GENERAL INTRODUCTION

General introduction

During the past decades, significant advancement has been made in the development of delivery systems for biomedical applications especially those involving drug delivery [1]. Polymer-based delivery systems provide a considerable platform for drug delivery technology primarily because of their ease of processing and the ability of researchers to readily control their chemical and physical properties via molecular synthesis. Numerous polymeric-carriers have been developed for the controlled delivery of biologically active molecules such as polymer-drug conjugates, micelles, particulate systems (capsules and spheres), sponges, films and fibers. Among polymeric drug delivery systems, researchers are now turning to advances in the worlds of micro- and nanotechnology [2].

1. Polymer-based delivery systems for topical biomedical applications

Polymers, the most versatile class of materials, have changed our day-to-day lives over the past several decades. However, the distinction between temporary and permanent biomedical applications of polymers was made only 30 years ago. Subsequently, the amalgamation of polymer science with pharmaceutical sciences led to a quantum leap in terms of 'novelty' (flexibility in physical state, shape, size and surface) in design and development of novel drug-delivery systems. Polymeric delivery systems are mainly intended to achieve temporal and/or spatial control of drug delivery [3].

Regarding topical biomedical applications, polymer-based delivery systems open up a number of opportunities with regard to controlled local drug delivery in many skin diseases, postoperative local chemotherapy, the management of wound-related conditions, prevention of post-surgical adhesions, tissue support and wound healing in tissue engineering [4]. In this context, polymer nanofibers are gaining growing attention as a multifunctional drug delivery scaffold of value in biomedical applications involving cell regeneration such tissue engineering and regenerative medicine [5] as well as the healing of cutaneous wounds [6].

2. Nanofibers as drug delivery scaffolds

Nanofibers (NFs) and ultrafine fibers are produced mainly from polymers treated in a specific manner to form threads of a few micrometers to nanometers in diameter. The three-dimensional structures with large surface to volume ratio and superior mechanical performance renders nanofibers the ideal matrix to develop super fine structures [7, 8]. These fibers have extremely high specific surface area due to their small diameters, and nanofiber mats can be highly porous with excellent pore interconnectivity. These unique characteristics plus the functionalities from the polymers themselves impart nanofibers with many desirable properties for advanced applications [9]. The possibility to immobilize antibiotics, enzymes, antimicrobial peptides, and growth hormones to nanofibers, either by encapsulation into fiber matrices or by surface treatment, opens a new field in biomedical engineering [10, 11].

The development of nanofibers has enhanced the scope for fabricating scaffolds that can potentially mimic the architecture of natural human tissue at the nanometer scale. The high surface area to volume ratio of the nanofibers combined with their microporous structure favors cell adhesion, proliferation, migration, and differentiation, all of which are highly desired properties for tissue engineering applications [12].

2.1. Nanofibers fabrication methods

Currently, there are three main techniques available for the synthesis of nanofibers: electrospinning, self-assembly, and phase separation, though new methods such as those based on nanospider technology [13] and ultrahigh-speed extrusion [14] have been developed. Of these, electrospinning is the most widely used technique for nanofibers fabrication [15]. Electrospinning represents an attractive technique for the processing of polymeric biomaterials into nanofibers. This technique also offers the opportunity for control over thickness and composition of the nanofibers along with porosity of the nanofiber meshes using a relatively simple experimental setup.

2.1.1. The electrospinning process

Electrospinning, schematically presented in Figure 1 , is the most cost effective and easiest way to produce large volumes of nanofibers. The process of electrospinning has been known for almost 100 years and first appeared in patent literature in 1902 [16]. The crucial patent was issued to Formhals [17] in 1934, in which the electrospinning of plastics was described for the first time. Later, Taylor [18] found that the pendant droplet attached to the spinneret develops into a cone (now called the Taylor cone) when the surface tension is balanced by electrostatic forces. Reneker and co-workers [19, 20], revived the interest in this technology in the early 1990s and described both fundamental and application-oriented research on electrospinning. The electrospinning method can be used for nanofibers fabrication using a polymer solution or polymer melt [7].

As presented in Figure 1, the electrospinning set-up consists of a pump to drive the solution into the spinneret and a high voltage electric source with positive or negative polarity. One electrode is placed in a polymer solution and the other electrode is linked to a conducting collector, which is usually a stationary or rotating metal screen, plate, or wheel. In addition, a syringe, and a conducting collector are used [21, 22].

The polymer solution or the melt to be spun is forced through a syringe pump to form a pendant drop of the polymer at the tip of the capillary. This droplet is subject to a high electrical voltage (8–50 kV) which leads to electrostatic charging of the fluid [23] As the applied potential reaches a critical value required to overcome the surface tension of the liquid, the droplet undergoes a cone-shaped deformation resulting in the formation of the so- called Taylor cone (Figure 2) [24]. A jet is ejected from the apex of the Taylor cone in the direction of the counter electrode and becomes narrower in the process [23, 25]. After the initiation from the cone, the jet undergoes a bending and whipping instability [26] and is field directed towards the oppositely charged collector. As the jet travels through the atmosphere, the solvent evaporates (or the melt

solidifies), leaving behind dry fibers with diameters ranging from micrometers to nanometers on the collecting device [27].

For integrated functionalization, the target bioactive molecule can be mixed with the polymer solution prior to electrospinning. This technique is known as blending electrospinning that is commonly employed for producing fibers with a drug releasing functionality [28, 29]. Drug release can be employed in tissue engineering scaffolds to enhance tissue regeneration, reduce the risk of infection [30] and to suppress adverse tissue reactions [31]. However, blend electrospinning has its own limitations, since most sensitive proteins and cytokines will denature in the presence of the solvents and lose their bioactivity. Nonetheless, many research groups have reported success using this strategy to functionalize fibers with biomolecules such as, heparin or plasmids [32, 33]. Alternatively, water soluble polymers such as poly (vinyl alcohol) (PVA) or poly(ethylene oxide) (PEO) have been used as NFs matrix materials for proteins [34, 35].

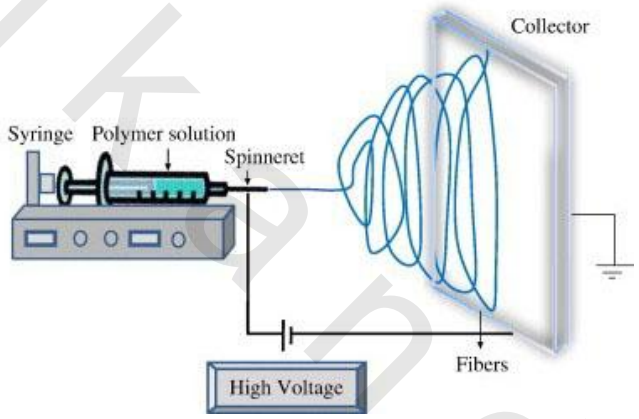

Figure 1: Schematic diagram of set up of electrospinning set-up [22].

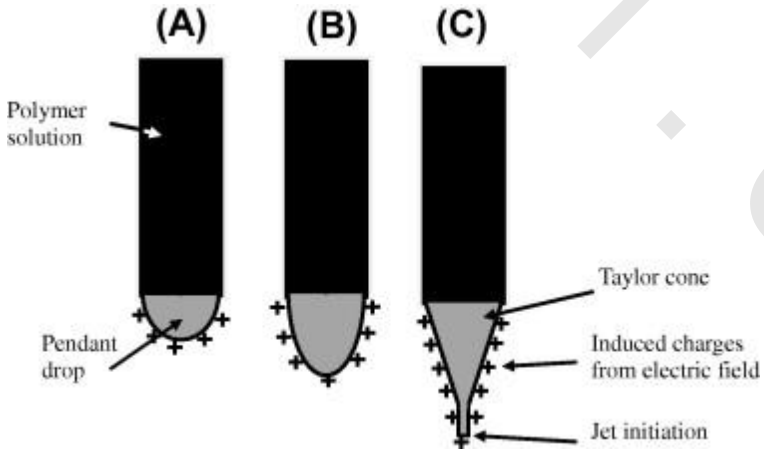

Figure 2: Schematic illustration of the Taylor cone formation: (A) Surface charges are induced in the polymer solution due to the electric field. (B) Elongation of the pendant drop. (C) Deformation of the pendant drop to the form the Taylor cone due to the charge-charge repulsion. A fine jet initiates from the cone [24].

2.1.2. Parameters affecting nanofibers formation by electrospinning

The electrospinning process is governed by many parameters, classified broadly into polymer solution parameters, process parameters and ambient parameters. Polymer solution parameters include viscosity, conductivity, as affected by the polymer molecular weight, viscosity and surface tension. Process parameters include the applied electric field, tip to collector distance and feeding or flow rate. Each of these parameters significantly affects the fibers morphology obtained as a result of electrospinning. By proper manipulation of these parameters, nanofibers of desired morphology and diameters can be obtained [36]. In addition to these variables, ambient parameters encompass the humidity and temperature of the surroundings which play a significant role in determining the morphology and diameter of electrospun nanofibers [37]. Table 1 shows the various electrospinning parameters and their effects on fiber morphology.

