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Electrospun nanofibers of a phosphorylated polymer—A bioinspired approach for bone graft applications

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ABSTRACT

Biomaterials based on bioinspired mineralization are expected to offer osteoconductive and osteoinductive scaffolds for bone regeneration. An important role in the mediation of *in vivo* biomineralization process is played by highly anionic non-collagenous phosphoproteins (NCP) bound to the collagen matrix. Inspired by this fact, synthetic analogues of the NCPs like polyvinyl phosphonic acid which provide surface nucleation sites have been employed successfully for mineralization of hard tissues. In this study, electrospun nanofibrous scaffolds of partially phosphorylated polyvinyl alcohol (PPVA) are prepared and studied for matrix mineralization and maturation of human pre-osteoblasts like MG63 cells. Partial phosphorylation was found to affect many solution properties of PVA like increase in surface tension, conductivity and semi-crystalline intermolecular hydrogen bond formations narrowing down the electrospinning window for PPVA. *In vitro* mineralization under SBF treatment was uniform along the length of fibers on PPVA nanofibers. Further, MG63 cells showed increased adherence and proliferation on PPVA nanofibers and the expression of alkaline phosphatase activity and cell-matrix calcium levels were about two times higher than PVA nanofibers. The study established fabrication of electrospun nanofibers of a partially phosphorylated polymer, PVA resulting in improved osteoconduction and expression of early markers of osteoinduction in MG63 cells.

1. Introduction

A series of seminal work in literature have demonstrated the role of polyvinyl phosphonic acid (PVPA) in guiding biomimetic remineralization of demineralized and compromised dental structures [1–3]. PVPA exhibits biomimetic functions by mimicking the non-collagenous phosphoproteins (NCP) like dentin matrix phosphoprotein, which in turn are natural mediators of *in vivo* mineralization [4]. Further evidences suggest that phosphorylation and the presence of phosphoric acid group is an essential biochemical feature of NCPs in their functions in mineral mediation [5,6]. It has been shown that NCP binding to calcium ions and the calcium salt precursors are non-specific in nature and suitable biomimetic analogues like PVPA and P-chitosan can be employed to replace function of NCPs [1,7,8].

Bone mineralization and formation of hierarchically arranged structures also share many processes similar to those observed in dentin matrix. Extracellular matrix (ECM) in bone comprises of type-1 collagen as the major organic component while inorganic component is represented by 4 nm thick carbonated apatite mineralites [9]. In fact, material properties of the bone are derived from its organic–inorganic hybrid structure which exhibits very precise array of the building blocks over a length scale ranging from macroscale of osteons to the nanometer level fibrillar-crystal structures [10]. It is of great interest to understand the process of formation of the organic–inorganic hybrid structure as it will allow design of osteoconductive biomaterials intended for bone regeneration in light of the fact that most of the present day bone grafts fail due to their inability to form a strong bond with the native tissue [11]. Like dentin matrix, bone tissue extracellular matrix also consists of many NCPs like osteocalcin, bone sialoproteins, and osteonectin which contribute to the abundance of signals in the immediate extracellular environment as well as play a crucial role as nucleators in biomineralization process [12–14]. These proteins are phosphorylated at some of their serine or threonine amino acid residues [15]. In NCP mediated mineralzation, pre-clusters of minerals exist in equilibrium with ions in solution, which are directed by templating molecules present on the surfaces to form loose clusters [16,17]. These clusters grow with time epitaxially to first form amorphous particles and ultimately resulting in characteristic ordered mineralization. In this polymer-induced liquid-precursor (PILP) process, surface functionalities play crucial role in first directing a site for generating the liquid-phase mineral precursors, facilitating hydroxyapatite growth and ultimately resulting in intrafibrillar and extrafibrillar mineralization of collagen matrix [10]. Thus the importance of mineralization in yielding

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an osteoconductive and mechanically strong matrix for bone tissue engineering is now well appreciated in bone tissue engineering [9,18,19]. However, since isolating NCPs and making use of them in biomaterial fabrication is a difficult proposition, attempts to mimic the mineralization function of phosphoproteins by phosphorylation of synthetic polymers present a promising approach [20,21]. In addition, electrospun nanofibers can most closely mimic the nanofibrillar matrix, present a high surface area, numerous surface nucleation sites, and presence of nanosized pores to aid infiltration of liquid phase-amorphous clusters to uniformly mineralize the whole matrix justifying the motivation to fabricate nanofibers of partially phosphorylated polymers for bone regeneration.

Present work attempts to fabricate matrix with improved osteoconducivity based on the principle of bioinspired mineralization. Polyvinyl alcohol, known for good mechanical properties, is selected as a model polymer to investigate the effect of electrospun phosphorylated polymers as PVA is easily electrospinnable and affords chemistry amenable for phosphate ester formation. An important distinction of the polymer used in the study is investigating phosphate esters of PVA compared to works on phosphonic esters cited previously in this paper. Further partially phosphorylated form of the polymer is synthesized in this work keeping in view the fact that throughout any non-collagenous protein, phosphorylated sites are few and dispersed over the length of macromolecule [22]. Though many studies have shown the increase in bone-like cell differentiation attained by partially phosphorylated polymers [23], to the best of our knowledge proof-of-concept nanofabrication of partially phosphorylated polymer and response of pre-osteoblasts like MG63 cells on these materials are not yet reported in literature.

