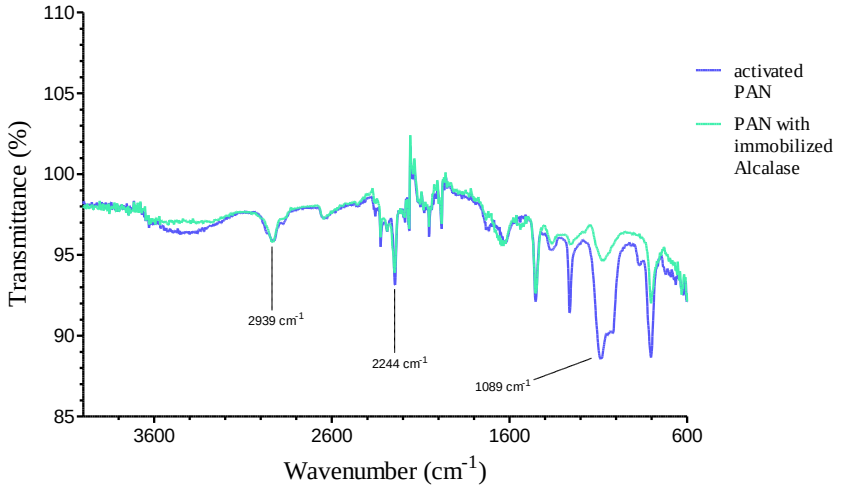
showed peaks at around 3400 cm^{-1}, representing a N-H stretch of the newly formed N-H groups at the site of enzyme immobilization as well as peaks near 1350 cm^{-1} representing the presence of C-N amine bonds. From the lack of a peak near 3400 cm^{-1} and 1350 cm^{-1} in fibers with Alcalase, it was suspected that Alcalase immobilization was less successful than that of BAN and Savinase. This needed to be confirmed by further analysis of enzyme activity.

Table 1. Summary of relevant IR spectra. (Harwood et al., 1998 ; Pavia et al., 2001)

Frequency (cm^{-1})	**Functional group**	**Comment**
2270-2200	C≡N	often a weak absorption band
3100-2700	CH_2	variable intensity
1300-1000	C-O	strong
1650-1500	C=N	variable intensity
1640-1560	N-H	broad
1350-1000	C-N	stretch



(a)



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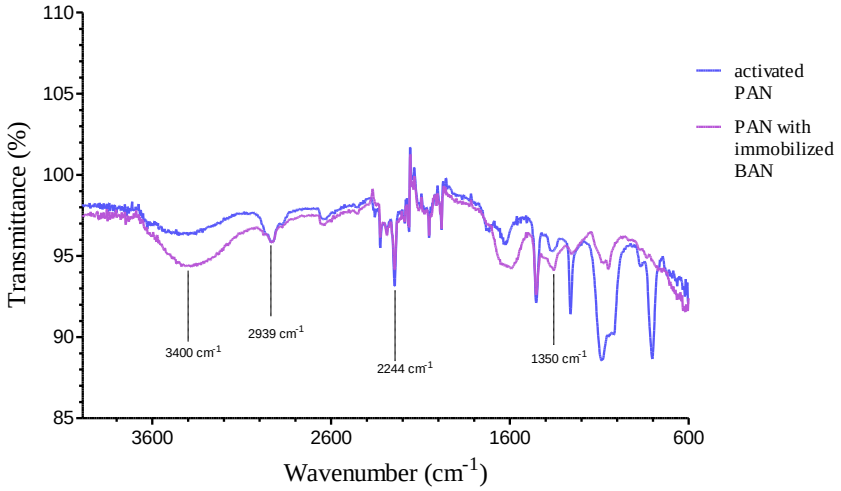
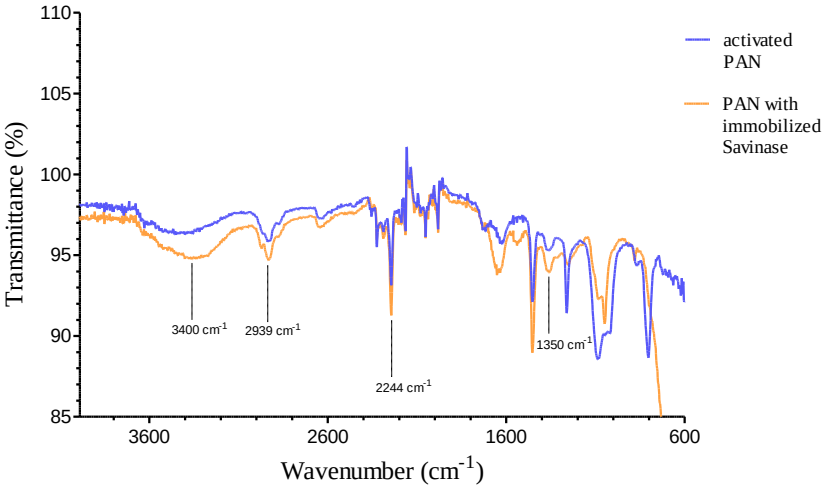