Table 1: Electrospinning solution, processing and ambient parameters and their effects on fiber morphology [22, 38].

Parameters	Effect on fiber morphology
Polymer solution parameters	
Viscosity	Low viscosity - beads generation High viscosity -increase in fiber diameter, disappearance of beads.
Polymer concentration	Increase in fiber diameter with increase of concentration.
Molecular weight of polymer	Reduction in number of beads and droplets with increase of molecular weight.
Conductivity	Decrease in fiber diameter with increase in conductivity
Surface tension	No conclusive link with fiber morphology, high surface tension results in instability of jets.
Solvent volatility	High volatility - Fibers exhibit microtexture (pores on their surfaces, which increase surface area)
Processing parameters	
Applied voltage	Decrease in fiber diameter with increase in voltage.
Distance between tip and collector	Generation of beads with too small and too large distance, minimum distance required for uniform fibers.
Feed rate/Flow rate	Decrease in fiber diameter with decrease in flow rate, generation of beads with too high flow rate
Ambient parameters	
Humidity	High humidity results in circular pores on the fibers.
Temperature	Increase in temperature results in decrease in fibers diameter.

2.1.3. Polymers used in fabrication of electrospun nanofibers

To date, it is believed that nearly one hundred different polymers have been successfully electro-spun, including natural and synthetic polymers, synthetic co-polymers, and blends of polymers [7].

***Natural biopolymers*:** proteins, polysaccharides, DNAs and lipids, have been fabricated into electrospun scaffolds. Protein fibers, mainly from collagen, gelatin, elastin and silk fibroin, have been well studied in recent years [39-41]. Recently, successful electrospinning of hyaluronic acid (HA) in aqueous solutions has been carried out [42]. Other polysaccharides, such as chitosan [43] and cellulose acetate [44] have also been electrospun into nanofibers. Beside proteins and polysaccharides, calf thymus Na-DNA in an aqueous solution was electrospun to form nanofibers with diameters of around 50–80 nm [45].

Synthetic polymers: Typical synthetic polymers used in biomedical applications are hydrophobic biodegradable polyesters, such as poly-lactide-co-glycolide (PLGA) [46] and poly(ε-caprolactone) (PCL) [47], and hydrophilic polymers, such as polyurethane [48], poly(vinyl alcohol) (PVA) [34] and polyethylene oxide (PEO) [35], have also been electrospun into nanofibrous scaffolds for biomedical applications.

Synthetic co-polymers: These have been used to manipulate the unfavorable properties of polymers such as hydrophilicity, degradation and cell adhesion, etc. Various copolymers such as polylactide-polyethylene glycol tri-block copolymer (PELA) [49] and block copolymers composed of PCL and PEG have been used for NFs formation for various biomedical applications [6].

Blends of polymers: Blending of hydrophilic polymers with hydrophobic ones such as gelatin or collagen with poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) [50] is an approach used to enhance hydrophilicity and water uptake properties. Even hydrophilic polymers can be blended to enhance their electrospinning properties as in case of blending alginate with PEG or PEO [51]**.**

2.1.4. Modified electrospinning methods

2.1.4.1. Co-axial electrospinning

Co-axial electrospinning is a modification of the traditional electrospinning process and involves two concentrically arranged nozzles connected to a high voltage source .Two solutions, the organic polymer solution and aqueous solution containing the bioactive agent are coaxially and simultaneously electrospun through different feeding capillary channels into one nozzle to generate composite nanofibers with a core–shell structure(Figure 3) [52]. Co-axial electrospinning keeps the two solutions separate, precluding contact between the two solutions until the last moment when they exit the nozzle [53]. Co-axial electrospinning is a dynamic process, and many factors such as flow rate of the inner and outer solutions, interfacial tension, and viscoelasticity of the two polymer solutions could affect entrainment with the production of

fibers without the required core-shell structure [54]. As aqueous biological solutions are unspinnable due to their low viscosity [55], hydrophilic polymers e.g. poly(ethylene glycol) are usually added to the aqueous biological solution to improve fiber forming properties. The addition of such hydrophilic polymers can also be helpful in modulating the release profiles of biomolecules as inclusion of hydrophilic polymers enhances water uptake by the electrospun scaffolds [56, 57].

Compared with blend electrospinning, coaxial electrospinning is relatively more complex, but it is beneficial in maintaining the functional activity of biomolecules. However, it may not be easy to set the parameters for stable coaxial electrospinning in order to form a uniform fibrous structure due to differences in the conductivities and viscosities of the two solutions [58].

2.1.4.2. Emulsion electrospinning

Emulsion electrospinning can be considered an extension of blend electrospinning because it requires the same basic set-up [52]. It produces core-shell structures that are generally more adaptive for incorporating hydrophilic small molecules [59, 60] and proteins [61, 62]. They could preserve an unstable biological agent from aggressive environments and functionalize the surface of nanostructures without affecting the core material. Emulsions for electrospinning, usually are of the W/O type. The oil phase is obtained by dissolving polymers particularly biocompatible and biodegradable polymers into an organic solvent, and the water phase is a small volume of an aqueous solution of the drug or protein with globule sizes of microns or sub-microns [63]. Xu *et al* [64] showed that the continuous phase rapidly evaporates during electrospinning, which increases its viscosity. The aqueous phase droplets thus migrate to the center of the jet due to the viscosity gradient. The droplets coalesce and elongate in the presence of the electric field due to mutual dielectrophoresis, which gives column-like structures that ultimately give a core-shell fiber morphology [65] (Figure 4). In some cases, the inner column breaks up into smaller droplets due to Rayleigh instability [66], similar to droplet formation in electrospraying. The exact reasons that cause one phenomenon to occur over the other are not properly understood. However, it is postulated that factors such as interfacial tension and viscoelasticity of the aqueous phase play a role in regulating this phenomenon [65, 66]. It is remarkable that unlike co-axial electrospinning which needs a special apparatus and careful selection of operational parameters to ensure the desired results, the basic equipment for emulsion electrospinning only needs a single nozzle [67]. Recently, preparing core-shell type nanofibers by emulsion electrospinning has attracted growing interests [64, 68, 69].

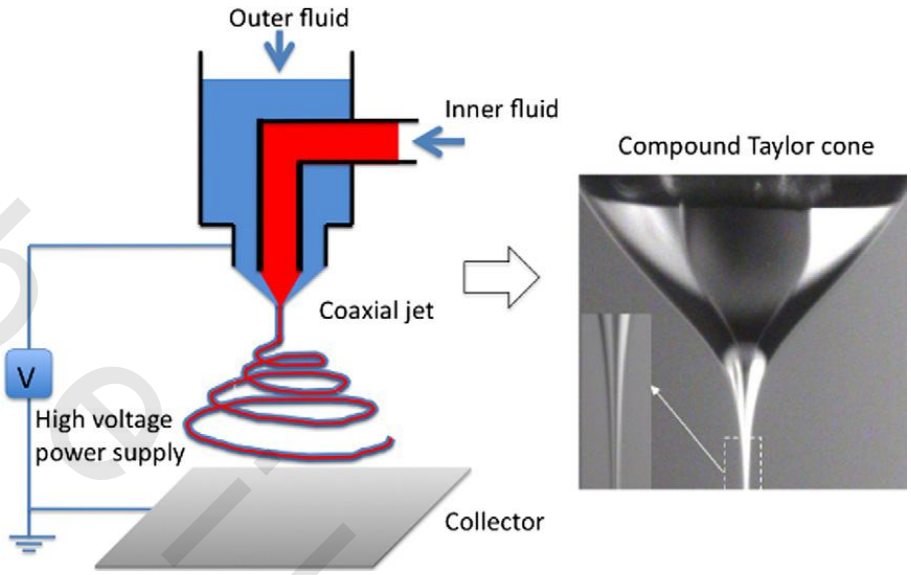

Figure 3: Basic setup for co-axial electrospinning and fabrication process of common core–shell nanofibers [70].

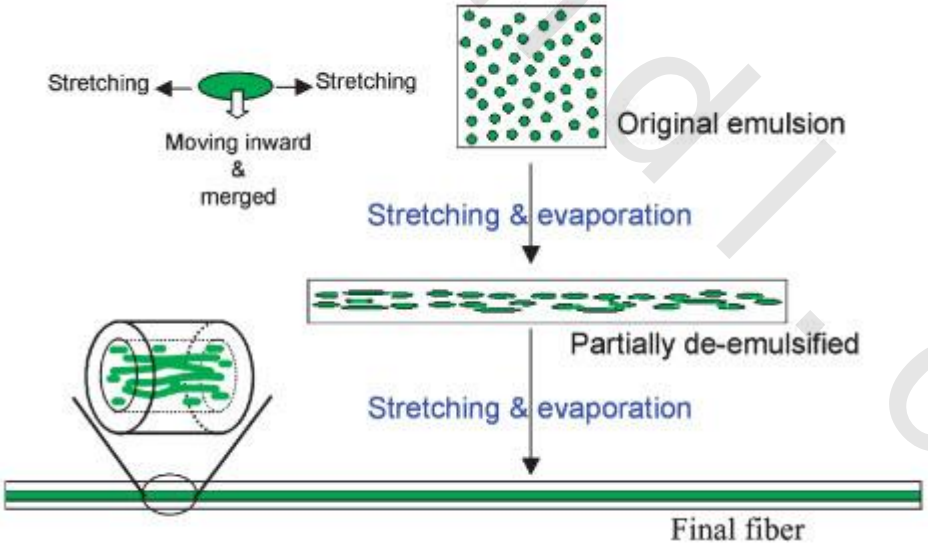

Figure 4: Schematic mechanism for the formation of core-sheath composite fibers during emulsion electrospinning [69].

