Synthesis and Surface Functionalization of Conducting Polymer Nanostructures for Biomedical Applications

Abstract

The π-conjugated conducting polymers (CPs) have attracted intensive interest in the past three decades because they display the physical and chemical properties of organic polymers and the electrical characteristics of metals due to the formation of nonlinear defects such as solitons, polarons, and bipolarons by the process of doping. In recent years, an enormous amount of research has been focussed on the development of 1D organic semiconductors such as CPs due to the potential advantages of combining an organic semiconductor with low dimensionality. Additionally, CPs possess very good electrical and optical properties, a high conductivity/weight ratio, reversible doping/de-doping processes, and can be made biocompatible, biodegradable and porous. The chemical, electrical and physical properties of CPs can be altered to suit specific applications by incorporating different functionality even after synthesis. Considering these versatility, several CPs such as polypyrrole (PPy), polythiophene (PT), polyaniline (PAni) and poly(3,4-ethylenedioxythiophene) (PEDOT)) have been investigated since the discovery of CP in 1977 for numerous applications such as microelectronics, polymer batteries, supercapacitors, and actuators and in biomedical applications as microsurgical tools, biosensors, drug delivery systems, and in tissue engineering.

Bioelectricity plays an integral role in maintaining normal biological functions via cranial, spinal and peripheral neural networks which signal for example muscle contraction and wound healing in bone, cartilage, skin, connective tissue. Specifically, the electrical signal in the peripheral nervous system accelerates axonal regeneration and elongation and enhances expression of neurotrophic factors and the biological activity of Schwann cells. In this regard, conducting polymers (CPs) offer excellent control over the level and duration of the electrical stimulus. CPs have a higher charge injection limit with improved charge-discharge characteristics leading to enhanced charge transportation to cells for membrane depolarization. This, in turn, can improve the adhesion and proliferation of nerve cells including the promotion of axonal growth. Therefore, there is the tremendous potential of development of conductive

tissue engineered scaffolds for modifying the regeneration, differentiation, or function of cells both *in vivo* and *in vitro* at a faster rate than conventional nonconductive scaffolds. In combination with the properties of novel scaffold and options for electrical stimulation, CPs lend themselves as one of the most promising biomaterials in future development of regenerative medicine to address worldwide crisis of organs and consequent rise of the risk of organ trafficking along with the shortcomings of the current clinical treatments for damaged tissue or organ and damaged nerves.

An ideal scaffold for regeneration of tissue should be biocompatible, biodegradable, bioactive, highly porous with large surface area to volume ratio, mechanically strong and capable of being formed into desired shapes. However, the development of nanostructured conducting polymers with tunable microstructures and controllable chemical/physical properties still remains a challenge and there are several issues such as toxicity, poor cell-biomaterial interactions due to the absence of cell interaction sites, poor hydrophilicity, non-biodegradability, poor solubility, and processability, as well as uncontrollable mechanical properties to mimic extracellular matrix (ECM) that need to be improved for realizing the true potential of these materials in tissue engineering applications. Therefore, synthesis of 1D nanostructured CP based biomaterials to mimic the nano-scaled patterns of chemical and topographical clues of natural ECM to tailor cell behaviors is one of the utmost interests of the present thesis. While surface functionalization of hydrophobic 1D CP based biomaterials is a promising option to confer necessary specific bioactivity to accelerate ECM secretion and regeneration of cultured cells. Surface functionalization can introduce polarized groups such as hydroxyl (-OH), aldehyde (-CHO), carboxyl (-COOH), amino ($-NH_2$) and sulfate ($-SO_4^{-2}$) groups on polymer surfaces to promote cell adhesion, proliferation, and to maintain cell normal phenotype and functions. Correspondingly, clear characterization of the chemical compositions and physical structures of the 1D CP based biomaterial surface has profound scientific importance, leading to insight understandings of cell-biomaterial interactions.