(c)



(d)

Figure 4. IR spectra of PAN fibers (a) non activated and activated (b) Activated with immobilized alcalase (c) activated with immobilized BAN (d) activated with immobilized Savinase.

Protein loading

The concentration of proteins on 3mg of nanofibers with immobilized Alcalase, Savinase and BAN were determined by comparing the absorbance at 562nm to that of a BSA standard. Fibers with immobilized Alcalase had 932µg of protein present, while fibers with Savinase had 1335µg of protein present and BAN had 1150 µg of protein present, confirming that immobilization of Alcalase was less successful than that of BAN and Savinase (Table 2).

Table 2. Protein immobilization

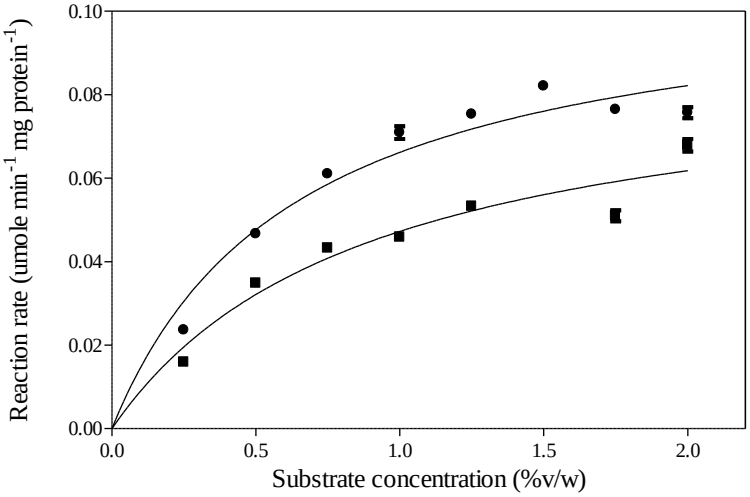
	protein / 3mg fibers (µg)	protein in free enzyme solution (µg/ml)	free enzyme solution equivalent in protein contents to 3g of PAN with immobilized enzyme (µl)
Alcalase	931.89	129927	7
BAN	1335.12	22894	58
Savinase	1150.17	15821	72