2.2. Drug loading strategies for electrospun nanofibers

2.2.1. Drug encapsulation

The solubility and compatibility of the drug in the drug/polymer/solvent system are the decisive factors for the preparation of the electrospun fiber formulation with controlled release of the drug [29]. As in blend electrospinning, in order to encapsulate the largest amount of a drug inside the polymer fibers, a lipophilic polymer should be chosen as the fiber material for a lipophilic drug while a more hydrophilic polymer should be employed for a hydrophilic drug and the solvents used should be suitable for both drug and polymer [71]. However, such medicated fibers cannot be used as drug delivery system because they can dissolve in blood or tissue fluid quickly and thus the drug release rate cannot be controlled. Therefore, successful incorporation and sustained release of the hydrophilic drugs is a great challenge [64].

Other strategies for loading sensitive biomolecules or hydrophilic charged drugs in hydrophobic polymers, would be by core-loading as in co-axial electrospinning [58, 72] and emulsion electrospinning [59, 61-63] as discussed previously.

2.2.2. Surface functionalization

For drugs that are unstable under the harsh electrospinning conditions, modified loading methods can be adopted as surface loading after the NFs have been fabricated. Surface functionalization is achieved by physical or chemical immobilization of the bioactive molecules post-electrospinning. Physical immobilization, particularly of therapeutic proteins and nucleic acids can be achieved by adsorption to the nanofibers surface via specific and nonspecific interactions for local and sustained delivery [73]. Chemical immobilization of bioactive molecules onto the surface of electrospun nanofibers is favored over physical immobilization in tissue engineering applications. Because the immobilized molecules are covalently attached to the nanofibers, they are not easily leached out from the surface-modified nanofibers when incubated over an extended period. However, it should be noted that partial inactivation of the immobilized molecules can occur upon the covalent modification when active sites are chemically modified [73]. Nanoparticles assembly on the surface of nanofibers was allowed due to the large interfacial area of the nanofibers. Such hierarchical nanostructure can also be constructed using therapeutically or biologically functional nanoparticles such as silver nanoparticles [74].

2.3. Antimicrobial nanofibers

Microbial contamination is of great concern, especially in hospitals. *S.aureus*, including methicillin resistant strains (*MRSA*), is the most prevalent microorganism in skin infections [75]. More than 95% of *S. aureus* strains are resistant to penicillin and 60–70% are resistant to methicillin [76, 77]. Organisms may become resistant to antimicrobial agents through production of enzymes that inactivate the antimicrobial agent, efflux pumps that remove an antimicrobial agent from the cell, down regulation (or alteration) of genes encoding an outer membrane protein channel, or through alteration of their cell walls [78]. If wounds are not treated effectively, pathogens form biofilms, rendering them resistant to antibiotics [79]. In severe cases, biofilms need to be surgically removed to prevent further infection [80].

Different strategies to introduce antimicrobial activity in polymer nanofibers have been adopted. These include surface modifications, inclusion of antimicrobial agents that leach from the polymer or chemical synthesis of non-leaching polymers of antimicrobial activity.

2.3.1. Antimicrobial polymer nanofibers with non-deliverable antimicrobial agents

2.3.1.1. Synthetic antimicrobial polymer conjugates

Antimicrobial polymers are synthesized by covalent binding of biocidal functional groups in a post-polymerization modification, providing antimicrobial or antiseptic properties [81]. Modification is either to the bulk polymer or selectively to the surface via available reactive moieties [82]. Another form of synthesis is the chemical modification of a biocidal molecule into a polymerizable compound that can subsequently be polymerized or co-polymerized with another monomer [83]. Both of these approaches have been valuable in establishing feasibility for the concept of non-leaching antimicrobial polymeric materials.

Ignatova *et al* [84] introduced nanofibers containing poly(vinyl pyrrolidone)-iodine complex (PVP–iodine) suitable as wound dressings. Different approaches were used: a one-step method based on electrospinning of PVP–iodine or poly (ethylene oxide)/PVP–iodine solutions and a three-step method based on electrospinning of PVP or poly (ethylene oxide)/PVP mixed solutions followed by photo-mediated crosslinking of the obtained nanofibers and subsequent complexation with iodine. Aqueous solutions of quaternized chitosan derivatives and poly (vinyl pyrrolidone) (PVP) were electrospun into fibers and crosslinked via UV irradiation. Crosslinked fibers were able to kill the majority of *S. aureus* cells within 30 min and after 1 hour all cells were killed. These fibers showed less reduction of *E.coli* numbers within 30 min, however, most of the cells were killed within 120 min. In another study conducted by Ignatova *et al* [85], it was shown that the PVP-iodine complex electrospun mats killed *S.aureus* cells within 30 min after contact. The same fibers were also able to kill 99.8% of *E. coli* and 98% *C. albicans* cells in 90 min and 60 min, respectively [86].

Well-defined antibacterial block copolymers of 4-vinyl pyridine (4VP) and pentachlorophenyl acrylate (PCPA) (P(4VP-*b*-PCPA)) were prepared via reversible addition–fragmentation chain transfer (RAFT) polymerization. Electrospinning of the P(4VP-*b*-PCPA)

from a solution in mixed tetrahydrofuran and dimethylformamide gave rise to fibers with diameters in the range of 0.5–4.0 μm. Quaternary ammonium salts (QASs) were generated by *N*-alkylation of pyridine groups of P4VP block and chloroaromatic compounds of PPCPA block (or self-quaternization of P(4VP-*b*-PCPA). The resulting nanofibers exhibited a high antibacterial efficiency, attributable to the hydrophobicity of the PPCPA blocks and the electrostatic interaction of QASs generated from the self-quaternization of P(4VP-*b*-PCPA). The antibacterial effect of the P(4VP-*b*-PCPA) nanofibers was assayed using *E.coli* and *S.aureus* cultures. It was found that 99.6% of *E. coli* and 99.1% *S. aureus* were killed after being in contact with 50 mg nanofibers in 10 min. The permanence of antibacterial activity of the self-quaternized P(4VP-*b*-PCPA) nanofibers was also demonstrated in repeat application [87].

Maleic anhydride copolymers proved to have good binding ability even to biological molecules such as nucleic acids and proteins [88]. Patel *et al* [89, 90] have prepared bioactive copolymers containing anhydride functionalities as drug carriers, whereby an antiseptic agent acriflavine was covalently bound on the surface of poly[styrene (S)-co-(maleic anhydride)] (PMSA) [89] and poly (methyl methacrylate-co-(maleic anhydride) [90]. The controlled release of acriflavine was evaluated by antibacterial assessment of the released drug against *B. subtilis*. The same group has also described the synthesis of a macromolecular prodrug of ampicillin bound to the anhydride groups of a matrix of PSMA via an amide bond [91]. The release profiles of ampicillin with different percentage of maleic anhydride in the polymeric carrier indicated that the rate of drug release could be controlled by the fraction of incorporated anhydride moieties in the copolymer.

2.3.1.2. Chitosan nanofibers

Chitosan is a linear polysaccharide composed of randomly distributed ß-(1-4)-linked D-glucosamine (deacetylated unit) and N-acetyl-D-glucosamine (acetylated unit. Chitosan is produced commercially by deacetylation of chitin, which is one of the most abundant natural polymers in the exoskeleton of crustaceans (crabs, shrimp, etc.) and in the cell walls of fungi and yeast [92]. Chitosan showed broad spectrum antimicrobial activity against a wide range of algae, bacteria, yeasts and fungi [93]. However, the activity of chitosan is limited to acidic conditions due to its poor solubility above pH 6.5 [94]. Furthermore, the antimicrobial properties of chitosan depend on the average molecular weight, the degree of deacetylation and temperature [95].

The polycationic nature of chitosan renders the polymer non-toxic, biocompatible, biodegradable and bioactive [96]. Chitosan derivatives (e.g. chitosan acetate and quaternized chitosan derivatives) enhance antimicrobial activity and solubility at neutral pH values [97]. There have been a few studies of the antimicrobial activity of chitosan nanofibers. In most cases the mechanism proposed was related to the dissolution of the nanofibers and release of positively charged amino groups [98, 99]. However, the molecular conformation may play an important role in explaining the antimicrobial activity of chitosan [100]. Nanofibers of chitosan–PVA showed a high antimicrobial activity against *E. coli* and *S. aureus* ,possibly due to a more effective contact surface area and, consequently, to a greater presence of amino groups to carry out the inhibition of microbial growth [101]. This implies that the release of amino groups from the fibers might not be the only mechanism of action. The antibacterial activity of chitosan nanofibers may also

be due to the release of small chitosan oligomers that could penetrate bacterial cells and interact with DNA. Also, a chitosan layer may be formed around the bacterial cell preventing the normal function of the membrane and eventually causing cell death [102].

