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Chitosan–hyaluronic acid hydrogel coated poly(caprolactone) multiscale bilayer scaffold for ligament regeneration

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HIGHLIGHTS

- Stacked Ch-HA hydrogel coated PCL aligned/random multiscale fibers were developed.
- The coated scaffold showed enhanced protein adsorption and cytocompatibility.
- Ligament fibroblast cells attached along the fiber direction in all the scaffolds.
- Higher cell retention was observed after hydrogel coating on fibers.
- Ch-HA hydrogel coating on PCL aligned multiscale would aid in ligament regeneration.

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ABSTRACT

Ligament tears, seen in young athletes to elderly people, pose a very challenging problem to achieve complete healing, due to its hypocellularity that decelerate regeneration of ligament after injury. Tissue engineering is an approach for the ligament regeneration that optimizes the response of cell–biomaterial interaction to fasten regeneration using engineered scaffolds that mimic the native environment. Electrospinning techniques and hydrogels are useful to engineer the structure of ligament. In this study, electrospun multiscale fibrous scaffold of PCL aligned microfibers/random nanofibers (PCL aligned multiscale fibers) and PCL random microfibers/nanofibers (PCL random multiscale fibers) were developed. Chitosan–hyaluronic acid hydrogel coating was done on these fibrous scaffolds and this was layered to form a bilayered construct. The developed scaffold was characterized by SEM, FTIR, and tensile testing. Protein adsorption studies show better protein adsorption on coated scaffolds compared to uncoated scaffolds which further improved the cell viability as determined by Alamar blue assay and DNA quantification by Pico green assay. Rabbit ligament fibroblast cell attachment and infiltration study conducted on the scaffolds showed cell elongation along the aligned fibers, which would be advantageous in the need to align cells along the direction of force in native ligament environment. Hydrogel coating on PCL random multiscale fibers show better cell infiltration. This study implies the use of hydrogel coated systems to provide a reservoir for cells and nutrients and further modifications of these systems would make it promising for ligament regeneration.

1. Introduction

Ligament, fibrous connective tissue which connects bone to bone, is one among the commonly injured tissue often addressed by an orthopedic surgeon. The functions of the ligament are to provide support and aid in joint movements. Basically ligament injuries were treated by using allografts, autografts or biological grafts [1]. The limited availability of tissue, donor site morbidity, chances to cause bacterial disease, transmit infection, unfavorable immunogenic response from the host and alteration of mechanical strength after sterilizing the graft and above all, the failure of graft leading to secondary surgery were the most recurring disadvantages faced [2,3]. Tissue engineering is the use of chemical, engineering and biological parameters to stimulate the regeneration of tissue with the aid of scaffolds that act as alternatives for the injured tissue and help in restoration of function of the tissue [1–4]. In ligament tissue engineering applications, scaffolds used should provide proper shape and required mechanical strength during reconstruction and degrade at a rate similar to the regeneration rate of tissue. For ligament reconstruction both natural and

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synthetic materials have been used in the form of membranes, gels or 3-D scaffolds [5–10]. Synthetic polymer biomaterials are widely used for ligament tissue engineering mainly because of their tunable degradation rate and effortless and cost effective large scale production, and also improved mechanical strength compared to natural biomaterials [3].

Electrospinning has been widely considered as a promising polymer handling technique for the construction of scaffolds to be used in tendon and ligament tissue engineering. The alignment of the fiber is determined by the rotation speed of the type of collector or target. Random fibers are collected using stationary collectors and aligned fibers are collected by wheel-like bobbin/metal frame or rotating drum [11]. Polyhydroxyesters degrade through hydrolysis and hence it is widely used for tendon/ligament tissue engineering. To mimic the hierarchical structure of natural ligament Lu.et al. [5] designed 3D braided scaffolds of polyglycolic acid (PGA), poly(lactic-co-glycolic acid) (PLGA), and poly(L-lactic acid) (PLLA). PCL fibers have been widely used in ligament reconstruction due to their excellent mechanical properties, low degradation time and non-toxic byproducts after degradation [12]. In this study a multiscale architecture using nano and microfibers have been designed for tendon or ligament tissue engineering. The importance of nanofibrous scaffolds is to provide proper environment and physical cues for cell attachment, proliferation and function than the traditional scaffolds. [13] Moreover the microfibers help in improving the porosity of scaffold for proper exchange of nutrients further aiding in cell infiltration [14–16].

