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Covalent cross-links in polyampholytic chitosan fibers enhances bone regeneration in a rabbit model

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ABSTRACT

Chitosan fibers were prepared in citric acid bath, pH 7.4 and NaOH solution at pH 13, to form ionotropically cross-linked and uncross-linked fibers, respectively. The fibers formed in citric acid bath were further cross-linked via carbodiimide chemistry; wherein the pendant carboxyl moieties of citric acid were used for new amide bond formation. Moreover, upon covalent cross-linking in the ionically gelled citrate–chitosan fibers, incomplete conversion of the ion pairs to amide linkages took place resulting in the formation of a dual network structure. The dual cross-linked fibers displayed improved mechanical property, higher stability against enzymatic degradation, hydrophobicity and superior bio-mineralization compared to the uncross-linked and native citrate cross-linked fibers. Additionally, upon cyclic loading, the ion pairs in the dual cross-linked fibers dissociated by dissipating energy and reformed during the relaxation period. The twin property of elasticity and energy dissipation mechanism makes the dual cross-linked fiber unique under dynamic mechanical conditions. The differences in the physico-chemical characteristics were reflected in protein adsorption, which in turn influenced the cellular activities on the fibers. Compared to the uncross-linked and ionotropically cross-linked fibers, the dual cross-linked fibers demonstrated higher proliferation and osteogenic differentiation of the MSCs *in vitro* as well as better osseous tissue regeneration in a rabbit model.

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1. Introduction

Surface modifications of biomaterials leading to differential interfacial phenomena such as wettability, protein adsorption, and bio-mineralization that in turn fine tune the cellular activities *in vitro*/*in vivo* are widely documented [1], [2] and [3]. For instance, competition between water molecules from aqueous biological environment and proteins for hydrophilic surfaces is reported to cause lower protein adsorption that is unfavorable for cellular adhesion kinetics [4], [5] and [6]. The hydrophobic materials adsorb relatively more protein than the hydrophilic surfaces [7]; nevertheless, it also leads to non-specific protein adsorption and eventually results in fouling and foreign body reactions [8], [9] and [10]. This is particularly detrimental for long-term cell cultures and *in vivo* applications. In this context, employing environmentally benign biomaterials that selectively binds to the protein of interest would be a major advancement in tissue engineering practices [11]. Zwitterionic materials are reported to mitigate non-specific protein adsorption and foreign body reactions owing to its ability to bind water molecules strongly via electrostatically induced hydration [12] and [13]. Mixed charged materials also demonstrate charge-induced hydration and have been shown to be equivalent to zwitterionic materials [14].

Ionic cross-linking of polycationic chitosan with carboxyl anions renders a polyampholytic (mixed charged) character to the polymer. In our previous study, glucosamine moieties of chitosan were ionically cross-linked with citrate ions to form strain reversible polyampholytic gels for applications in living tissues experiencing cyclic load [15]. Chitosan-citrate gels were cyto-compatible and resulted in faster subchondral bone regeneration upon implantation in femoral condyle of rabbits. This may be because of the fact that citrate is one of the components of bone and plays a pivotal role in stabilizing the existing apatite nanocrystals [16]. However, ionically cross-linked structures suffer from mechanical instability owing to pH change and solvent exchange [17]. The introduction of stable covalent bonds in ionically gelled network structure significantly improves the physico-chemical properties [18]. For instance,

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Bakarich et al. reported extrusion printing of hydrogels prepared from ionically cross-linked gellan gum and covalently cross-linked gelatin that demonstrated improved mechanical characteristics [19]. However, no study has attempted so far to investigate the effect of ionic-covalent linkages in a single polymer network in terms of mechanical stability, interfacial phenomena and its utility in tissue engineering.

The present study investigates the fiber forming ability of chitosan with citric acid at physiological pH via instantaneous ionotropic complexation. The citrate–chitosan fibers were further cross-linked via carbodiimide chemistry to introduce amide bonds in the network structure forming a dual cross-linked network. The fibers were assessed in terms of physico-chemical properties, biodegradation, bio-mineralization, cellular behavior and *in vivo* response towards bone tissue regeneration.

2. Experimental

2.1. Materials

Chitosan (MW 710000; > 90% deacetylated, Marine chemicals, India), citric acid (Merck), sodium hydroxide (Merck), N-Hydroxysuccinimide (NHS; Sigma), 1-Ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC; Sigma), Florescamine (Sigma), lysozyme (hen egg-white, ~50,000 units/mg protein; Sigma), BCA kit (Sigma), dexamethasone (Sigma), ascorbic acid (Sigma), Alcian blue (Sigma), Masson trichome staining kit (Sigma), 5% aqueous solution of povidone-iodine (Hixadine, Hicks Thermometers Limited) were used as received. Cell culture medium (alpha-MEM), fetal bovine serum (FBS), antibiotics, rhodamine–phalloidin (catalog no. R415), DAPI (4′,6-diamidino-2-phenylindole, catalog no. D1306), MTT ([3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide], catalog no. M-6494) and osteocalcin ELISA kit was purchased from Invitrogen, India. For the preparation of aqueous solutions, deionized Milli-Q water (18.2 MΩ cm) was used.