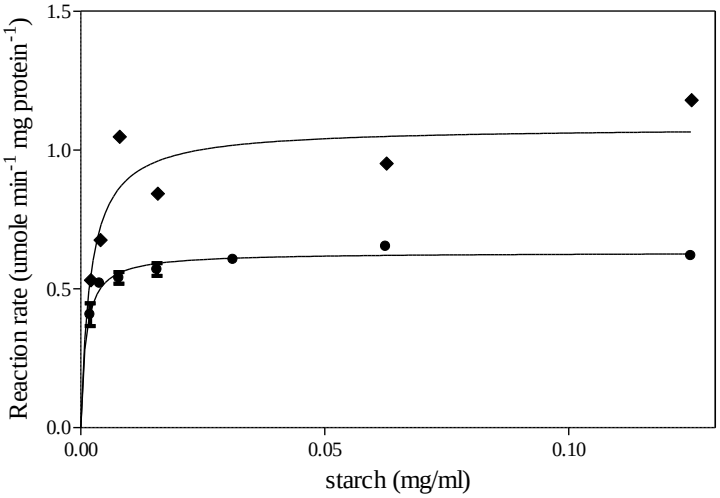
Enzyme activity assays

An increase in K_m after immobilization is usually caused by conformational changes brought about by the immobilization process, and means the enzyme now has a lower affinity for its substrate compared to that of the free enzyme. This is the case with immobilized Alcalase, Savinase and BAN (Figure 5 (a-c)). V_{max} usually decreases after enzyme immobilization (Figure 6 (a-c)). This was not the case with BAN, which showed an increase in V_{max} after immobilization. This observation could indicate that the maximum reaction velocity increases after enzyme immobilization. It should be noted however, that BAN does not show a good fit to Michaelis Menten kinetics, and may thus falsely indicate an increase in V_{max}. However, of the three enzymes, BAN was immobilized most efficiently onto PAN, and also had a higher specific activity after immobilization (Table 3). This makes BAN more effective in its immobilized state as opposed to the free enzyme. Alcalase retained 90% of its specific activity after immobilization and Savinase retained 83%.

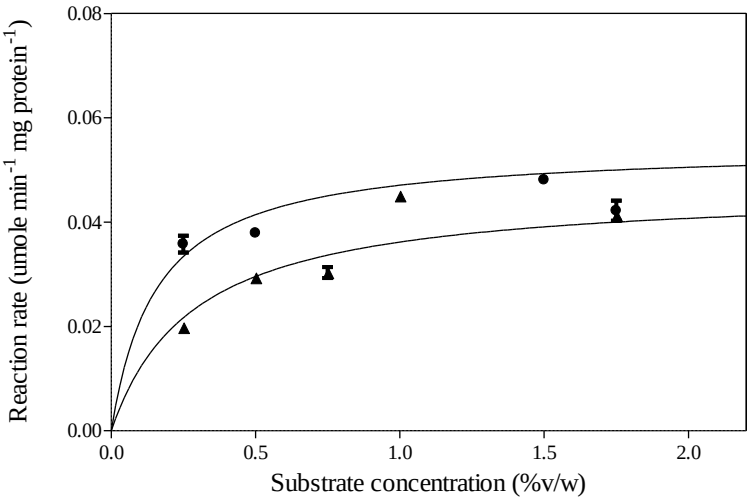
Enzyme activity assays for free as well as immobilized enzymes were carried out. Table 2 indicates the volume of free enzyme that contains the equivalent amount of proteins that are immobilised onto the nanofibers. For each enzyme, equivalent protein amounts of the free and immobilized forms were assayed for activity and Michaelis Menten kinetics.

5(a)



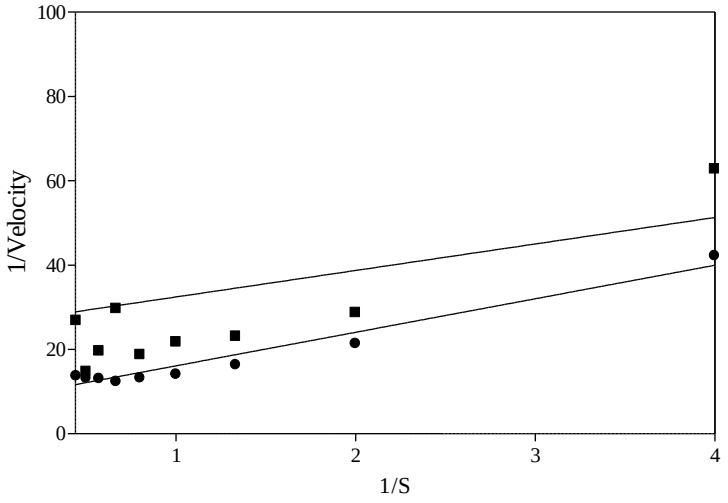


5(b)

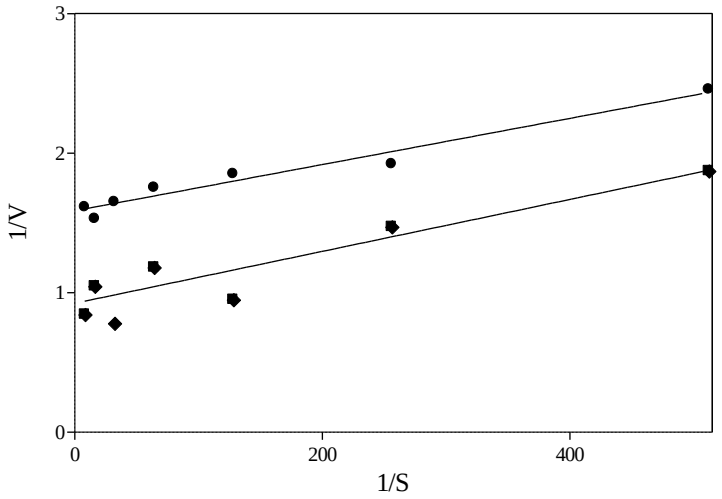


5(c)