The present doctoral thesis is a detailed description of synthesis and modification strategies of conducting polymer based nanostructured biomaterials along with a bunch of biological experiments with several living cell types to overcome the above drawbacks. Effect of surface modification through the incorporation of the different polar functionality of conductive polymer based nanocomposites on its physicochemical properties and its consequences on cell-

biomaterial interactions have not been studied extensively. Moreover, only a handful of strategies have been explored for the development of PAni and PAni based composites with good biocompatibility, conductivity, and mechanical properties. While MEH-PPV has not been accessed previously in tissue engineering applications although it offers an interesting property for biological application due to its high-density holes-traps and better solubility than the other CPs such PPy, PAni, PEDOT etc. In the present work, efforts have been made to achieve a better understanding in the fabrication and modification strategies of nanostructured CP based biomaterials and to explore subsequently, the potential of these materials towards biomedical applications such as biosensors and tissue engineered scaffolds. It also tries to gain deeper insight into synthesis and optimization of ECM analogue nanostructured CP based biomaterials with considerable focus on the improvement of cell-biomaterial interactions. After incorporating adequate optimization, the present thesis work further emphasizes on the potential of CPs in neuronal stimulation upon the application of an external electrical field for nerve repairing applications. In the present thesis, three different systems viz. polyaniline (PAni) nanofibres, polyaniline nanofibres:chitosan (PAni:Ch) nanocomposites and electrospun MEH-PPV:PCL nanofibres have been synthesized and their physicochemical and biological properties have been investigated. All these systems have been also surface functionalized to confer bioactivity through incorporation polar functionalities such as aldehyde (-CHO), carboxylic (-COOH), amine ($-NH_2$), hydroxyl (-OH) etc. for better cell-biomaterial interactions. All the systems, before and after surface modification, have been investigated using different sophisticated analytical tools. Transmission electron microscopy (TEM) and scanning electron microscopy (SEM) have been used to study the morphology and structural details of the different nanostructured CP based materials. X-ray diffraction (XRD) studies have been carried out to investigate the degree of crystallinity, domain length and strain in the nanostructured CP based materials. The optical properties of the PAni nanofibres system have been explored using ultra-violet visible (UV-Vis) absorption spectroscopy and fluorescence spectroscopy. The tensile test has been carried out to study the mechanical properties of the materials. The thermal stability and electrical properties of the materials have been investigated using thermogravimetric analysis (TGA) and current-voltage (*I-V*) characteristics. The surface chemistry of the materials has been explored using Fourier transform infrared (FT-IR) and X-ray photoelectron (XPS) spectroscopy.

Wettability and surface energy measurements of PAni and its composites have been carried out by using contact angle measurements. Nuclear magnetic resonance (NMR) spectroscopy has been performed to study the chemical structure of PAni nanofibers before and after surface functionalization. In addition to the above physicochemical characterizations, we have also performed biological characterizations of the nanostructured CP based materials with different biomolecules and living cell types for their potential biomedical applications.

The present thesis is comprised of seven chapters and each chapter has been again subdivided into several sections, subsections for clarity of the contents. In **Chapter I**, beginning with a general introduction on conducting polymer, primary features of 1D conducting polymer nanostructures and its advantages in regard to biomedical applications have been reviewed. The synthesis and surface modification strategies of 1D conducting polymer for biomedical applications particularly for improved performance as tissue engineered scaffold and biomolecule immobilization have been extensively discussed. A substantial literature review on biomedical applications of conducting polymers and its nanostructures with special focus in tissue engineering has been presented. At the end, the motivation of the thesis, scope of the thesis and statement of the thesis problems have been enumerated.

In **Chapter II**, the polymerization mechanisms of PAni nanofibers and basic theory of electrospinning have been discussed. The theories that governed the charge transport in conducting polymers have been explained in brief. The methods for determination of surface energy and its components of the conducting polymer based biomaterials' surface have been also detailed. In addition, **Chapter II** also includes the theory of Fluorescence resonance energy transfer (FRET) and the description of the method of determination of number of binding sites and binding constants using fluorescence enhancement effect. The theories and equations of enzyme kinetics have been discussed in brief at the end of the chapter.

The materials and methods employed in the synthesis and surface functionalization of PAni nanofibres, PAni:Ch nanocomposites and MEH-PPV:PCL nanofibres have been discussed in details in **Chapter III**. The principles of the various physicochemical characterization tools such as TEM, SEM, XRD, Tensile test analyzer, TGA, *I-V* measurement, Contact angle measurement, XPS, FTIR spectroscopy, NMR spectroscopy, UV-Vis spectroscopy, and Fluorescence spectroscopy, have been explained herein. The assays for biological characterization

of the synthesized conducting polymer based materials viz. urease immobilization and activity assay, Hemolysis activity assay, MTS cell proliferation assay, acridine orange/ethidium bromide (AO/EtBr) staining, live/dead assay including beta (III) tubulin immunochemistry have been expounded in details. The experimental details for electrical stimulation of neuronal PC12 cells have been discussed as well.