2.3.2. Antimicrobials-loaded nanofibers

Polymers impregnated with antimicrobial agents that are released over time. In all of these polymers, the slow release of the antimicrobial compound, and hence low concentrations, may lead to the development of resistance in microorganisms [103]. Faster initial release of antimicrobial agent is for essential eradication of planktonic bacteria and considerable suppression of biofilm [46].

2.3.2.1. Antibiotics-loaded nanofibers

The traditional oral antibiotic therapy is not effective all the time, and it can have adverse side effects. In order to reduce associated surgical site infections when bio-implants are involved, a growing interest among material scientists is to provide antimicrobial properties to the implantable biomaterials. Therefore, in addition to serving its primary function, the implant will also help prevent the formation of bacterial biofilms and the released antimicrobial agent would kill or inhibit the growth of bacteria [104]. Nanofibrous mats perform as potential carrier system for the controlled delivery of antibiotics to control or prevent localized infections for biomedical applications such as wound healing [105, 106], and anti-tissue adhesion barriers [30, 47], surgical sutures [104] .. etc

Tetracycline hydrochloride was incorporated into electrospun PEG–PLA nanofibrous membrane without loss of bioactivity. The medicated nanofibrous membrane demonstrated sustained release of tetracycline over 6 days and was found to be effective in inhibiting growth of *S. aureus*. They were suggested as new wound dressings for malignant wounds and ulcers caused by diabetes or other diseases [105]. A dual drug release electrospun nanofibrous scaffold wound healing was fabricated containing an anesthetic, lidocaine, and an antibiotic, mupirocin. Two drugs with different lipophilicities were electrospun from a poly-l-lactic acid (PLLA) solution with a dual spinneret electrospinning apparatus into a single scaffold [106].

Cefoxitin sodium, a hydrophilic antibiotic was encapsulated in electrospun poly(lactide-co-glycolide) (PLGA)-based nanofibrous scaffolds without the loss of structure and bioactivity. The antibiotic drug released from these electrospun scaffolds was effective in their ability to inhibit *S.aureus* growth (>90%). The combination of mechanical barriers based on non-woven nanofibrous biodegradable scaffolds and their capability for local delivery of antibiotics increases their desired utility in biomedical applications, particularly in the prevention of post-surgical adhesions and infections [30].

Ampicillin sodium salt was encapsulated in poly (ε-caprolactone) (PCL) electrospun fibers that have been twisted into nanofiber yarns. A burst release of ampicillin from the yarns has been observed in the first hour, and the release is almost completed in 96 hours. The burst release is believed to be due to the low compatibility of ampicillin with PCL, the accumulation of

ampicillin on fiber surface and the small fiber diameters. The results from the zone of inhibition test of the yarns against both gram-positive *S. aureus* and gram-negative *K. pneumonia* indicate that the released ampicillin retains its effectiveness after the production processes. The results indicate that the electrospun nanofibers yarns will have a great potential to be used for biomaterials, such as surgical sutures, to decrease the surgical site infection rate [104].

2.3.2.2. Antimicrobial peptides-loaded nanofibers

Bacteriocins are ribosomally synthesized proteins or protein complexes with bacteriostatic or bactericidal activity that are produced by lactic acid bacteria which are a diverse group of organisms with GRAS (generally regarded as safe) status and have been consumed over decades as probiotics [107]. The peptides have a net positive charge (cationic) and are amphiphilic or rather more hydrophobic. They intercalate into the cell membrane of sensitive cells, form pores and disrupt the proton motive force [108, 109]. Bacteriocins are thus attractive natural alternatives to antibiotics, which can be used in the treatment of bacterial infections. A localized delivery system is, however, required to control the level and rate of bacteriocins delivered to the wound. A novel approach would be to encapsulate bacteriocins into electrospun nanofibers and use this as wound dressings. The feasibility of encapsulating bacteriocins into electrospun nanofibers was recently reported by Henuis *et al* [110]. Bacteriocin plantaricin 423, encapsulated in polyethylene oxide nanofibers, retained activity after electrospinning and inhibited the growth of *E. faecium* HKLHS and *Lactobacillus sakei* DSM 20017 that served as target strains.

2.3.2.3. Nanobiocides-loaded nanofibers

Nanobiocides are anti-microbial nanoparticles that generally fall into one of two categories, metals and metal oxides, (e.g. silver nanoparticles, copper, zinc and titanium oxides) and Engineered/synthesized nanoparticles (e.g. fullerenes) and naturally occurring anti-microbial materials such (e.g. chitosan nanoparticles). Currently, the most commonly used nanobiocides are noble metal nanoparticles, and in particular, silver nanoparticles [111]. 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 [112]. The advantageous characteristics of silver nanoparticles as biocides can be expanded for further applications by incorporating it into other materials, especially polymer nanofibers.

Silver nanoparticles

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 [113]. Silver is the most commonly used biocide in electrospun nanofibers [114]. Recently, electrospun nanofibers containing silver nanoparticles have successfully been fabricated for antimicrobial applications using polymers such as poly(vinyl alcohol) [115], gelatin [116] and cellulose acetate [44]. They

were incorporated in cellulose acetate nanofibers by electrospinning cellulose acetate with 0.5 wt% $AgNO_3$ [44]. Silver particles were generated on the surface of the nanofibers after irradiation at 245 nm. Almost all viable cells (99.9%) of Gram-positive bacteria, *E.coli*, *K.pneumoniae*, and *P.aeruginosa* were killed after 18 hours of exposure to the encapsulated silver nanoparticles. Silver-loaded zirconium phosphate was incorporated into poly ε-caprolactone fibers [117]. Growth inhibition of up to 99.27% of *S.aureus* and up to 98.44% of *E.coli* was recorded when bacteria were cultured in the presence of these nanofibers. Human dermal fibroblasts attached to these nanofibers and continued to proliferate, suggesting that the fibers may be used as wound dressings. Similar findings have also been reported by Rujitanaroj *et al* [116] who fabricated ultrafine gelatin fiber mats containing silver nanoparticles. These were further cross-linked with moist glutaraldehyde vapor to improve their stability in an aqueous medium. Antibacterial activity was investigated against some common bacteria found in burn wounds. The antibacterial activity was greatest against *P.aeroginosa*, followed by *S.aureus*, *E.coli*, and methicillin-resistant *S.aureus*, respectively. However, it has been reported that silver may elicit toxic side effects on human cells [118].

Titanium dioxide nanoparticles

Titanium dioxide or titania has well-known photoactive antimicrobial attributes for the killing or growth inhibition of bacteria under UV and visible light [119]. Titanium dioxide nanofibers were successfully electrospun as a polymeric composite from an inexpensive titanium precursor, titanyl nitrate and polyvinyl pyrrolidone by Azad *et al* [120]. They also developed nonwoven nanofibers of pure and iron-doped titanium dioxide and evaluated their antimicrobial attributes for use as disinfectant gauze for wound healing upon brief activation by UV/IR illumination [121]. Sheikh *et al* [122] developed a good combination consisting of electrospun titanium dioxide nanofibers incorporated with high purity hydroxyapatite nanoparticles and antimicrobial silver nanoparticles introduced for hard tissue engineering applications. Antimicrobial assessments indicated high bactericidal effect.

2.4. Biomedical applications of antimicrobial nanofibers

2.4.1. Tissue engineering

Tissue engineering approaches typically involve three key elements: scaffolds, cells, and biochemical and/or mechanical stimuli. Scaffolds generally serve as the foundation for many strategies to promote tissue formation. Although a wide range of scaffold materials are available, polymeric scaffolds are commonly employed to support tissue growth and to serve as carriers for bioactive factor delivery. The unique properties of polymeric nanofibers make them a valuable tool to tissue engineers [22]. Nanofibers offer great potential in this respect due to their high porosity, high surface-to-volume ratio and most importantly, architectural analogy to natural extracellular matrix (ECM) with suitable mechanical properties [123, 124]. The structural and functional properties of the natural extracellular matrix (ECM) are crucial for the proliferation, differentiation and migration of cells [58]. These unique mechanical properties are useful for modulating cell behavior as well as providing adequate tension and strength to resist the forces from the cell cytoskeleton [125]. Apart from structure-based functionalities, nanofibers can

deliver suitable bioactive agents, including antimicrobial agents [126]. Since polymeric nanofibers are well suited for such applications, they are gaining popularity in tissue engineering and have been used in attempts to regenerate a variety of tissues including cartilages, dermal tissue, bones, arterial blood vessels, heart and nerves etc. [22]. Ruckh *et al* [127] developed rifampicin-loaded PCL NFs to prevent incidence of peri-prosthetic infections after major orthopedic surgeries such as total joint arthroplasty and internal fracture fixation

2.4.2. Reduction of post-surgical tissue adhesion

Post-operative adhesions are an almost inevitable occurrence after most abdominal and pelvic surgery procedures. The incidence of intraperitoneal adhesions ranges from 67 to 93% after general surgical abdominal operations and up to 97% following open gynecological pelvic procedures [128].