Figure 5. Michaelis Menten plots of free (●) vs. immobilized enzyme kinetics (a) Alcalase (■) (b) BAN (♦) (c) Savinase (▲)



6(a)



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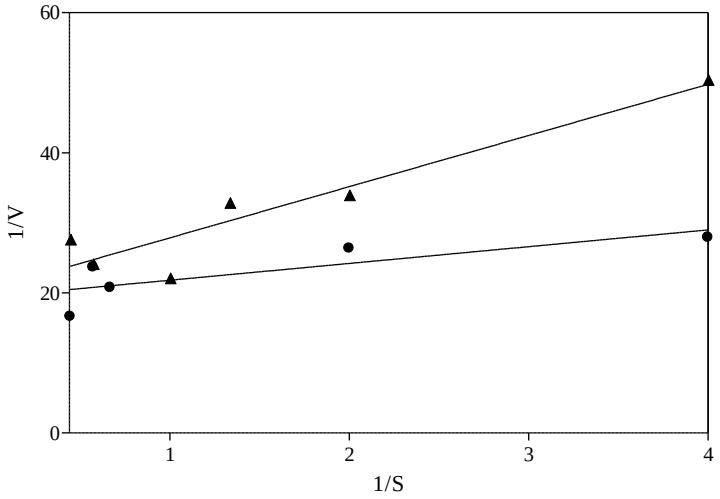
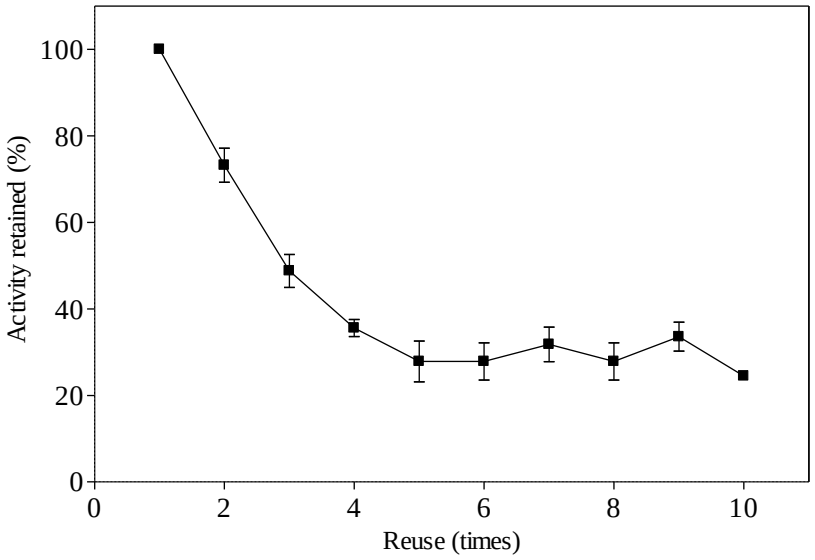


6(c)

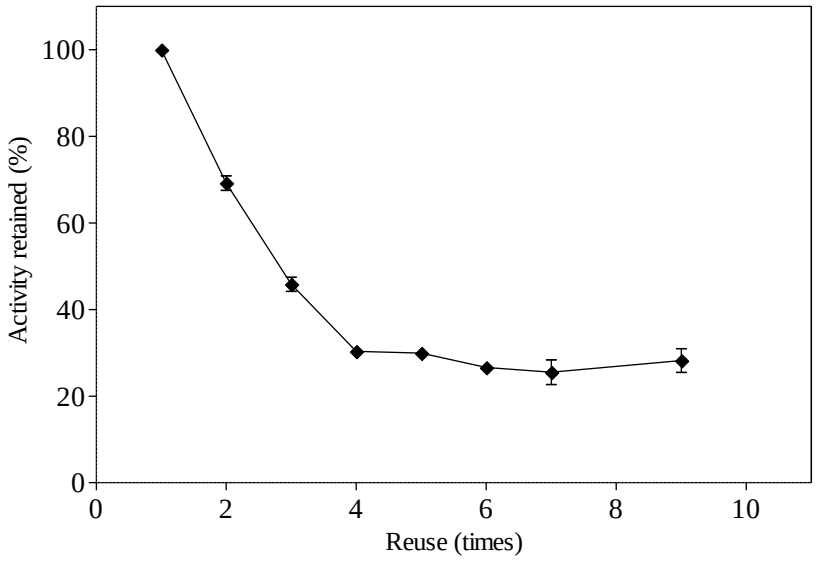
Figure 6. Lineweaver Burke plots of free (●) vs. immobilized enzyme kinetics (a) Alcalase (■) (b) BAN (♦) (c) Savinase (▲)