Chapter IV deals with the synthesis and surface functionalization of polyaniline nanofibres including the physico-chemical and biological characterizations. PAni nanofibres (PNFs) have been synthesized by dilute polymerization method using HCl as dopant and APS as the oxidant. PAni nanofibres have been surface functionalized by 1% glutaraldehyde solution in phosphate buffer solution (PBS) solution (pH=7.4) to introduce polar functionalities such as aldehyde (-CHO), hydroxyl (-OH) groups on the surface. The effects of surface functionalization of PAni nanofibers on their structural, conformational and optical properties have been investigated using TEM, SEM, XRD, *I-V* characteristics, tensile strength test, TGA, UV-Vis absorption spectroscopy, fluorescence spectroscopy, ATR-FTIR, NMR spectroscopy and contact angle analysis using sessile drop technique. Surface functionalization has been found to be performed with incorporation of polar hydroxyl and aldehyde functionalities on the surface as demonstrated by FT-IR and ^{1}H NMR spectroscopy, while surface energy calculations using OWRK (Owens, Went, Rabel and Kaelble) and AB (Acid-base) methods suggest enhanced surface polarity after surface functionalization. Surface functionalized PAni nanofibers (SF-PNFs) demonstrate enhancement in fluorescence due to partial reduction of quinoid to benzenoid units, indicated by UV-Vis and fluorescence spectroscopy. Fluorescence spectroscopy has been used as an analytical tool with SF-PNFs as a substrate to monitor any biochemical reactions involving three aromatic amino acids viz. Tyrosine, Tryptophan and Phenylalanine. ^{1}H NMR spectroscopy suggests covalent interactions of SF-PNFs with aromatic amino acids and possible reaction mechanisms have been proposed based on these results. Remarkable enhancement in fluorescence signals of SF-PNFs in presence of aromatic amino acids has been observed and the apparent binding constant (K_A) and the number of binding sites (n) have been calculated using fluorescence enhancement equation. The K_A value has been found to be highest for SF-PNFs + Tyrosine and n is two for all the polymer amino acid complexes, which are in agreement with the FT-IR and ^{1}H NMR results. Fluorescence resonance energy transfer (FRET) efficiency has been

found to be highest for SF-PNFs + Tyrosine giving maximum fluorescence enhancement. The study of interaction mechanisms by means of an extremely sensitive technique like fluorescence using SF-PNFs as a substrate may provide a promising analytical tool for detection and monitoring any biochemical reactions involving these three aromatic amino acids. SF-PNFs, which are mechanically strong and electrically conductive, have been found to exhibit enhanced blood compatibility, cell viability including greater MDA-MB-231 cell count, spreading and adhesion when compared to the non-functionalized nanofibers as revealed by hemolysis activity assay, MTS proliferation assay, AO/EtBr staining and cell adhesion test (by SEM). The PAni nanofibres were found to be stable in physiological solution since there was no significant change in surface resistivity and fiber diameters after keeping PAni film in PBS for 30 days

Chapter V describes detailed study of physico-chemical and biological characterizations of polyaniline nanofibres:chitosan nanocomposites before and after functionalization. In this embodiment, PAni nanofibres synthesized by dilute polymerization method have been blended with chitosan, a natural biopolymer, at a concentration of 4 % and 6 % (w/v) to confer bioactivity and biodegradability for improved function as biomaterial scaffold. Within this study, a conductive PAni:chitosan nanocomposite based biodegradable material has been surface functionalized with glutaraldehyde and glycine N-hydroxysuccinimide (NHS) ester, separately, in order to investigate the hypothesis that surface functionalization of these conductive materials will enhance bioactivity and improve cell-biomaterial interactions. SEM reveals layers of interconnected networks of PAni nanofibres soaked in chitosan matrix after formation of nanocomposites with rough and porous when compared to that of pure chitosan. PAni nanofibres appeared to be distributed more uniformly in the PAni:chitosan nanocomposites with 6 wt% PAni content than in those with 4 wt% PAni content. The amorphous nature of the nanocomposites has been confirmed from XRD analysis. Stability test demonstrates that after incubation in physiological solution such as phosphate buffered saline (PBS, pH=7.4) for 15 days shows remarkable enhancement in porous morphology on the surface due to degradation of chitosan matrix as confirmed from SEM analysis, whereas surface resistivity measurement reveals no significant change in conductive properties indicating no degradation occurs to PAni nanofibers in the nanocomposites. The distribution of pore diameters after 15 days of incubation in PBS has been increased

