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Melanin incorporated electroactive and antioxidant silk fibroin nanofibrous scaffolds for nerve tissue engineering

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ABSTRACT

Nerve restoration and repair in the central nervous system is complicated and requires several factors to be considered while designing the scaffolds like being bioactive as well as having neuroinductive, neuroconductive and antioxidant properties. Aligned electrospun nanofibers provide necessary guidance and topographical cues required for directing the axonal and neurite outgrowth during regeneration. Conduction of nerve impulses is a mandatory feature of a typical nerve. The neuro-conductive property can be imparted by blending the biodegradable, bioactive polymers with conductive polymers. This will provide additional features, i.e., electrical cues to the already existing topographical and bioactive cues in order to make it a more multifaceted neuroregenerative approach. Hence in the present study, we used a combination of silk fibroin and melanin for the fabrication of random and aligned electrospun nanofibrous composite scaffolds. We performed the physicochemical characterization and also assessed their antioxidant properties. We also evaluated their neurogenic potential using human neuroblastoma cells (SH-SY5Y) for their cellular viability, proliferation, adhesion and differentiation levels. Designed nanofibrous scaffolds had adequate physical properties suitable as neural substrates to promote neuronal growth and regeneration. They stimulated the neuroblastoma cell attachment and viability indicating their biocompatible nature. Silk/melanin composite scaffolds have specifically exhibited high antioxidant nature proven by the radical scavenging activity. Additionally, the melanin incorporated aligned silk fibroin scaffolds promoted the cell differentiation into neurons and orientation along their axis. Our results confirmed the potential of melanin incorporated aligned silk fibroin scaffolds as the promising candidates for effective nerve regeneration and recovery.

1. Introduction

The central nervous system (CNS) consists of the brain and spinal cord which provides excitatory stimuli and conducts and interprets the signals of the peripheral nervous system. Spinal cord injury (SCI) is the major type of damage to CNS whose major causes are motor vehicle accidents, diving injuries, falls, gun shots and knife injuries etc. [1,2]. SCI leads to dysfunction of the cord, with sensory and motor function loss distal to the point of injury. Based on the location of the injury, SCI can lead to paraplegia or quadriplegia and causes devastating neurological deficits and disabilities which affect the quality of the patient's life [3,4]. Repair and restoration of nerves upon damage in CNS is very challenging owing to the factors like the inability of CNS neurons to regenerate in their native environment. Also, the glial cells of the CNS, astrocytes will proliferate and become reactive leading to the glial scar formation impeding the axon growth. Moreover, the primary injury in CNS is followed further by secondary injury due to secretion of free radicals from the blood via the blood-brain barrier which hampers the regeneration [3]. Thus, various attempts have been made to develop an alternative to nerve grafts for CNS where research is focussed to create a suitable environment for regeneration. Many strategies have been developed to improve the functional recovery; each, in turn, restores

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the function with varying degrees of success [5]. Several tissue engineering approaches with moderate success were developed using novel synthetic and semi-synthetic biomaterials [6]. These designed materials known as scaffolds provide the support for three-dimensional cell cultures and mimic the natural extra cellular matrix (ECM) aiding in the tissue regeneration.

Nanofibrous scaffolds have been extensively studied for the similarity with the fibrous architecture of the native ECM at the nanometer scale. Nanofibers can be produced through diverse processes such as electrospinning, phase-separation, and self-assembly. Among all the fiber forming techniques, electrospinning has been widely used due to their ease of fabrication and production of long and continuous fibers. Also, manipulation of the fiber diameter, morphology, physical and mechanical properties, etc. is possible as there is a high degree of control over the process parameters [7,8]. Several applications were found for electrospun nanofibrous scaffolds. Various natural and synthetic polymers of the wide range have been fabricated by electrospinning technique and used for neural regeneration. Electrospun PLGA nanofibers showed a larger number of neural stem cells (NSC's) attached on their surface than the PLGA microfibers and solvent cast films [9]. Cross-linked collagen nanofibers have promoted the NSC proliferation in vitro in a study [10].

In order to achieve either functional recovery or successful nerve regeneration, an ideal nerve conduit must meet several requirements such as (1) It should mimic the structure of native ECM, (2) the topography should favor and guide the axons along their direction, (3) must provide essential bio-recognition signals for the cells to attach, proliferate and perform their normal functions and (4) provide electrical cues for neurite stimulation and growth. Additionally, the antioxidant property which scavenges free radicals at the site of injury will minimize the scar formation and aid in regeneration [11,12]. The aligned electrospun nanofibrous scaffolds fulfill the first two criteria, where the fibrous morphology of the nanofibers provides structural similarity and the orientation along with necessary guidance for the regenerating axons in a particular direction [10,13–16]. Aligned nanofibrous scaffolds have shown significant improvement in maintaining neural cell functions in vitro when compared to their random nanofiber counterparts. Additionally, axial orientation of nanofibrous tubular scaffolds could promote the contact guidance via the establishment of focal adhesion complex thereby directing axonal outgrowth to the distal end of the nerve [17–19]. To achieve the third criteria, i.e., to provide biochemical signals, several attempts have made by blending the natural and synthetic polymers during electrospinning. Also, the incorporation of conducting polymers like polyaniline (PANi), polypyrrole (PPy) into biodegradable polymers has shown enhanced cell orientation and improved nerve regeneration [20,21]. Lee et al. demonstrated that PLGA electrospun nanofibers coated with polypyrrole have shown longer and extensive neurite formation upon electrical stimulation [22]. In another study, PLLA blends with polyaniline showed similar results on NSC neurite outgrowth [23]. PLLA nanofibers when conjugated with polypyrrole as well as NGF and has promoted the PC12 neural cell growth and extension synergistically [24]. Poly (3, 4- ethylenedioxythiophene) doped with poly [(4-styrene sulfonic acid)-*co*-(maleic acid)] (PEDOT: PSS-coMA) were coated on carbon microfibers and functionalized with N-Cadherin and L1 recombinant proteins that stimulated the axonal growth and guidance [25]. However, despite the successful use, synthetic and conducting polymers possess issues related to biocompatibility, immune response, biodegradability, and bioresorbability and thus require an additional process of biofunctionalization to facilitate the cellular attachment.