2. Materials and method

2.1. Synthesis of partially phosphorylated polyvinyl alcohol (PPVA)

PPVA was synthesized following procedures reported in literature with some modifications [24,25]. 10 g polyvinyl alcohol (GHOSENOL GH 17-R, DD-86–89%, Singapore), was charged into a three-necked round bottom flask containing 4.5 M orthophosphoric acid (Merck, India) solution with vigorous mechanical stirring. Completely homogenous melt mixture was refluxed at 85 °C for 3 h, cooled to room temperature and product precipitated in excess of methanol (Himedia, India). The precipitate was repeatedly resolubilized in deionized water, reprecipitated and washed with methanol to remove unreacted molecules. The product was then vacuum dried at 60 °C for 3 days. Degree of phosphorylation was calculated by the ammonium molybdo–vanado–phosphoric acid method for five batches of products synthesized to achieve control over the degree of phosphorylation in laboratory [24].

2.2. NMR spectra of PPVA

Polymers were dissolved in D_2O by heating at 60 °C for 90 min at concentration of 100 mg/ml and proton NMR spectra were recorded on an AVANCE DAX-400 (Bruker, Sweden) 400 MHz NMR spectrometer.

2.3. Solution state physicochemical properties of PPVA

Weighed polymer samples were allowed to swell in water for 1 h and dispersions were heated at 60 °C for about 90 min followed by mechanical stirring for 2 days at room temperature. pH and conductivity were measured for samples synthesized in three batches by Cyberscan PC 510 (Eutech Instruments, Singapore). Surface tension was measured on tensiometer with platinum plate (DCAT 100, Dataphysics GmBH, Germany) and the Bohlin CVO D100 (Malvern, UK) with cone and plate geometry (CP 4/40, Gap size 150 μm, 25 °C) rheometer was used for viscosity measurements.

2.4. Fabrication of electrospun nanofibers of PPVA

Electrospun nanofibers were obtained from 10% (w/v) aqueous solutions of PPVA using a conventional electrospinning set up. The conditions used were flow rate of 10 μl/min (KD Scientific, USA) fed into a 2.5 ml disposable syringe, 18 KV voltage (E 23 Glassman High Volatge, Japan), and a tip-collector (aluminium foil covered copper ground, 5 cm × 5 cm) distance of 10 cm. The deposited sheet was detached from the aluminum foil and cross-linked in acetone-HCl (0.1 M)–glutaraldehyde (50 mM) solution (all Merck, India) for 3 h at room temperature and washed thoroughly in acetone and ethanol. Both uncrosslinked and crosslinked fibers mats were sputter coated with gold (Polaron, UK) and observed under SEM (EVO 60, Carl Zeiss SMT, Germany). Similar procedure was adopted for PVA nanofibers and solvent cast films. The fibrous mats were checked for their stability in aqueous and 70% ethanol solvent systems.

2.5. FTIR and contact angle characterization

Vibration spectrum of the as spun and cross-linked samples was acquired from a PerkinElmer (USA) FTIR GX spectrophotometer with the ZnSe (45°) HATR attachment. Average of 32 spectral scans in 4000–650 cm^{-1} at resolution of 4 cm^{-1} were obtained for each sample and analyzed by the Spectrum software. Contact angles were measured in water (only crosslinked samples) by DCAT tensiometer (Dataphysics, Germany) at room temperature.

2.6. In vitro mineralization in simulated body fluid

Ethanol treated nanofibrous scaffolds (30 mm × 30 mm × 1 mm) were immersed in simulated body fluid (SBF) prepared as per formula of Kokubo and Takadama [26] and incubated at 37 °C. After 5 days of incubation, samples were removed, washed thrice with deionized water, vacuum dried, and examined under SEM. Compositional analysis was performed by EDX (Inca PentaFET-3, Oxford Instruments, UK) without any gold coating.

2.7. Protein adsorption study

The protein adsorption measurements were performed on the PVA nanofibers and PPVA nanofibers for 1, 2, 4, and 8 h by incubation with 10% FBS (Himedia, Mumbai Cat. No. RM1112) solution at 37 °C following an established procedure [27].

2.8. Cell culture experiments

Human osteoblast-like MG63 cells were cultured in DMEM media supplemented with 3.7% $NaHCO_3$, 10% FBS, 2 mM sodium pyruvate, 1 mM L-glutamine and 1% antibiotic antimyotic solution (All Himedia, India) in controlled environment of 37 °C, 95% humidity and 5% CO_2. The cells were detached from culture flasks (Nunc, Denmark) by EDTA-trypsinization and centrifuged. Cell count was performed by Countess (Invitrogen, USA).

2.9. Cellular viability on PPVA scaffolds

Four samples of identical dimensions were sterilized and presoaked in medium overnight and cells were seeded at 4×10^5 cells/scaffold concentrations. Cellular content and viability were assessed using MTT assay on days 1 (12 h after seeding), 3, 5 and 7,