from 20-100 nm to 50-1000 nm, providing an essential porosity of tissue engineered scaffolds. Therefore, we speculate that PAni nanofibers took a lead role in maintaining the electrical and structural stability of the nanocomposites as compared to chitosan. PAni:Ch nanocomposites with 6 wt% PAni content show improved *I-V* characteristics than the nanocomposites with 4 wt% PAni content. I-V characteristics of all the nanocomposites demonstrate. Surface resistivity calculations derived from *I-V* characteristics reveal no significant change in the conductive properties of the glutaraldehyde functionalized and glycine NHS ester functionalized nanocomposites from their non-functionalized counterparts. The mechanical strength test reveals that tensile strengths of all the nanocomposites match the tensile strength of softer tissues. Contact angle analysis indicates improvement in wettability and surface polarity of the nanocomposite after surface modification. The appearance of C=O stretching at about 1724 cm^{-1} and a broad band centered at about 3017 cm^{-1} corresponding to strongly H-bonded O-H stretching vibration, in the FTIR spectra of surface functionalized nanocomposites, confirms the presence of carboxylic acid. XPS results suggest the enrichment of the surface of PAni:Ch nanocomposites with higher fractions of O-C=O, C-OH along with the higher percentage of O1s and N1s confirming the incorporation of glycine onto the surface of the nanocomposites making it a carboxyl functionalized surface after functionalization by glycine NHS ester. Similarly, in the case of glutaraldehyde functionalized nanocomposites, FTIR results indicate the presence of aldehyde (1720 cm^{-1}) and hydroxyl (1040 and 3440 cm^{-1}) functionalities after surface functionalization. This is further confirmed by XPS results which show higher fractions of C=O, C-OH and higher atomic percent of O1s on the surface of the nanocomposites after functionalization. Both types of surface functionalized nanocomposites show very less hemolytic activity (less than 5%) when compared to the non-functionalized counterparts, indicating improved blood compatibility of the materials. MTS proliferation assay indicates the improved viability of 3T3 fibroblasts and a neuronal rat pheochromocytoma (PC12) cells on glycine NHS ester functionalized nanocomposites, indicating their non-cytotoxic effect. Glycine NHS ester functionalized nanocomposites demonstrate improved 3T3 cell adhesion, spreading, proliferation and morphology as confirmed by calcein-AM/ethidium homodimer live/dead assay and SEM analysis. The glutaraldehyde functionalized nanocomposites exhibit higher urease activity immobilized on it owing to improved surface hydrophilicity due to the incorporation of polar aldehyde (-CHO)

and hydroxyl (-OH) functionality when compared to its non-functionalized counterpart. The Michaelis constant, K_m has been determined to be 5.41 mM, 13.93 mM and 21.5 mM from the Lineweaver-Burk plot for free and immobilized urease on glutaraldehyde treated and untreated films, respectively. Furthermore, MDA-MB-231 cell seeded on glutaraldehyde functionalized nanocomposites exhibit a higher percentage of viability, adhesion, spreading and morphology as confirmed after AO/EtBr staining and cell adhesion test by SEM. This study further demonstrates that several factors such as surface functional groups and charge as well as wettability and material stiffness, can be moderated by surface functionalization of PAni:chitosan nanocomposites with polar functional groups to improve cell adhesion, spreading, and growth on conductive polyaniline based biomaterials. The collagen coated nanocomposites supported the growth and differentiation of PC12 cells to sympathetic neurons comparable to control collagen-coated glass plate suggesting that PAni:Ch nanocomposites may be suitable as conductive scaffolds for nerve repair. Electrical stimulation of PC12 cells under potential of 500 mV/cm for 2h/day through the conductive collagen coated PAni:Ch nanocomposites demonstrates more neurite formation and longer neurite outgrowth than the unstimulated cells on the same scaffolds and indicates its potential in nerve repair.

Chapter VI epitomized the synergistic effect of nanofibre feature, surface functionalization of electrospun MEH-PPV based materials and electrical stimulation in neuronal growth for potential application in neural tissue engineering. In the third experimental system, a conducting polymer, Poly[2-methoxy-5-(2-ethylhexyloxy)-1,4-phenylenevinylene] (MEH-PPV) along with a biodegradable polymer Polycaprolactone (PCL) has been utilized to prepare conductive, biocompatible, bioactive and biodegradable nanofibrous scaffold to investigate the synergetic effect of nanofibre structure, surface functionalization and electrical stimulation on neuronal growth for possible use in nerve repair. Nanofibres have been produced by electrospinning of blended $FeCl_3$ doped MEH-PPV with polycaprolactone (PCL) at a volume ratio of 20:80, 40:60, 50:50 and 60:40. In order to achieve nanofibres with more conductivity for effective electrical stimulation of cells, coaxial electrospinning of PCL (core) and $FeCl_3$ doped MEH-PPV (sheath) has been performed at a flow rate of 0.6 and 1 mL/h. The electrospun nanofibres have been surface-functionalized using (3-Aminopropyl) triethoxysilane (APTES) and 1,6-Hexanediamine to achieve amine functionalized surface for better cell-biomaterial interactions with an aim to replace

