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# Physico-chemical/biological properties of tripolyphosphate cross-linked chitosan based nanofibers

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ABSTRACT

In this study, chitosan-PEO blend, prepared in a 15 M acetic acid, was electrospun into nanofibers (~78 nm diameter) with bead free morphology. While investigating physico-chemical parameters of blend solutions, effect of yield stress on chitosan based nanofiber fabrication was clearly evidenced. Architectural stability of nanofiber mat in aqueous medium was achieved by ionotropic cross-linking of chitosan by tripolyphosphate (TPP) ions. The TPP cross-linked nanofiber mat showed swelling up to ~300% in 1 h and ~40% degradation during 30 day study period. 3T3 fibroblast cells showed good attachment, proliferation and viability on TPP treated chitosan based nanofiber mats. The results indicate non-toxic nature of TPP cross-linked chitosan based nanofibers and their potential to be explored as a tissue engineering matrix.



## 1. Introduction

The architecture of extra-cellular matrix (ECM) and its micromechanics modulate morphogenesis of the native tissues *in vivo* [1]. Nano/microfibrillar arrangement of ECM components allows formation of a highly interconnected porous network with adequate structural resilience essential for cellular function. To mimic this fibrillar structure in tissue engineering scaffolds, nanofiber matrices offer a good choice [2]. Nanofiber morphology facilitates good cellular response owing to their high surface area and favor cell-material interaction [3]. Of the various techniques used for nanofiber fabrication, electrospinning seems to be promising due to its simplicity, environmentally friendly nature, cost-effectiveness and scalability [4]. Electrospun matrices provide an interconnected porous network with tailorable mechanical properties desirable for tissue engineering applications.

However, a major concern for electrospun matrices is their inherent small pore size that restricts cellular infiltration, thereby limiting their potential application as three dimensional tissue engineering constructs. This difficulty may be addressed by various techniques such as incorporation of leachable agents into polymer solutions [5], controlling fiber packing density by tuning spinning parameters [6] or by using optimally designed collector modules [7]. In some cases small pores may be desirable, for example in specific tissue engineering applications like vascular graft reconstruction. Soletti et al. (2010) have successfully developed a bilayered vascular graft scaffold with highly porous cellular inner layer acting as tunica media and an outer electrospun layer with limited porosity mimicking tunica adventitia [8].

From the perspective of skin tissue engineering, electrospun scaffold may be utilized for basement membrane reconstruction to treat skin injuries. In native skin, basement membrane (mainly composed of collagen IV, laminin, nidogen, and glycosaminoglycans) acts as a separating layer for the two distinct cellular compartments, namely, the epidermis and dermis [9]. Trans-membrane migration of epidermal and dermal cells is restricted to maintain the compartmentalized anatomy of skin. The epidermal compartment contains no blood vessels and completely relies on underlying dermis for nutrient supply and metabolic waste disposal via diffusion across basement membrane. While mimicking skin basement membrane, scaffolds with nanometer scale pore sizes may play key role in restricting inter-compartmental migration of epidermal and dermal cells (especially fibroblasts and keratinocytes with average diameter of 3–10 μm) while allowing diffusion of nutrients and metabolic wastes. It may also be noted that dermal fibroblasts can attach and organize well around fibers with diameter less than the cellular diameter [7]. In this respect, a submicron scale fibrous scaffold may be advantageous for better cellular attachment, proliferation and related ECM deposition.

Looking into the current trends of tissue engineering, more emphasis is being given for development of scaffolds using natural and bio-degradable materials like chitosan, collagen, hyaluronic acid, silk and fibrinogen [10–14]. Amongst various biopolymers used, chitosan (N-deacetylated derivative of chitin) is promising due to its biocompatibility, tailorable biodegradability, non-antigenicity, antimicrobial activity and wound healing potential [15]. For tissue engineering application, chitosan seems attractive due to its similarity

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with glycosaminoglycans (GAG)—a major content of the ECM [16]. The slow biodegradation of chitosan in lysozyme environment releases nontoxic end products (e.g. N-acetyl glucosamine, glucosamine and related oligomers), that trigger native macrophages for guided collagen deposition, essential for wound healing and functional tissue regeneration *in vivo* [17].

Electrospinning of chitosan has been attempted by various researchers and was found to be challenging owing to its limited solubility, rigid crystalline structure and hydro-colloid nature [18]. Geng et al. (2005) have reported electrospinning chitosan nanofiber, using a less deacetylated polymer (degree of deacetylation, DA < 54%) in 15 M acetic acid [19]. Unfortunately, chitosan with lower deacetylation exhibit lower tensile strength and poor cell attachment/proliferation potential, that restricts its applicability in tissue engineering. Min et al. (2004) and Ohkawa et al. (2006) have also fabricated chitosan (DA ~ 80%) nanofibers using solvents like 1,1,1,3,3,3-hexafluoro-2-propanol and tri-fluoroacetic acid, which have toxic physiological effects [20,21]. In order to overcome the difficulties of chitosan electrospinning, nanofiber fabrication has been attempted using blends of chitosan with other biocompatible polymers like poly (vinyl alcohol) (PVA) and PEO [22,23]. In fact, addition of co-spinning polymers facilitates entanglement of the polymer molecules, while reducing the solution viscosity necessary for fiber formation. In a chitosan-PEO blend, flexible PEO molecules associated with rigid chitosan chains promoted solution flowability along with providing adequate entanglement needed for electrospinning.