Separation of raw peritoneal surfaces during early days of the healing process is the ideal method to prevent postoperative adhesions. A number of natural and synthetic graft materials have been employed in an effort to reduce adhesion formation between traumatized surfaces. These barriers are placed over traumatized tissues at the conclusion of surgery in order to separate tissue surfaces. The ideal barrier, besides being safe and effective, should persist during the critical re-mesothelialisation phase, stay in place without sutures or staples, remain active in the presence of blood and be biodegradable [129]. There are many trials on the usage of polymeric nanofibrous membranes as anti-adhesion barriers in animal models as PLGA-based membranes [130, 131], polylactide-polyethylene glycol tri-block copolymer (PELA) [49], PCL [47], chitosan [132], poly(3-hydroxybutyrate-co-3-hydroxyhexanoate) [133] and others. Antibiotic-loaded NFs have been investigated for prevention of peritoneal adhesions by preventing the incidence of infections during surgeries that may provoke adhesion formation [47, 131].

2.4.3. Wound healing

A wound is a disruption of normal anatomic structure and function of the skin [134]. On the basis of wound healing processes, there are two types of wounds: acute and chronic wounds. Wound healing is a special biological process which is related to physiological parameters. Selection of a suitable wound dressing material for a specific type of wound requires comprehensive knowledge of the wound healing process [135-138]. These can be summarized into five consecutive cascades of events of hemostasis, inflammation, migration, proliferation, and maturation.

Wound dressing materials produced by electrospinning technology have special properties as compared to the dressings produced by conventional methods. These properties are as follow [4, 138]:

1. **Hemostasis:** Nanofibrous wound dressings with their small holes and high effective surface area can promote the hemostasis phase. The promotion of this phase is due to nanofibrous structure of the dressing material without using any hemostatic agent.

2. **Absorbability:** Due to the high surface area to volume ratio of the nanofibers, they exhibit water absorption of 17.9–213% whereas typical film dressings show water absorption of 2.3% only. Thus, if hydrophilic polymers are employed, the nanofibrous dressings will be able to absorb wound exudates more efficiently than the typical film dressings.
3. **Semi-permeability:** The porous structure of a nanofiber dressing is excellent for the respiration of cells which does not lead the wound to dry up. This indicates appropriate control of a moist environment for the wound. Also, the small pore size can effectively protect the wound from bacterial infection.
4. **Conformability (3 D-dressing):** Finer fiber fabrics are easier to fit to complicated 3-D contours. Therefore, dressing materials made of ultra-fine fibers can provide excellent conformability and result in better protection of the wounds from infection.
5. **Functionability:** Multifunctional bioactive nanofibrous dressings may contain antiseptics, antifungal, vasodilators (e.g. minoxidil used to promote wound epithelialization and neovascularization), growth factors, and even cells (e.g. keratinocytes).
6. **Scar-free:** Ultimately, nanofibers also hold promise of healing wounds without scarring, as the biodegradable fibrous scaffolds would give the skin cells a better road map for self-repair.

Different polymeric materials were studied for fabrication of NFs for wound dressing applications, these include hydrogels as alginate [139], chitosan [43, 99, 140-142] and polyvinyl alcohol (PVA) [43], biodegradable polyesters such as PCL [36, 143] and PLGA [144], natural and proteins as gelatin [36, 116], collagen [139, 141, 142] and silk fibroin [139, 140]. Composite nanofibrous membranes could be a good candidate for wound dressing application. Chen *et al* [141] introduced electrospun NFs made of type I collagen, chitosan, and polyethylene oxide, which could be further crosslinked with glutaraldehyde vapor. NFs showed no cytotoxicity toward growth of 3T3 fibroblasts and had good *in vitro* biocompatibility. From animal studies, NFs were better than gauze and commercial collagen sponge wound dressing in wound healing rate. Cai *et al* [140] fabricated composite chitosan and silk fibroin NFs and tested for their antibacterial activities against *E.coli* and *S.aureus* and biocompatibility to murine fibroblast. Blending with hydrophilic polymers could enhance the properties of wound dressing matrices. For instance, Han *et al* [50] investigated the effect of blending of gelatin or collagen with poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) (PHBV) to increase water absorption ability of the matrix. In cell attachment and growth on matrices, dermal sheath cells attached to hydrophilic PHBV/collagen and PHBV/gelatin faster than hydrophobic PHBV. Accordingly, co-cultured matrices were used as a cell-seeded biological dressing that regenerates the epidermis efficiently during the early stage of wound healing. Choi *et al* [6] used biodegradable polymeric NFs to immobilize recombinant human epidermal growth factor (EGF) for the purpose of treating diabetic ulcers. Wound healing effects of the EGF nanofibers were confirmed in diabetic mice with dorsal wounds.

2.5. Potentials of electrospun nanofibers in antimicrobial photodynamic therapy (APDT)

2.5.1. Antimicrobial photodynamic therapy (APDT)

Photodynamic therapy (PDT) and antimicrobial photodynamic therapy (APDT), also known as antimicrobial photodynamic chemotherapy (PACT) is based on the concept of killing cancer cells or microorganisms using a photosensitizer (PS) activated by harmless visible light of an appropriate wavelength in the presence of oxygen to generate reactive oxygen species (ROS) that are toxic to target cells [145, 146].

The ability of light-drug combinations to kill microorganisms has been known for over 100 years [147] with the observation that light and an acridine dye in the presence of oxygen could kill microorganisms [148]. Since the middle of the last century, anti-microbial photodynamic therapy was forgotten because of the discovery of antibiotics [145]. The rapidly increasing emergence of antibiotic resistance amongst many species of pathogenic bacteria may be bringing to an end a period extending over the past 50 years, termed "the antibiotic era" [149]. Recently, there has also been a large interest in APDT to treat localized infections [150].

2.5.1.1. Principle and mechanisms of APDT

The treatment of infections with APDT includes three major parameters: administration of a PS, subsequent exposure to light and the presence of molecular oxygen.

$$PS + O_2 \xrightarrow{\lambda\nu} \text{Singlet oxygen} \rightarrow \text{Cell damage}$$

The photoactivation process of a photosensitizer is illustrated in Figure 5. The initiating step of the photosensitizing mechanism is the absorption of a light photon by the PS, causing a promotion of the drug molecule from its singlet ground state (S_0) to the extremely unstable excited singlet state (S_1) [151] with lifetime approximately nanoseconds [152]. From here, the molecule has two possibilities to return to S_0, either the molecule returns directly to S_0 by emitting a photon (fluorescence) or by internal conversion with excess energy lost as heat; or the molecule may take the inter-system crossing and be transferred to the lowest triplet state (T_1) [153] with a longer lifetime (approximately microseconds) and then to S_0. PSs in the triplet state return to the ground state by emitting phosphorescence, and also can transfer energy to other molecules. The triplet state PS has lower energy than the singlet state, but has a much longer lifetime. This increases the probability of energy transfer to other molecules. The triplet state lifetime influences the probability for the interaction with surrounding molecules. The energy of the excited PS molecule can then be transferred to molecular oxygen or other molecules by the two reaction mechanisms described below [151-153]. The type I pathway is a radical or redox reaction while type II pathway is an energy transfer process

Type I pathway involves electron or hydrogen atom transfer from the excited PS to another molecule, producing radical forms of the PS or the substrate. These intermediates may react with oxygen to form peroxides (O_2^{2-}), superoxide (O_2^-) ions, and hydroxyl radicals, which initiate free

radical chain reactions. These reactive oxygen species (ROS) react in turn with cellular targets and causes irreversible damages [153].

In type II reaction, PSs in the excited triplet state directly transfer energy to molecular oxygen in a triplet ground state ($^3O_2$), resulting in the formation of highly reactive singlet oxygen ($^1O_2$). The PS is not destroyed in this process, but returns to its ground state (S_0) without chemical alteration and is able to repeat the process of energy transfer to oxygen many times. In other words, the PS essentially acts as a catalyst. It may eventually be destroyed (photobleached) by interaction with $^1O_2$. Diffusion distance of singlet oxygen in biological tissue has been estimated in the order of 0.01 μm, corresponding to a lifetime of about 0.01- 0.04 μs [154]. Since the diffusion distance of singlet oxygen in cells during its lifetime is less than 100 nm, local damage can be expected at its sites of origin. Therefore, in principle, the photodynamic activity of PS directly depends not only on their $^1O_2$ quantum yields but also on their different uptake mechanisms and subsequent intracellular concentration and localization [155]. Although there is probably a contribution of both type I and II processes, the *in situ* generation of singlet oxygen via type II pathway appears to play the central role in photodynamic cytotoxicity because of the highly efficient interaction of the $^1O_2$ species with different biomolecules [151, 156].