Reusability

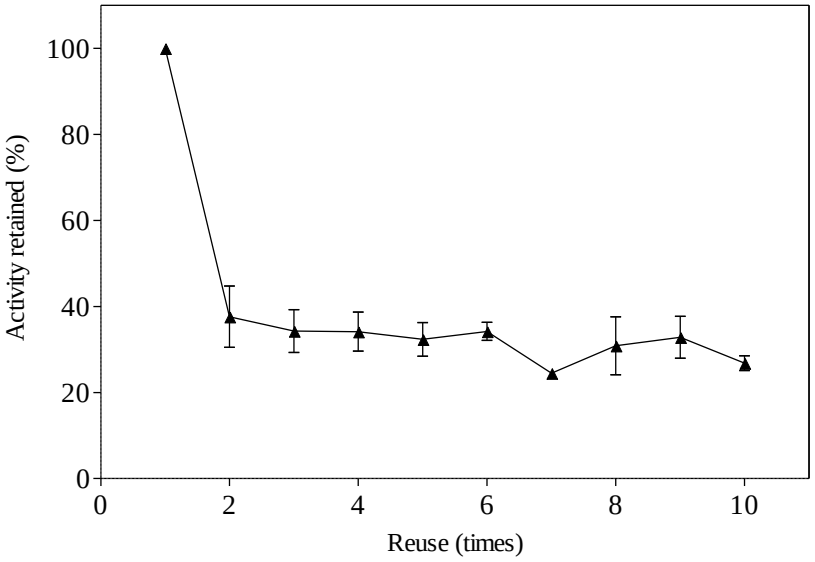
It is clear that the specific activity of Alcalase decreased by roughly 30% after each of the first 3 uses (Figure 7a). After that, a constant specific activity of just below 30% was retained. This was similar for BAN (b). Savinase (c) lost most of its specific activity after the first use, but retained about 30% of activity after subsequent usage (Table 3).



7(a)



7(b)



7(c)

