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Antioxidative study of Cerium Oxide nanoparticle functionalised PCL-Gelatin electrospun fibers for wound healing application

Hilal Ahmad Rather [a], Ria Thakore [b], Ragini Singh [b], Dhwani Jhala [a], Sanjay Singh [b], Rajesh Vasita [a, *]

[a] Biomaterials & Biomimetics Laboratory, School of Life Sciences, Central University of Gujarat, Gandhinagar, 382030, Gujarat, India
[b] Division of Biological and Life Sciences, School of Arts and Science, Ahmedabad University, Navrangpura, Ahmedabad, 380009, Gujarat, India

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ABSTRACT

Skin wound healing involves a coordinated cellular response to achieve complete reepithelialisation. Elevated levels of reactive oxygen species (ROS) in the wound environment often pose a hindrance in wound healing resulting in impaired wound healing process. Cerium oxide nanoparticles (CeNPs) have the ability to protect the cells from oxidative damage by actively scavenging the ROS. Furthermore, matrices like nanofibers have also been explored for enhancing wound healing. In the current study CeNP functionalised polycaprolactone (PCL)-gelatin nanofiber (PGNPNF) mesh was fabricated by electrospinning and evaluated for its antioxidative potential. Wide angle XRD analysis of randomly oriented nanofibers revealed ~2.6 times reduced crystallinity than pristine PCL which aided in rapid degradation of nanofibers and release of CeNP. However, bioactive composite made between nanoparticles and PCL-gelatin maintained the fibrous morphology of PGNPNF upto 14 days. The PGNPNF mesh exhibited a superoxide dismutase (SOD) mimetic activity due to the incorporated CeNPs. The PGNPNF mesh enhanced proliferation of 3T3-L1 cells by ~48% as confirmed by alamar blue assay and SEM micrographs of cells grown on the nanofibrous mesh. Furthermore, the PGNPNF mesh scavenged ROS, which was measured by relative DCF intensity and fluorescence microscopy; and subsequently increased the viability and proliferation of cells by three folds as it alleviated the oxidative stress. Overall, the results of this study suggest the potential of CeNP functionalised PCL-gelatin nanofibrous mesh for wound healing applications.

1. Introduction

Reactive oxygen species (ROS) are critical players of the wound healing process. Low levels of ROS play physiological role in normal wound healing, notably act as cellular messengers to stimulate cell migration, inflammation and angiogenesis associated with wound healing. In the inflammatory phase, neutrophils and macrophages arrive at a wound lesion and secrete large amounts of ROS along with pro-inflammatory cytokines [1]. The ROS directly attack invading pathogens, kill them and aid their phagocytosis [2]. Furthermore, moderate levels of ROS accelerates angiogenesis by upregulating the production of the vascular endothelial growth factor in keratinocytes [3,4]. ROS are also involved in re-epithelialization. They trigger the activation of epidermal growth factor receptors and the keratinocyte growth factor receptors [5,6] and induces the production of TGF-α in fibroblasts. Hence, ROS can support the migration and proliferation of epidermal cells and therefore enhance wound healing. However, uncontrolled production of ROS produces superoxides which damages the tissue by reduction in antioxidant production and activity [7]. In early stages, wounds with impaired healing have elevated levels of ROS, however, the activity of anti-oxidant enzymes are not elevated, which leads to increased oxidative stress in the wound environment [8]. The early generation of ROS in presence of nicotinamideadenine-dinucleotide-dependent oxidases that are produced by resident endothelial cells and fibroblasts causes impaired wound healing [9]. According to Rodriguez et al., both hypoxia and hyperoxia increases ROS levels which transcends the beneficial effect and

* Corresponding author.
E-mail address: rajesh.vasita@cug.ac.in (R. Vasita).
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causes additional tissue damage [10]. Furthermore, elevated ROS in the wound environment have decelerating effects on angiogenesis and lead to a stagnant inflammatory phase, which further damages tissue through excessive production or activation of reactive oxygen intermediates, inflammatory cytokines, proteases, pro-apoptotic proteins causing increased cell death [11] that lead to wounds with impaired healing [12,13]. Therefore, controlling the levels of ROS at the wound environment may be a viable option to enhance the wound healing.

Different nanoparticle based approaches for decreasing the ROS levels have been used to enhance wound healing [14–16]. Recently, cerium oxide nanoparticles (CeNP) have gained attention for applications in wound healing. This is because CeNPs have a capacity to alternate between two oxidation states: 3^+ and 4^+ due to an oxygen vacancy in their crystal structure [17]. Due to this autoregenerative cycle, they can be used as antioxidant agents [18–20]. Their ROS scavenging ability alleviates the oxidative stress experienced by cells in the wound environment [14,15,21,22].