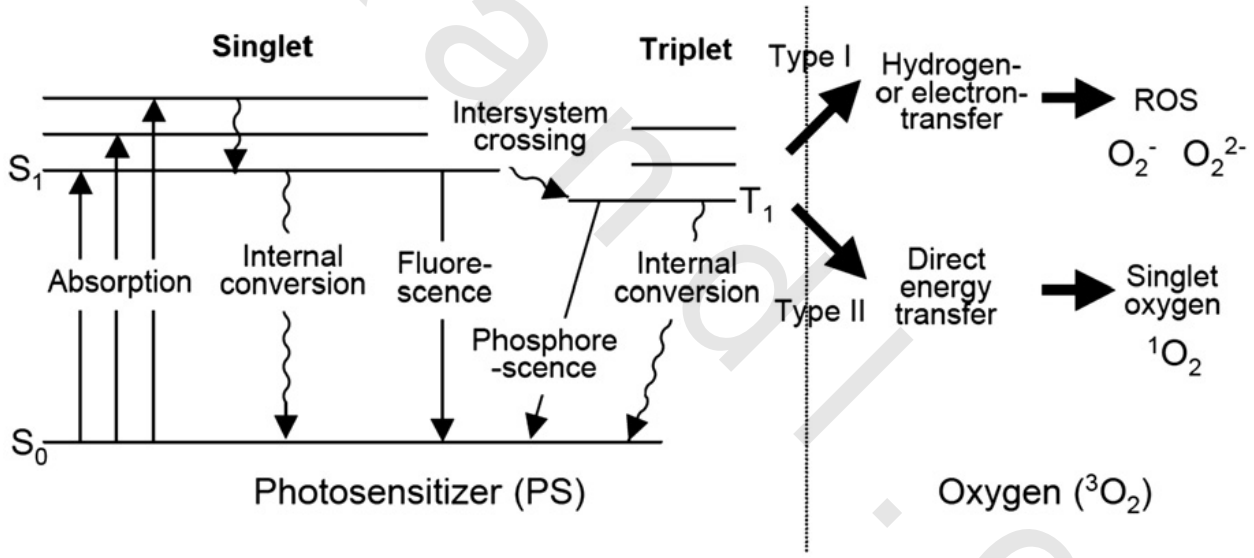

Figure 5: Jablonski diagram showing the energy transfer from PSs to molecular oxygen [153].

*S_0: PS ground state; S_1: PS excited singlet state; T_1: PS excited triplet state

2.5.1.2. Photosensitizers

Photosensitizers are usually aromatic molecules which are efficient in the formation of long-lived triplet excited states [157]. Photosensitizers used in PDT should exhibit the following physico-chemical properties:

- Suitable lipophilicity (log P) and ionization (pKa) [157] for localization in specific cellular or microbial compartment. It is particularly useful if the photosensitizer is amphiphilic, water soluble but containing a hydrophobic moiety which should facilitate the crossing of cell membranes [158].
- Limited *in vivo* stability is preferred to allow for rapid removal from tissues [158].
- High extinction coefficient ($\varepsilon \geq 50000\ M^{-1}\ cm^{-1}$) to increase the number of photons absorbed and to take advantage of the increase in the penetration depth of light into tissue at longer wavelengths [158].
- Activation (maximal absorption) at wavelength with optimal tissue penetration with little absorption at other wavelengths within the spectrum. This is to minimize the side effect of skin photosensitivity [157].
- High quantum yield of the triplet state ($\Phi_T > 0.4$) combined with high triplet lifetime ($t_T >$ 100 µS) as photochemical reactions predominantly occur in the excited triplet state [155].
- High quantum yields for the generation of singlet oxygen (Φ_Δ) [159].
- Absorption in the long-wave region ($\lambda > 630$ nm, preferably $\lambda > 680$ nm) [155].
- Strong absorption at the photoactivation wavelength [155].
- Low dark toxicity of the PS and their degradation products [155].
- Relatively stable against photobleaching [155].

2.5.1.2.1. Types of commonly used photosensitizers

Phenothiaziniums

Phenothiaziniums are compounds which have a core structure consisting of a planar tricyclic heteroaromatic ring with the formula $S(C_6H_4)_2NH$ [160], with an absorption range of λmax = 620-660 nm [157]. The lead compound of phenothiaziniums is methylene blue (MB). The minimal toxicity of these dyes to human cells and their ability to produce high quantum yields of singlet oxygen generated a great interest in testing the potential of these PSs as photo-activated antimicrobial agents [161].

Methylene blue (MB) and toluidine blue (TBO) are the most extensively studied phenothiazinium-based PSs for their antibacterial activity. These compounds are generally cationic at physiological pH, which enables them to target cell membranes of both Gram-positive and Gram-negative bacteria [162]. Most of them have amphiphilic properties which are important for interaction with the membrane and enable the molecules to become internalized into the bacterial cell [160]. Phenothiazinium dyes and derivatives are effective against *E. coli* and *S. aureus*. The dyes showed inherent dark toxicity (minimum lethal concentrations: 3.1-1000 µM) [163]. The phototoxicity of the commercially available phenothiaziniums is high against both

Gram-positive and Gram-negative bacteria, indicating clinical potential in local disinfection. Antibacterial activity of phenothiazinium dyes were translated into practical applications such as photo-decontamination of blood plasma [164].

Macrocyclic photosensitizers:

Porphyrins

Porphyrins are oligomers of hematoporphyrin (complex mixture). The principal porphyrin-based drug used in both pre-clinical and clinical work is photofrin. The absorption spectrum of Photofrin shows five peaks, the strongest at about 400 nm and the weakest at about 630 nm [165].

Chlorins

Chlorins constitute a group of molecules very similar to porphyrins. In contrast to porphyrins, chlorins have the strongest absorption peaks in the red part of the spectrum, which gives the compounds a green color. Beside the beneficial absorption wavelength at 690 nm, it also has a relatively short half-life *in vivo*, with skin photosensitization lasting for less than a week. Chlorin e6 is a compound that has a strong absorption peak at 664 nm. It has a very short-term phototoxicity [165].

Phthalocyanines

Phthalocyanines strongly absorb red light with maxima around 670 nm and 770 nm, respectively. When chelated with metal ions, such as Zn and Al, they exhibit high triplet yields and longer triplet lifetimes. The dyes can be sulphonated to various degrees to enhance water solubility [166].

Naturally occurring photosensitizers:

Psoralens

Psoralens are tricyclic furocoumarins which absorb UV light in the range of 320-400 nm. The use of psoralens for the phototherapy of psoriasis is well established [167]. Due to the absorbance of blue light by psoralens, they are not appropriate for use in human infections. Other applications such as blood disinfection may be relevant due to the ability of psoralens to target nucleic acids and produce singlet oxygen upon activation with UVA [168].

2.5.1.3. Activating Light

2.5.1.3.1. Light sources

Photodynamic therapy as a treatment modality is governed by the accessibility of light to the target area. Any light source with the appropriate wavelength spectrum can be used. For PDT,

both coherent (lasers) and non-coherent (lamps and LED) lights are suitable. Both types have their own advantages and disadvantages [169] (Table 2).

Lasers

Laser is considered as an ideal light source for photodynamic therapy due to its coherence and monochromaticity. Monochromaticity permits the irradiation with a wavelength near to the maximum absorption of the photosensitizer [169] in the region where its penetration in tissue is also at its largest, thus avoiding thermal damage of the tissue with light that is not active in the photochemical reaction. Coherence implies that the light waves are all in phase and possess a minimal angle of divergence which permits the spot-on application of laser beam onto the target tissue. There is a possibility to use optic fibers for the transmission of light to cavities of the body via endoscopy. Thus, laser has been considered an ideal choice as light source for PDT [169]. Common examples of clinically used lasers are; argon laser, copper vapors laser, gold vapor laser, dye lasers, and laser diodes such as gallium-aluminium- arsenide laser [169]. The major drawbacks to the use of laser systems are their high cost and the continuing need to replace and maintain optical parts. The development of diode lasers has become a promising field in the past few years, as these lasers are cheaper and more reliable. Moreover, they are much smaller compared with dye lasers [170].

Lamps

Lamps have proven useful in photodynamic therapy especially in the cure of skin diseases [171]. Their main advantages are linked to the low cost and reduced dimensions. In principle, lamps can only be used for superficial illumination. When filtered, the wavelength band achieved is quite broad unlike the narrow lasers. The emitted light outside the absorption band of the photosensitizer was earlier presumed to have no other purpose than inducing hyperthermia of surrounding tissues. Later this hyperthermia was revealed to give an increased tumouricidal effect [172]. Metal halogen lamp and short arc xenon lamp were used in photodynamic therapy [169].

Light emitting diodes (LEDs)

These are non-coherent light sources that are used in photodynamic therapy. They emit in wavelength bands that are much broader than those from a laser, usually around 25 nm and look promising in the future as they are predominantly simpler and portable [173]. Additionally, light sources with a broader emission spectrum permit the use of different photosensitizers with different absorption maxima. Another advantage of non-coherent light sources is the low cost [170].