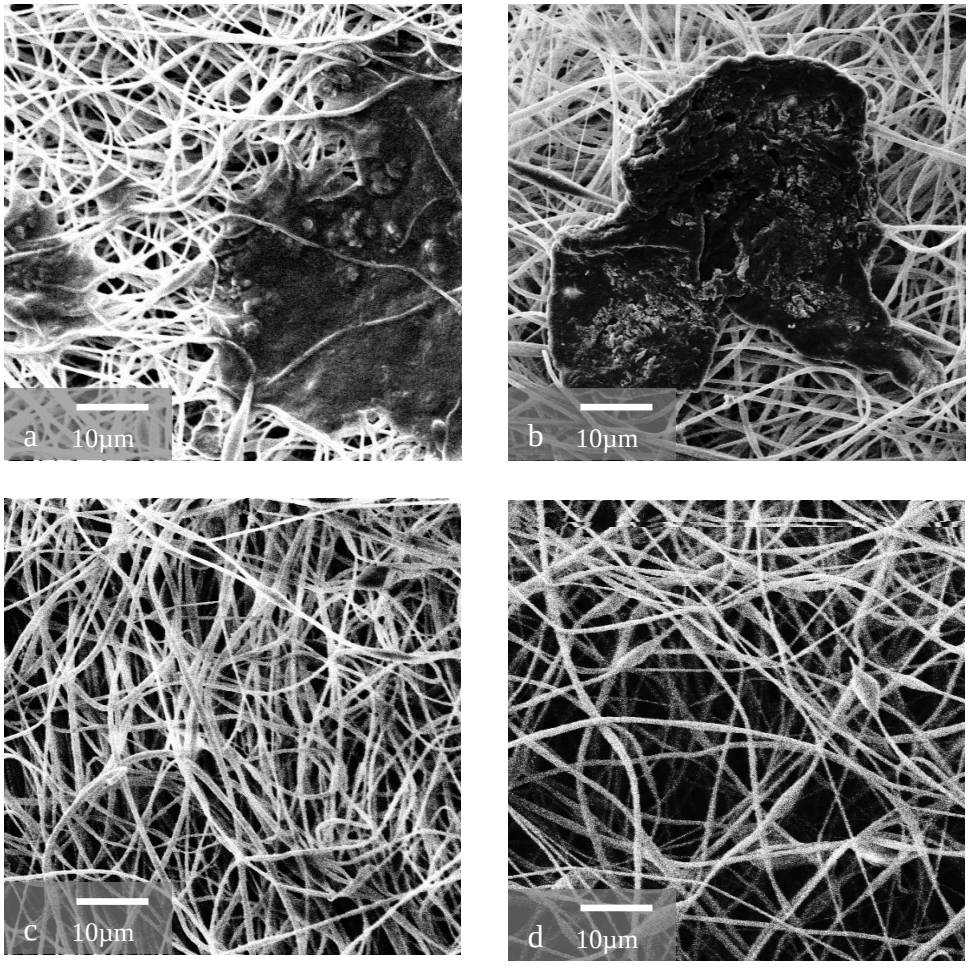
Figure 7. Reusability of immobilized enzymes (a) Alcalase (■) (b) BAN (♦) (c) Savinase (▲)

Table 3. Summary of the effects of immobilization on Alcalase, BAN and Savinase.

Enzyme immobilized	Specific activity (µmol ml-1 min-1 µg protein-1)		Specific Activity retention	Protein loading (mg/g matrix)	Vmax (U/mg)		Km (mM)		Activity retained after 10 times use
	Free enzyme	Immobilized enzyme			Free enzyme	Immobilized enzyme	Free enzyme	Immobilized enzyme	
Alcalase	$8.12x10^{-5} \pm 1.42x10^{-6}$	$7.28x10^{-5} \pm 1.57x10^{-6}$	90%	931.89 ± 236.77	0.108 ± 0.005	0.089 ± 0.007	0.638 ± 0.093	0.890 ± 0.172	25%
BAN	$4.89x10^{-4} \pm 6.39x10^{-6}$	$7.15x10^{-4} \pm 2.97x10^{-5}$	100%	1335.12 ± 209.37	0.631 ± 0.013	1.082 ± 0.0461	0.001 ± 0.0002	0.002 ± 0.0004	28%
Savinase	$5.76x10^{-5} \pm 1.57x10^{-6}$	$4.78x10^{-5} \pm 8.16x10^{-6}$	83%	1150.17 ± 66.47	0.055 ± 0.003	0.046 ± 0.003	0.157 ± 0.058	0.28 ± 0.0833	27%

Anti-biofilm activity

Functionalized PAN nanofibrous mats incubated with biofilm forming bacterial cultures were studied by SEM imaging the 2 x 2cm fibre squares in quadrants. Visible patches of biofilm growth was present in at least 3 out of 4 quadrants on both activated and non activated PAN fibers with no enzymes immobilized on them (Figure 8 (a) and (b)). No biofilm growth was detected on fibers functionalized with Alcalase (c) BAN (d) or Savinase (e).