2.2. Fiber formation

6 wt% Chitosan stock solutions were prepared according to our earlier established protocol [20]. Chitosan solution was extruded in a coagulant bath using viscose type stainless steel spinneret (50 holes, 0.1 mm) to generate continuous fibers, which were subsequently collected on a rotating bobbin. The coagulant bath consisted of different concentrations of citric acid, pH 7.4 or 5% w/v NaOH, pH 13. Fibers were rinsed thoroughly with DI water, incubated in 100% ethanol for 2 h followed by drying in a humidified chamber. The chitosan fibers formed in citric acid bath were additionally cross-linked covalently with NHS-EDC at pH 5.5. Hence, there are three types of fibers–(a) chitosan fibers formed in citric acid bath, (b) citrate-chitosan fibers further cross-linked using NHS-EDC and (c) fibers formed in NaOH bath. The surface features of the dried chitosan fibers were examined using scanning electron microscopy (SEM, Zeiss EVO 60) after gold coating using plasma-sputtering unit.

2.3. Chemical characterization

Chitosan fibers formed under different conditions were chemically characterized by FTIR and ninhydrin assay as given in the Supplementary data. Florescamine assay was performed to detect % of free primary amine on the surface of the chitosan fibers as reported in a previous study [21]. Briefly, glycosidic linkages in the chitosan fibers were completely cleaved in lysozyme leaving amine bonds intact for fluoroscopic detection. Chitosan solution (20 μL) was reacted with 10 μL fluorescamine dye (3 mg/mL in DMSO stock solution) in 170 μL of sodium borate buffer, pH 8.5. The solutions were incubated for 5 min and the end-point emission was recorded at 460 nm by using the excitation wavelength of 400 nm in the fluorescence plate reader. Uncross-linked chitosan was assumed to contain 100% amine groups and served as control on which the relative amount of primary amine was calculated. The formation of amide bonds in the citrate–chitosan fibers cross-linked with NHS-EDC was investigated using BCA assay as provided in the Supplementary data.

2.4. Nanoindentation and mechanical stability

Nanoindentation on chitosan-based fibers was performed to obtain load–displacement curve, modulus and hardness using nanoindenter (TI 950 TriboIndenter, Hysitron Inc., USA) following our previous study [15]. A maximum load of 100 μN was applied at 10 different points on the fibers at a distance of 4 mm. Fibers were further subjected to loading–unloading cycles under a fixed load of 100 μN to investigate the differential energy dissipation of the fibers. Thermal drift was measured and corrected for each indentation. Biodegradation study was performed as stated in the Supplementary data.

2.5. Interfacial studies

2.5.1. Contact angle

Fiber wettability was investigated using sessile drop technique as reported in a previous study [22]. The equilibrium angle formed between a 2 μL of milliQ water drop and the surface of the fibers was calculated using Protractor software ($n = 3$).

2.5.2. Protein adsorption

Chitosan fibers ($n = 5$, each experiment was repeated twice) were investigated for protein adsorption after 1 and 16 h as provided in the Supplementary data.

2.5.3. In vitro mineralization

Ethanol treated fibers were immersed in simulated body fluid (SBF) prepared as per formula of Kokubo and Takadama [23] and incubated at 37 °C. After 5 days of incubation, samples were removed, washed thrice with DI water and vacuum dried. Preparation of serum supplemented SBF was performed as mentioned before [24]. Briefly, 400 mL of SBF was mixed with 100 mL of fetal bovine serum. The mixture was sterilized using 0.22 m filter. The solution was stored at 4 °C and warmed to 37 °C prior to use. The samples were incubated with the serum supplemented SBF for 3 days. The samples were imaged with FESEM (FESEM Supra 55, Make–Zeiss, Germany). Compositional analysis was performed by EDX (Oxford Liquid Nitrogen free SDD X MAX 50 EDSInca, UK).

2.6. Cell culture studies

Bone marrow derived MSCs were procured from Advanced Neuro-Science Allies (ANSA) Bangalore, India, where routine characterization for surface markers expression is studied. BM-MSCs were certified positive for CD 90, CD 73, and CD 44 (Supplementary data, Fig. S1). MSCs were expanded in complete medium consisting of alpha-MEM supplemented with 10% FBS, 1% penicillin–streptomycin solution and 3.7% sodium bicarbonate and cultured at 37 °C in a 5% CO_2 incubator. Cytocompatibility assay was evaluated with hMSCs in two different ways: culturing cells in media containing degradation products of the fibers and on the surface of the fibers to directly evaluate polymer–cell interface. The biodegradation products of the chitosan fibers after 4 weeks were centrifuged and 1 mL supernatant with 4 mL complete media was incubated with hMSCs to evaluate the cytotoxic effects of the fibers

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