Table 2: Comparison of different light sources for PDT [170].

Light source	Advantages	Disadvantages
Sunlight	Free	Dosimetry
Lasers (metal vapor, gas, dye laser)	• Can be coupled to fibers • Dosimetry	• Expensive • Requires maintenance • Stationary systems • Often limited to one wavelength
Diode lasers	• Can be coupled to fibers • Dosimetry • Relatively inexpensive	• Currently only available at higher wavelengths (630 nm and above) • Limited to one wavelength
Broad-band lamps	• Inexpensive • Can be used with several photo-sensitizers • Large illumination fields	• Only surface illumination possible • Dosimetry
LED	• Inexpensive • Can be used with several photo-sensitizers • Dosimetry	• Only surface illumination possible

2.5.1.3.2. Light penetration and interaction with tissue

There are many factors that affect light penetration through body tissues especially in a wound. The wound environment contains a mixture of debris, necrotic tissue, exudates and colonizing bacteria. Light dissemination in such a medium involves processes of refraction, reflection, absorption and scattering. To minimize the loss of light intensity, when light passes through the interface of two media, as a result of reflection and refraction, the light is applied perpendicularly to the tissue [174]. Scattering of light in tissue has the most pronounced effect on light intensity and distribution. Both scattering and refraction control light beam width, and result in a loss of the light power delivered per unit area (i.e. reduction of light intensity). Absorption of light is the most relevant factor that involves the loss of light intensity with increasing penetration depth. However, the presence of chromophores in the tissue such as oxyhaemoglobin, deoxyhaemoglobin, melanin and cytochromes play an important role in light absorption. The absorption spectra of these molecules define the optical window for PDT in tissue. The absorption maxima of oxyhaemoglobin and deoxyhaemoglobin lie in the range of 500–600 nm, therefore a longer wavelength should be used for PDT [174]. Most of the PSs in clinical use are excited at 630-670 nm, where light can penetrate tissue as deep as 3–5 mm (Figure 6). The use of a PS with an absorption peak at wavelengths of 800 nm or more should double the penetration depth and thus enable the treatment of deep wounds and burns. Finally, the factors by which light penetration is limited such as optical scattering, the light absorption by endogenous chromophores or by the high concentration of sensitizing agent (self-shielding) should be taken into consideration when selecting a PS [175].

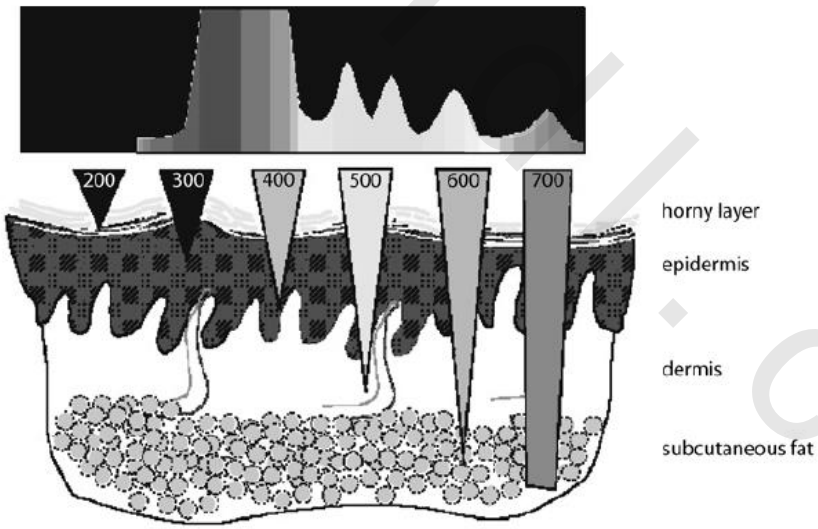

Figure 6: Penetration of light of different wavelengths into tissue [170].

2.5.1.4. Antimicrobial activity of APDT

APDT was proven to be active against many different bacterial strains. The photosensitivity of bacteria is affected by the physiological state: in general, cells in the logarithmic phase of growth are appreciably more susceptible to photodynamic inactivation than the corresponding cells in the stationary phase [17]. There are many advantages of APDT compared to antimicrobial drugs. APDT is safe because of its double selectivity obtained by targeting the PS, derived from its higher affinity for microbial cells, and the light, implying that only the infected area is irradiated and, consequently, treated [162, 176]. The results are instantaneous while antibiotics take several days to act. APDT cannot easily induce the development of microbial resistance. The therapeutic window of PDT is broader than other antimicrobial therapies, even against pathogenic biofilms [177]. Besides, It can be used to treat damaged or dead tissue, e.g. burns [178, 179].

Thus, there are many different possible applications of antimicrobial PDT against a wide range of pathogenic microorganisms including bacteria [162], fungi [180], protozoa [181] and viruses [168, 182]. It also can be applied to many different infectious diseases. A wide variety of localized infections could be clinically treated with antimicrobial APDT. The technique is also applied to blood sterilization [158].

2.5.1.4.1. Activity against Gram positive and Gram negative bacteria:

The response of bacterial cells to photosensitized processes is controlled by structural factors of cell membrane. The difference between Gram-positive and Gram-negative bacteria relate to differences in their cell wall structure and chemical composition. In Gram-positive bacteria, the cell membrane (15– 80 nm thick) consists of peptidoglycan layers. This wall displays a relatively high degree of porosity [183]. Thus, in this class of bacteria, the outer wall does not act as a permeability barrier for the most commonly used photosensitizers, whose molecular weight does not generally exceed 1,500–1,800 Da. On the contrary, the cell membrane of Gram-negative bacteria possesses an additional 10–15 nm thick outer membrane, which is external to the peptidoglycan network and has densely packed negative charges. Such a highly organized system inhibits the penetration of most molecules. Only relatively hydrophilic compounds with a molecular weight lower than 600–700Da can diffuse through the membrane channels [184].

Gram-positive and Gram-negative bacteria react differently to APDT. Unlike Gram-positive bacteria, Gram-negative bacteria are less susceptible to APDT due to the membrane barrier that prevents uptake of anionic and neutral PS [185]. Regarding the uptake pathways of anionic and cationic PS, George *et al* [186] reported that the uptake of anionic PSs by bacterial cells may be mediated through a combination of electrostatic charge interaction and by protein transporters, while the uptake of cationic PSs is mediated by electrostatic interactions and "self-promoted" uptake pathways. Alternate strategies have been developed to overcome this barrier and enhance the permeability of the outer wall in order to make Gram-negative bacteria sensitive to the action of photodynamic processes [157, 187]. These include the use of positively charged (cationic) PS, or by coupling or combining the PS with positively charged entities such as poly-L-lysine [188].

2.5.1.4.2. Selectivity and resistance development

An important observation about cationic antimicrobial PSs concerns their selectivity for microbial cells compared to host mammalian cells [189]. The positive charge allows the cationic antimicrobial PSs to strongly attach to the bacterial surface, since the bacterial cells have negative superficial charges [186]. Cell membranes of both eukaryotic and prokaryotic cells have residual negative surface charges mainly due to the presence of phosphate groups on the membrane phospholipids [190]. However, the presence of negatively charged molecules such as peptidoglycans, lipoteichoic acid and lipopolysaccharide in the bacterial surfaces confers a more negative residual charge in bacterial cells compared to eukaryotic cells [186, 191]. It is thought that cationic molecules are only slowly taken up by host cells by the process of endocytosis, while their uptake into bacteria is relatively rapid. If illumination is performed at short intervals after PS application (minutes) PDT damage to host tissue is minimized [176]. There have been a relatively few *in vitro* studies demonstrating selective killing of microbes under conditions in which mammalian cells were unharmed. Zeina *et al* [192] reported that human keratinocytes resisted killing by methylene blue-mediated PDT under conditions that killed several cutaneous microbial species. Further, porphyrin-based PSs exerted effective killing of different *MRSA* strains via reactive oxygen species without harming fibroblasts or keratinocytes cells at the same concentrations [193].

Despite the large number of studies on the effect of APDT against different microorganisms, the development of resistance to PDT has not been reported [194, 195]. Interestingly, cancer cells also do not develop resistance to PDT [196].

2.5.1.4.3. APDT efficacy in infected wound models in animals:

APDT has been used for the treatment of infections in animal models including infections resulting from burns [178, 179], surgical wounds [197] or soft-tissue infections [198]. In animal models, the results revealed that APDT is a promising treatment against a variety of infectious microbes *in vivo.* Bennett *et al* [199] reported for the first time the use of a light-activated antimicrobial agent, methylene blue, to kill an epidemic methicillin-resistant Staphylococcus aureus (*EMRSA*-16) strain in two mouse wound models, excisional and superficial. Following irradiation of wounds with laser light (670 nm) in the presence of methylene blue, a 25-fold reduction in the number of viable *EMRSA* was seen. This was independent of the increase in temperature of the wounds associated with the treatment. Histological examination of the wounds revealed no difference between the photodynamic therapy (PDT)-treated wounds and the untreated wounds, all of which showed the same degree of inflammatory infiltration at 24 hours. Hamblin *et al* [197] monitored the topical application of a targeted polycationic photosensitizer conjugate between poly-L-lysine and chlorin *e6* on *E.coli* infecting excisional wounds in mice, and subsequent activation with 660 nm laser light. There was a light-dose dependent killing of bacteria in the wounds, not seen in untreated wounds. Both treated and control wounds healed with no signs of damage to host tissue.