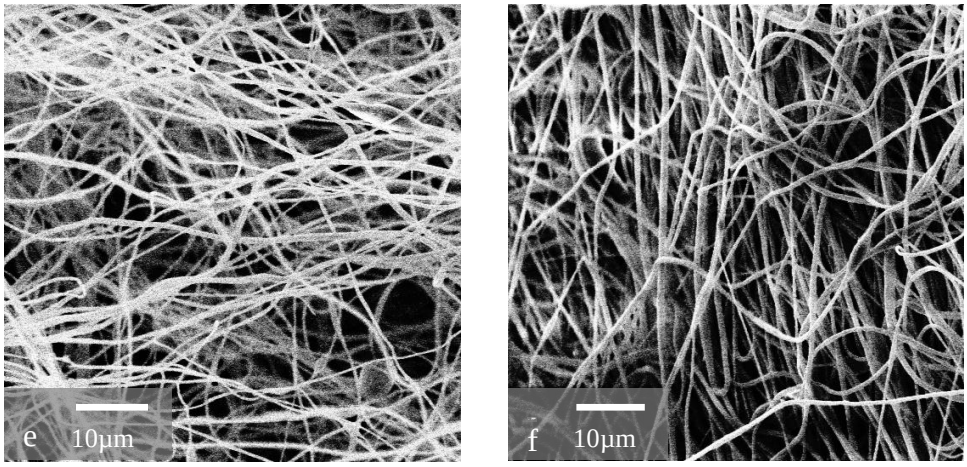


Figure 8. SEM micrographs of non activated PAN nanofibers (a), activated PAN nanofibers with no immobilized enzymes (b) and PAN nanofibers with immobilized enzymes (c) Alcalase (d) BAN (e) Savinase and (f) a cocktail of Alcalase, Savinase and BAN, after incubation with biofilm forming organisms (magnification 1K X).

Conclusion

Non-woven mats of PAN were successfully fabricated through both the processes of needle electrospinning and bubble-electrospinning. Confirmed by FTIR, these nanofibers were successfully activated by amidination, allowing the covalent immobilization of respectively two serine proteases and an α-amylase onto the fibers. When inspected visually, fibers largely retained their original morphology after activation and enzyme immobilization. Immobilized enzymes were, however visible as aggregated particles on the nanofiber surfaces. The large surface area to volume ratio provided by the nanofibers as immobilization surface, allowed sufficient amounts of enzymes to be immobilized onto the fibers so that all enzymes retained above 80% of the specific activity of the free enzymes. For each of the immobilized enzymes, just below 30% of initial activity was retained after 10 repeated cycles of use. When considering the combined advantages of this effective immobilization process, the robustness of the enzymes used in this study, and their effectiveness against biofilms in their immobilized state, a valuable addition has been made to technology available for the control of biofilm formation on filtration membranes, and could potentially be employed to control biofilm formation in water filtration systems.

Acknowledgements

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CHAPTER 5

General discussion and conclusions

In the light of the urgent need for development of new, more efficient, accessible, economically viable and environmentally friendly techniques of water sanitation products, current research developments in the field of nanobiocides, nanofiltration, enzymatic control of biofouling and the efficient immobilization of enzymes onto nanofibers offer promising solutions.

Conventional disinfection methods in water treatment often include the use of large amounts of chemical disinfectants, which produce harmful by products (Krasner *et al*., 2006). Nanobiocides, such as noble metal nanoparticles, and silver nanoparticles in particular, offer an alternative method of disinfection without reacting with the water itself, meaning no harmful by products are added to the water.

The use of silver nanoparticles as biocide and the incorporation of silver nanoparticles into electrospun nanofibers to yield anti-microbial nanofibers are well researched fields. Silver is the most commonly incorporated biocide in electrospun nanofibers (Teo and Ramakrishna, 2009), and a wide range of polymers have been used in the fabrication of polymer nanofibers that include silver nanoparticles.

In Chapter 3, a study was conducted to determine how using different types of polymers in the fabrication of anti-microbial nanofibers, influences the properties of the final product. Different types of polymer including a silver salt for the formation of silver nanoparticles by *in situ* reduction were used to fabricate anti-microbial fibers. The fibers were then compared based on the morphology of the fibers, the toxicity of the fibers and most importantly, the anti-microbial efficacy of the fibers when specifically applied in water disinfection.

Two types of polymers, namely PVA and PAN were used to synthesize nanofibers by electrospinning, incorporating $AgNO_3$ into the polymer solutions, which were reduced to

silver nanoparticles by UV irradiation. PVA, a water soluble, non-toxic, biodegradable synthetic polymer that can be cross-linked to be water insoluble has successfully been used to fabricate highly effective anti-microbial nanofibers containing silver nanoparticles (Chun et al., 2010). PVA was chosen for this study due to its environmentally friendly characteristics, and the ease with which nanofibers are fabricated from it by electrospinning. PAN which is not biodegradable, and is dissolved in DMF, on the other hand, was chosen for this study because of its durability in water without the need for stabilization via cross-linking, and the ease with which nanofibers can be spun from PAN. Anti-microbial PAN nanofibers containing silver nanoparticles were also previously successfully fabricated (Lala et al., 2007).