the need of costly biomolecules such as collagen, fibronectin, laminin and RGD peptide for surface modification. The diameter of the electrospun nanofibres [324 ± 70 nm (20:80), 280 ± 82 nm (40:60), 198 ± 30 nm (50:50) and 132 ± 53 nm (60:40)] obtained from a blend of MEH-PPV and PCL decreased with increasing concentration of MEH-PPV as confirmed by SEM. The core-sheath nanofibres have been characterized by TEM and SEM, which indicate the production of larger diameter nanofibres (630 ± 137 nm) using higher flow rate than lower flow rate (526 ± 60 nm). The highly porous electrospun meshes exhibit higher mechanical properties such stiffness constant *(E)* and ultimate tensile strength (UTS). Surface functionalized meshes demonstrate enhanced stiffness constant *(E)* and UTS as compared to their non-functionalized counterparts due to the cross-linking between the polymer chains occurred after functionalization. The core-sheath nanofibres are mechanically strong when compared to the nanofibres prepared from a blend of MEH-PPV and PCL, which is assigned to larger diameters of the core-sheath fibres. The core-sheath electrospun meshes exhibit improved *I-V* characteristics than the nanofibres prepared by simple electrospinning of blend of MEH-PPV and PCL, which can be attributed to the presence of conductive MEH-PPV in the sheath of core-sheath nanofibres. There was no significant change in conductive properties of the electrospun meshes after surface functionalization with APTES and 1,6-Hexanediamine indicating that functionalization was performed without much affecting the conductive properties of MEH-PPV. Stability test results suggest that all the electrospun nanofibres were found to be stable enough in physiological solution due to the non-degradable nature of MEH-PPV and slow degradation rate of PCL, where fibrillar diameter and surface resistivity have been found almost constant after 45 days incubation in PBS (pH=7.4). FTIR and XPS show successful incorporation of amine functionality after surface functionalization. C1s XPS spectra and XPS survey scanconfirm the presence of nitrogenous chemical groups such as C-N and atomic nitrogen (N1s) on the surface of the functionalized electrospun meshes, which are not present in the non-functionalized meshes. Lower water angle values indicate improved wettability or hydrophilicity of the amine functionalized electrospun meshes as confirmed from contact angle analysis. The surface functionalized electrospun meshes showed significant improvement in viability of 3T3 fibroblasts and a neuronal model rat pheochromocytoma 12 (PC12) cells as confirmed by MTS assay. Live/dead assays

and cell adhesion study by SEM demonstrate that 3T3 fibroblasts adhered, spread and proliferated well on the surface functionalized electrospun meshes as compared to non-functionalized meshes. PC12 cells were found to adhere and differentiated well on collagen I coated meshes followed by surface functionalized meshes as compared to non-functionalized meshes. Electrical stimulation of PC12 cells through the electrospun nanofibres under the potential of 500 mV/cm for 2 h for 3 days demonstrates significant improvement in neurite formation and outgrowth than the unstimulated PC12 cells, indicating the potential of MEH-PPV:PCL in the fabrication of nerve guidance cells for nerve regeneration applications. However, the effect of electrical stimulation on PC12 cells cultured on core-sheath nanofibrous meshes has been found to be more prominent owing to their better conductive properties than the nanofibres prepared from blended MEH-PPV with PCL. It is also noteworthy that surface amination of the core-sheath nanofibres along with electrical stimulation comes out as a promising scaffold to replace the need of coating the scaffold with costly biomolecules such as collagen, laminin, fibronectin etc. The enhanced cellular activities and improved neurite growth and differentiation suggest the potential use of these scaffolds for tissue engineering applications including repairing of damaged nerves.

Chapter VII summarizes the major conclusions drawn from the thesis work. The conclusions drawn from the each of the systems have been recapitulated in the context of biomedical applications. An interpretation has been tried to make to figure out the best biomaterial scaffold out of the three synthesized material systems in terms of their physicochemical properties and biological performance in modulating the cell behaviour. At the end of this chapter, the future scope of research in the field of conducting polymer-based biomaterials has been briefly elucidated.