2.5.2. Photosensitizers delivery strategies for different antimicrobial photodynamic therapy (APDT) applications:

2.5.2.1. Membranes

Several researchers aimed to prepare photosensitizers-loaded polymeric membranes to control infection in wounds [200] or for disinfection of implanted catheters [201-203]. The non-shedding surfaces of catheters frequently become colonized by microbes resulting in biofilm formation and, ultimately, an infection. Such catheter-related infections are a major cause of morbidity and mortality [204]. To activate the PS, wound site would be superficially irradiated with LED or lamps or even laser light. For catheters, laser irradiation at the catheter entry site and along the catheter length would be effective.

Donnelly *et al* [200] developed Poly(vinyl alcohol)–borate hydrogels loaded with MB and meso-tetra (N-methyl-4-pyridyl) porphine tetratosylate for PDT of wound infections. Both PSs were released and found to be phototoxic to planktonic and biofilm-grown *MRSA* following suitable illumination. These PS-loaded hydrogels would be capable of conforming to the shape and contours of a wound whilst maintaining structural integrity. Perni *et al* [201-203] developed light-activated antimicrobial silicone elastomers for fabricating catheters by a simple swell-encapsulation-shrink method using toluidine blue [203] or methylene blue [201, 202] with nanogold. These polymers were effective in reducing survival of bacteria on their surfaces by generating singlet oxygen and other ROS. The presence of gold nanoparticles enhanced the hydrophobic properties of the polymer and its bactericidal activity, possibly by increasing the production of ROS other than singlet oxygen.

2.5.2.2. Nanocarriers:

Hydrophobic PSs tend to form inactive aggregates in physiological solutions. When aggregated, the PSs are far less efficient in translating light energy into a chemical potential [205, 206]. This happens because the effect of quenching is amplified in these aggregates, i.e., the aggregated molecule is not able to absorb light or, even though it absorbs light, the photo-excited PS decays to the ground-state before producing singlet oxygen Moreover, due to their low water solubility, hydrophobic PS cannot be simply be injected intravenously for the delivery of the PS to targeted tissues [207]. However, hydrophobic PS can be associated with specific drug delivery systems, such as the nanoparticulated carriers. For this purpose, some specific nanoparticles have hydrophobic compartments that maintain the PS molecules in a monomeric non-aggregated form inside or at the surface of the nanoparticle, keeping the PS active for properly absorbing light [208]. PS encapsulation can also enhance the PS solubility [209]. In addition, the nanoparticle surface can be chemically modified in order to target the PS to prokaryotic cells and prevent their uptake by eukaryotic cells [210]. This strategy may reduce possible side effects of the therapy and the ability of the target cell to pump out the PS, hence reducing the possibility of multidrug resistance [211].

2.5.2.2.1. Liposomes

Liposomes are micro- or nano-structured vesicles formed by a phospholipid bilayer around an aqueous core with a size ranging from 50 to 1000 nm. Liposomes vesicles are interesting and useful drug carriers because they can carry both hydrophilic molecules in their aqueous core and hydrophobic drugs among the fatty acid chains in the phospholipid bilayers [212]. For photodynamic applications, liposomes have been used in association with different PS types and have been successfully applied against several bacterial strains in both *in vitro* tests [191, 210] and clinical studies [213, 214].

Zeta potential of liposomes is an important parameter used in targeting of liposomes to bacterial cells in APDT. If zeta potential is close to zero, liposomes tend to aggregate with reduction in antimicrobial activity [215]. A negative zeta potential results in repulsion between bacterial cells and liposomal nanocarriers [210]. Positively charged liposomes, however, were reported to efficiently disrupt the bacterial outer wall [216]. Tsai *et al* [217] encapsulated hematoporphyrin in liposomes and micelles; liposomal encapsulation prevented the PS aggregation resulting in a higher antimicrobial effect against a series of Gram positive bacteria.

2.5.2.2.2. Nanoparticles

Nanoparticles have been recently used in antimicrobial APDT to enhance the effectiveness of the treatment. The PS is either embedded in polymeric nanoparticles or bound to their surface. Nanoparticles can be divided further into biodegradable (PLA and PLGA) and non-biodegradable types (gold, silica) [218]. Klepac-Ceraj *et al* [219] developed MB-encapsulated PLGA nanoparticles with negative and positive surface charge. Cationic PLGA nanoparticles showed greater uptake by bacteria and phototoxicity compared to neutrally charged nanoparticles. PLGA [220] and polyacrylamide [216] nanoparticles containing MB have been shown to inactivate both planktonic and biofilm cells. Pagonis *et al* [220] demonstrated the accumulation of these nanoparticles on the surface of *Enterococcus faecalis* enhancing delivery of PS through the cell wall.

Apart from encapsulation, PSs have been also covalently bound to the surface of nanoparticles to prepare what, in essence, is a new PS with better properties than the original PS. This is the main difference between this approach compared to PS encapsulation, which is an improved delivery method. A few reports of antibacterial PS linked to nanoparticles are available [221]: e.g. toluidine blue was bound to the surface of gold nanoparticles, resulting in inactivation of *S.epidemidis* [222]. In order to bind the PS to the nanoparticle surface, both the PS and the nanoparticle surface have to exhibit some reactive groups where the linking can occur. Generally, nanoparticles do not have such moieties; therefore, the synthesis of such conjugates has been carried out first by functionalizing the surface of the nanoparticles with tiopronin [222], amine groups [223] or carboxylic groups (acid-functionalization) [224]. The second step is the reaction between the functionalized group on the nanoparticle surface and the PS. In the case of porphyrins, the PS needed to be preliminarily functionalized [224].

2.5.2.3. Photoactive nanofibers

Nanofibers could be a good candidate as a delivery system for APDT for localized and controlled effect especially in treatment of superficial wounds in response to light. A few reports have been published concerning the use of NFs for APDT applications.

A research group led by Jirí Mosinger [48, 225, 226] developed polyurethane nanofibrous textiles that were prepared by electrospinning followed doping with tetra-phenylporphyrin and/or zinc phthalocyanine photosensitizers. These matrices showed the ability to kill bacteria on their surfaces when exposed to visible light. Later, they applied tetra-phenylporphyrin-doped NFs clinically to patients with leg ulcers. The application of the textiles resulted in a 35% decrease in wound size, 42 days post-treatment [227].

Lim *et al* [228] prepared Nylon 6 nanofibers containing organic photosensitizers in the application of the material as protective clothing and home appliances. Benzophenone, 4, 4′-bis (dimethylambenzophenone) and thioxanthen-9-one were used as photosensitizers and the nylon 6 nanofibers were prepared using electrospinning. Antimicrobial properties of the prepared nanofibers were tested against *S.aureus* and *E.coli* upon exposure to UV irradiation.

This review of the literature indicates that polymer-based delivery systems for topical biomedical applications offer great promises in improving patient care. However, further development of drug delivery strategies, enhancement of the activity and specificity of pharmacologically active agents in addition to the integration of the potentials of carrier systems, active agents and devices or techniques would greatly enhance performance and the translation of research activities into clinical practice.

OBJECTIVES OF THE THESIS

Objectives of the thesis

The objective of the thesis was to develop MB-eluting electrospun polymer nanofibers as a controlled delivery matrix that combines the structural and functional advantages of nanofibers and the multiple biological effects of methylene blue for biomedical applications, reducing their limitations. As a structured polymer matrix, nanofibers play an important role as a substitute for the extracellular matrix in applications involving cell regeneration such tissue engineering, regenerative medicine and wound healing as well as a mechanical nanofibrous reinforcement structure for various membranes. In engineering nanofibers for such applications, the functions of NFs can be further enhanced by loading with drugs and bioactive agents. On the other hand, loading of drugs and bioactives in NFs as a delivery matrix may allow protection against chemical and enzymatic degradation in the biological milieu, temporal control on their release in harmony with in-use requirements, prolongation of the localized biological effect at the application site which reduces the shortcomings of using these agents in the free form.

In this context, development of MB-eluting NFs aimed at proposing a new type of antimicrobial NFs as a biomaterial based on antiseptic dyes rather than conventional antibiotics and biocides. These NFs could provide a platform for a wide range of biomedical applications that could diversified by effective exploitation of the various biological effects of MB and the modulation of the polymer matrix compositional and structural characteristics. To this end, polyhydroxybutyrate-based electrospun matrices were developed for selected biomedical applications involving the antimicrobial, antioxidant and light-activation potentials of MB. Compositional and structural modulation of the MB-eluting PHB matrix was undertaken in order to enhance the performance of the matrix in the respective applications.