The results obtained in Chapter 3 showed that when PVA and PAN nanofibers containing silver nanoparticles were compared based on the morphology of the fibers, PVA and PAN nanofibers showed similar morphology, with a more even distribution of silver nanoparticles in the PAN fibers. PAN fibers also had higher silver nanoparticle content than PVA fibers. Neither type of fibers showed significant signs of silver leaching in water, thus no toxicity. When considering anti-microbial activity, both PVA and PAN nanofibers showed excellent anti-microbial activity, with PVA nanofibers killing between 91% and 99% of bacteria in a contaminated water sample, and PAN with a slightly improved anti microbial activity, killing 100% of bacteria in water samples contaminated by 3 different pathogens.

From these results it was concluded that either type of polymer nanofibers have sufficient anti-microbial activity, with only minor differences with respect to silver nanoparticle distribution and content. It can be noted that the milieu in which the fibers will be applied will be the deciding factor in which type of fiber to use. In an environmentally sensitive milieu, for instance when applied in a disposable filtration product, the use of PVA fibers are recommendable due to their biodegradability, whereas in a high throughput system, the use of PAN nanofibers will be more favorable because of better robustness.

There is growing evidence that silver nanoparticles exhibit cytotoxic effects on higher organisms, raising the need for further investigation into the impact of the use of silver as a nanobiocide on the environment and human health (Marambio-Jones., 2010), especially when used in water treatment. Furthermore, the exact mechanism of silver nanoparticles as biocides has yet to be fully elucidated. Future research will include further studies into the environmental impact of silver nanoparticles when released into the environment, the cytotoxicity of silver nanoparticles in mammalian cell lines and further studies into the anti-microbial mechanism of action of silver nanoparticles.

Silver nanobiocide can be included into nanofibers through a simple process, yielding efficient anti-microbial nanofibers. Equally successful is the immobilization of hydrolytic enzymes onto the surface of polymer nanofibers. When using enzymes targeted specifically against the components of a biofilm, anti-biofouling nanofibers are created. Anti-microbial nanofibers will eradicate the viability of contaminant cells, but will not remove the biomass remaining from dead cells when used to filter contaminated water. The remaining biomass is likely to accumulate on the nanofibers and in the pores of the nanofiber mat, blocking filtration efficiency, and providing substrate for biofouling.

In Chapter 4, a study was done to address the problem of biofouling on filtration membranes. Polymer nanofibers with immobilized hydrolytic enzymes on the surface were fabricated and tested for hydrolytic activity and resistance against biofilm formation. PAN nanofibers, with only inert groups on the surface were chemically activated by amidination for the covalent immobilization of respectively two serine proteases and an α-amylase onto the fibers. The large surface area to volume ratio provided by the nanofibers as immobilization surface, allowed sufficient amounts of enzymes to be immobilized onto the fibers so that all enzymes retained above 80% of the specific activity of the free enzymes, and could be re-used. Nanofibers with enzymes immobilised on the surface did not support the growth of biofilms, as opposed to nanofibers with no enzymes on the surface.

From the results in Chapter 4, it was concluded that covalent immobilization of serine proteases and amylase onto PAN nanofibers was possible through prior chemical activation of PAN by amidination. Furthermore, the enzymes retained activity and prevented biofilm formation on PAN nanofibers. Thus, anti-biofouling nanofibers have been developed.

Future research can include the investigation of other enzyme immobilization methods onto polymer nanofibers, and how they compare to covalent immobilization. Furthermore, the process of PAN activation through amidination can be further optimized to improve in immobilization efficiency, and thus enzyme activity retained. The use of enzymes with different target substrates in different combination ratios can also be investigated for improved activity against biofilms.

When considering the combined advantages of this effective immobilization process, the robustness of the enzymes used in this study, and their effectiveness against biofilms in their immobilized state, a valuable addition has been made to technology available for the control of biofilm formation on filtration membranes, and could potentially be employed to control biofilm formation in water filtration systems.

Further studies need to be done into the combination of antimicrobial nanofibers with nanofibers with immobilized enzymes into a single nanofiltration product, which will have both anti-microbial and anti-biofouling properties. Such a product will be highly applicable in water treatment systems.

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