Although recent efforts of chitosan based nanofiber fabrication are encouraging, their crosslinking under physiological conditions is still a challenge. To prevent fiber dissolution in aqueous medium [24], chitosan mats are commonly cross-linked using various covalent cross-linkers like glutaraldehyde [25], glyoxal [26], ethylene glycol diglycidyl ether [27], etc. However, this covalent crosslinking takes long reaction time and has potential cytotoxicity *in vitro* [28]. The ionic cross-linkers like tripolyphosphates (TPP), on the other hand, can facilitate kinetically driven cross-linking of chitosan in aqueous medium under physiological conditions [29]. However, effect of TPP mediated ionotropic cross-linking on physico-chemical and biological properties of chitosan based nanofiber mats has not been explored largely, especially in context of skin tissue engineering application.

The present study investigates potential of ionically cross-linked electrospun chitosan based nanofibers for tissue engineering. Electrospinning of chitosan and chitosan-PEO blend solutions in acetic acid is reported for nanofiber fabrication. Preliminary investigation of different solution parameters including polymer loadings was carried out to understand their effect on electrospinning of chitosan. The nanofiber mats were cross-linked with TPP ions to enhance their architectural stability in aqueous medium. Further cell attachment, proliferation and viability of cross-linked nanofibers were studied to validate *in vitro* cytocompatibility of the matrices.

## 2. Materials and methods

### 2.1. Preparation of solutions for electrospinning

In this study, glacial acetic acid (Merck, India) and de-ionized water were used as solvents for dissolving two different polymers, chitosan (<90% de-acetylated, average $M_w$ ~ 710,000, Marine Chemical, India) and poly(ethylene oxide) (PEO, average $M_v$ ~ 200,000, Sigma Aldrich, US). The stock solutions of 3% (w/w) chitosan and 10% (w/w) PEO were prepared using 0.5 M and 15 M dilute acetic acid by overnight stirring. The prepared solutions were filtered using filter mesh (~88 μm pore size) followed by centrifugation to remove air bubbles present in it for uninterrupted spinning. Chitosan-PEO blends in different weight ratios (3:1, 4:1, 6:1 and 9:1) were thus prepared using 3% (w/w) chitosan and 10% (w/w) PEO followed by overnight stirring and centrifugation. Freshly prepared solutions were used for physico-chemical analysis and attempted for electrospinning. Samples were labeled as *x*C*y*P where '*x*' and '*y*' refer to the weight ratio of 3% (w/w) chitosan and 10% (w/w) PEO, respectively. Details of all sample solutions prepared are given in Table 1.

### 2.2. Solution conductivity and pH

In this study, pH and conductivity of chitosan, PEO solutions and their blends were examined using a conductivity/pH meter (Orion, USA) at 25 °C.

### 2.3. Dynamic surface tension measurement

The dynamic surface tension of chitosan, PEO stock solutions and their blends were measured based on Wilhelmy plate method as described by Kriegel et al. (2009) using a standard platinum probe mounted on a computer controlled surface tensiometer (Data Cat, Germany) [30]. The surface tension (σ) of the fluid was calculated from the following equation-

$$\sigma = F/L\cos\theta$$

where *F*, *L*, and θ are force acting on probe, length of meniscus, and contact angle, respectively.

### 2.4. Rheological properties of solutions

In this study, viscosity and dynamic yield stress of chitosan, PEO and their blends were measured using a Bohlin CVO and Gemini 200 rheometers (Malvern, UK), respectively, using parallel plate geometry (20 mm diameter) at 25 °C. The shear rate was varied between 0.2 and 100 $s^{-1}$.

### 2.5. Electrospinning of chitosan, PEO stock solutions and their blends

For electrospinning, a high voltage DC power supply (30 KV, Glass Mann, Japan) assembled with a syringe pump (KD Scientific, Switzerland) was enclosed in a wooden chamber having additional safety switch. A custom made wooden cylindrical rotary mandrel (~7 cm diameter, 200 rpm), used as collector, was connected to ground for charge dissipation from the deposited polymer solution. A syringe, fitted with a blunt end stainless steel needle (26 G), was fed with the polymer test solutions. The syringe was housed horizontally on the syringe pump that maintains solution flow rate in microliter range. The metal capillary of syringe was attached to positive electrode of the high voltage DC power supply. The rotary mandrel was wrapped with aluminum foil and placed at a distance of ~15 cm from the capillary tip. The syringe pump released polymer solution at a flow rate of 8 μl/min. A 20 kV electric field was maintained between the two electrodes. All experiments were carried out at 25 °C. The drop of polymer solution present in the capillary tip was monitored for formation of the cone-shaped jet (Taylor cone), under applied electric field, which further travelled towards the collector causing fiber/bead formation. The samples were vacuum-dried and stored in desiccators prior to any further use.

### 2.6. Scanning electron microscopy

The morphologies of electrospun samples, prepared using a range of solutions (Table 1), were examined under scanning electron microscopy (SEM, EVO 60/ Zeiss, Germany) at an accelerating voltage of 10–20 kV. Prior to microscopy, all samples were gold coated for 90 s using a plasma gold sputter. Fiber diameters of each electrospun sample were measured from SEM micrographs using IT3 software. 20 random fibers were considered for this measurement.