Electrospun nanofibers have been widely used for skin tissue engineering applications due to their ECM mimicking property, biodegradability and biocompatibility [23]. Blended natural and synthetic biomaterials including PCL-gelatin [23], polyurethane-gelatin (28), poly(l-lactic acid)-*b*-poly(ε-caprolactone)-gelatin [24], PCL-collagen [25], poly(lactic-*co*-glycolic acid)-collagen [26], were extensively explored for skin tissue engineering applications. Blending combines the biological properties of natural polymers and the physicochemical properties synthetic polymers [27,28]. Nanofibers can also be functionalised with certain agents like drugs [29], growth factors [30] and nanoparticles [31] that enhance the wound healing mechanism. Nanofibers incorporated with nanoparticles like silver [32–35], zinc oxide, chitosan [36], gold [37–39] have been assessed for wound healing applications. In a study using spirulina extract derived from blue-green algae as an antioxidant and anti-inflammatory agent. Spirulina extract-loaded PCL nanofiber wound dressings have been found to increase the fibroblast viability *in vitro* by suppressing the function of ROS. *In vivo* assays carried out using these nanofibers have showed increased rate of wound healing and skin regeneration [40].

Therefore, in current study PCL-Gelatin nanofibers have been fabricated as a reservoir of CeNPs to reduce the ROS levels produced by the oxidative stress of mouse fibroblasts. The CeNPs loaded nanofibers were characterized for their degradation behaviour and crystallinity. At multiple time points released nanoparticles were used for superoxide dismutase (SOD) mimetic activity. For biological characterisation direct contact assay (cells were grown on CeNPs loaded nanofiber) and indirect assay (leached nanoparticles was included in cell culture medium) were performed with 3T3L1 mouse fibroblast cells.

2. Materials and methods

2.1. Materials

Polycaprolactone (PCL) (average M_n 80 kDa), gelatin powder (type A from porcine skin), cerium nitrate hexahydratre ($Ce(NO_3)_3.6H_2O$), ferricytochrome C, xanthine oxidase, resazurin sodium salt, poly(2-hydroxyethyl methacrylate) (polyHEMA) and 2,7-dichlorofluorescein diacetate (DCFDA) were purchased from Sigma Aldrich (USA). 1,1,1,3,3,3-Hexafluoro-2-propanol (HFIP), 99% was obtained from Alfa Aesar (India). Dulbecco's modified eagle's media-high glucose (DMEM-HG) and fetal bovine serum (FBS) were obtained from Gibco (India). Hypoxanthine was purchased from Hi-Media Pvt. Ltd. (India). Hydrogen peroxide 30%w/v (H_2O_2) was purchased from Nice Chemicals Pvt. Ltd. (India). 3T3-L1 cell line was obtained from National Centre for Cell Sciences (NCCS) (Pune, India).

2.2. Synthesis of nanoparticles

Cerium nitrate hexahydrate was used for synthesis of cerium oxide nanoparticles (5 mM, 30 mM) using previously published method [41]. Briefly, specific quantity of cerium nitrate hexahydrate was dissolved in 49 mL of deionized water and 1 mL hydrogen peroxide was added. The solution was then aged at room temperature for 10–15 days in order to obtain nanoceria with a high $3^+/4^+$ state. This transition was also signified by conversion of color of solution from yellow to colorless.

2.3. Dynamic light scattering (DLS)

Zeta potential of CeNPs was measured by using dynamic light scattering measurements from Zeta Sizer Nano (Malvern Instruments, United Kingdom) which uses a laser with wavelength of 633 nm.

2.4. Transmission electron microscopy (TEM)

Sample was prepared by adding drop of CeNPs on carbon coated copper grid. After drying the images of CeNPs were acquired by using transmission electron microscope JEOL 2010-F TEM, Japan.

2.5. Fabrication of PCL-Gelatin nanofibers loaded with cerium oxide nanoparticles (CeNPs)

A polymer solution of PCL-Gelatin was prepared by mixing 10% w/v PCL and 20% w/v gelatin in HFIP solvent system for 6–12 h. For CeNP loaded nanofibers (PGNPNF), a 25% v/v 30 mM CeNP solution was added to the polymer solution; whereas for control samples nanoparticle solution was replaced with similar amount of distilled water. Both samples were electrospun using E-spin Nanotech, India electrospinning unit. Briefly, the polymer solution was filled in a 5 mL BD syringe fitted with a 26 gauge blunt end needle. The polymer solution was oozed out at a continuous flow rate of 1 mL/h using a syringe pump. A fixed electrical potential of 1 kV/cm was applied across a distance of 15 cm between the tip of the needle and the collector. The resulting electrospun nanofibers were collected on aluminium foil and glass coverslips (18 × 18 mm) for characterisation and cell culture studies, respectively.

2.6. Scanning electron microscopy

The morphological study of the nanofibers was performed using scanning electron microscopy (SEM) (EVO 18, Zeiss, Germany). After fabrication, the nanofibers were lyophilized and then sputter coated with gold-palladium followed by SEM imaging. The diameters of the PGNF and PGNPNF were determined by measuring randomly selecting 50 fibers from each, using ImageJ software (National Institute of Health, USA).

2.7. Fourier transform infrared spectroscopy

The chemical characterisation of nanofibers was performed using attenuated total reflection Fourier transform infrared spectroscopy (ATR-FTIR) (Agilent, USA). FTIR spectra of PGNF, PGNPNF, gelatin powder and PCL film were recorded in a range of 400–4000 cm^{-1} with a resolution of 4 cm^{-1} at data intervals of 1 cm^{-1}. Multiple spectra were recorded for each nanofiber sample by taking sections from different areas of the sample.

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