In order to make an ideal neural device, the material interface should mimic the biophysical and the biochemical properties of neural tissue [26]. So several modifications were attempted by Abidian et al., where the conducting polymers physical surface chemistries can be modified and patterned with bioactive molecules [27]. Polypyrrole patterned CP films were generated through hydrogels stamps which were further entrapped with bioactive molecules creating attractive platforms for cell studies [28]. PEDOT nanofibers were immobilized with glucose oxidase for fabricating a glucose biosensor which showed higher sensitivity and a lower limit of detection in comparison with control PEDOT film [29]. In another study, PEDOT was doped with either polystyrene sulfonate (PSS) or phosphate buffered saline (PBS) ions and results show that the conducting substrates support myoblast proliferation and differentiation [30].

Silk fibroin (SF) has emerged as an excellent biomaterial for the tissue engineering applications due to the unique mechanical properties of these fibers as well as biocompatibility [31]. Silk fibroin electrospun nanofibers have shown improved Schwann cell adhesion, proliferation [32]. Nerve guidance channels made up of electrospun and woven silk fibroin/poly (lactic-*co*-glycolic acid) were biocompatible and had a favorable mechanical strength which was strong enough to resist the suture being pulled out of them [33]. SF/collagen scaffolds were seeded with Schwann cells and adipose-derived stem cells to construct a tissue-engineered nerve conduit (TENC) which showed accelerated nerve regeneration [34]. Electrospun aligned silk fibroin fibers functionalized with growth factors Brain Derived Neurotrophic Factor (BDNF) and Ciliary Neurotrophic Factor (CNTF) promoted retinal ganglion cells (RGC's) axonal growth which indicates their growth-stimulating effects on axons of CNS [35].

Melanin is a biologically derived pigment which has electrically conductive [36] and anti-oxidant properties [37,38]. Eumelanins are extended heteropolymers of 5,6-dihydroxyindole and 5,6-dihydroxyindole-2-carboxylic acid which can stack to form aggregates with strong π–π interactions. The presence of these aromatic systems is thought to offer the source for the unique observed electrical conductivity and photoconductivity [36,39]. Bacterial melanin (BM), obtained from the mutant strain of *Bacillus thuringiensis* favored the structural and functional recovery after peripheral nerve injury [40]. BM has also exhibited neuroprotective action and showed enhanced nerve regeneration followed by conduction recovery after damage to corticospinal tract [41] and facilitated recovery of limb movements after motor tract lesions [42].

Neurorestoration is a crucial phenomenon required for CNS regeneration which is an amalgamation of mechanisms that include axonal regeneration/sprouting, neuroprotection, neuroplasticity, neuromodulation, and neuroregeneration [43]. Efforts to treat traumatic CNS injuries can be broadly divided into two categories: (a) neuroprotection, the minimization of cell damage and death and axonal degeneration caused by the cascade of secondary events, and (b) neuroregeneration, the promotion of plasticity and axonal growth. The main factor to be considered is that the sprouting axons need to be guided to reach their targets. So there is a requirement of a topographical and contact guidance which could be provided by the electrospun nanofibers which act as structural bridges reconnecting the regenerating axons from the lesioned proximal part to the distal end. Neuroprotection can be provided by conferring the antioxidant property. Melanins have the capacity to break free radical chain reactions and execute antioxidant protection. So the melanin incorporated scaffolds designed in the current study can offer the dual neurorestoration property for the CNS regeneration.

We anticipated that silk fibroin/melanin composite would make the ideal nerve biomaterial with electroactive, antioxidant and bioactive properties. Hence in the present study, we used a combination of silk-fibroin and melanin for the fabrication of random and aligned electrospun nanofibrous composite scaffolds with biocompatible, bioactive and conductive properties. We performed the physico-chemical characterization and assessed their antioxidant properties. We also evaluated their neurogenic potential using human neuroblastoma cells (SH-SY5Y) for their cellular viability, proliferation, adhesion and differentiation levels. Our in vitro cell culture results confirmed the potential of silk/melanin composite aligned nanofibrous scaffolds as the favorable candidates to be employed for neuronal cell cultures and thus