Hydrogels are water swollen polymeric networks, mainly consisting of crosslinked hydrophilic polymers that are not water soluble but can swell. It plays a very important role in tissue engineering due to their soft nature that provides tissue like environment for cell growth and allow diffusion of nutrients and cellular waste through the elastic hydrogel network. Hydrogels have been developed as scaffold materials due to their property of structurally mimicking the extra cellular matrix of many tissues [17]. Natural polymers were used to make hydrogel scaffolds for the tissue engineering applications due to their biocompatibility, biodegradability and acute biological roles. Major type of natural polymer or polysaccharides viz chitosan and hyaluronic acid were used to make hydrogels for tissue engineering [17–20]. Chitosan is a deacetylated form of chitin and is a favorable polymer for tissue engineering owing to its nontoxic, nonallergenic, mucoadhesive, biocompatible, biodegradable property and also as it accelerates cell proliferation [18]. Further, chitosan gradually depolymerize to release N-acetyl-β-D glucosamine, which initiates proliferation of fibroblast, also helps in collagen deposition and stimulates increased level of natural hyaluronic acid synthesis at wound sites [19]. HA is a glycosaminoglycan (GAG) and is a linear polysaccharide composed of repeating disaccharide unit of glucuronic acid and N-acetyl glucosamine. HA has structural similarity to the ECM of tissue and is said to support wound healing. Hyaluron plays a significant role in the inflammation, granulation, and remodeling phases that the body undergoes [20–22]. Hayami et al. had designed a biodegradable composite scaffold composed of electrospun poly(epsilon-caprolactone-co-D,L-lactide) (PCLDLLA) fibers embedded in a noncell-adherent photocrosslinked N-methacrylated glycol chitosan (MGC) hydrogel seeded with primary ligament fibroblasts [23]. In another study Thayer et al. compared PLGA and poly(ester–urethane–ester) meshes loaded with mesenchymal stem cells with an interpenetrating polyethylene glycol (PEG) hydrogel network [24]. In another study a scaffold composed of PLLA fibers in braid-twist structure was combined with a polyethylene glycol diacrylate (PEGDA) hydrogel to improve viscoelastic properties [25]. Hydrogel concentration and amount were varied to obtain good porosity. In all these studies synthetic polymeric hydrogel was employed, which may reduce the functionality of the system. In ligament regeneration, the development of scar tissue as a result of lack of cell proliferation leading to lack of or impaired deposition of collagen extracellular matrix, could be rectified to an extend by providing a platform for initial cell attachment and proliferation by using an ECM mimicking natural polymer based hydrogel coating. Therefore with the aim of developing a scaffold that could mimic the native ligament fibrous morphology, along with an ECM mimicking coating that can act as a cell or nutrient reservoir, we developed aligned PCL micro/ random nano (PCL aligned multiscale) and random micro/nano (PCL random multiscale) fibers through electrospinning following which a coating with chitosan–hyaluronic acid hydrogel was given. The hydrogel coating provided over the electrospun membrane would act as an initial cell holding platform. The macroporous nature of the developed coating would initiate proliferation of cell and by subsequent degradation of the coating the cell reach the fibrous layer, which then would help in the maturation of cells. Stacked hydrogel coated electrospun fibrous scaffold would be analyzed for ligament regeneration.

2. Materials and methods

2.1. Materials

Chitosan (molecular weight: 100–150 kDa, degree of deacetylation-85%) was purchased from Koyo chemical Ltd, Japan. Hyaluronic acid (HA) was purchased from Qingdao Haitao Biochemical, China. N,N-(3-dimethylaminopropyl)-N-ethyl carbodiimide (EDC) and Acetic acid were purchased from Sigma–Aldrich, India. PCL (molecular weight: 43,000–50,000) was purchased from Polysciences Inc, USA, Warrington, PA. Chloroform was purchased from Merck, USA. Methanol was purchased from Emplura®, USA. Syringes and needles for spinning were purchased from BD Sciences, India. Alamar blue reagent for cell viability was received from Invitrogen, India. Pico green reagent was purchased from Life technologies, India.

2.2. Preparation of PCL multiscale fibrous scaffold

Spinning of all the concentrations of PCL was done at room temperature [26,27]. Electrospinning setup as shown in (Fig. 1) was fabricated using a high voltage DC power supply [Model RR30P, 0–30 kV, Gamma High Voltage Inc., USA], syringe pump [KDS 220, (KD Scientific Inc., USA], motorized rotational target [Holmarc auto-mechatronics, India], grounded static target and 10 ml syringe with blunt end metal needle of 21 gauge.

PCL aligned multiscale fibrous scaffolds were developed as follows. Aligned PCL microfibers were prepared from 30 wt% of PCL solution in chloroform at a voltage of 7 kV by spinning onto a rotating mandrel having the speed of 5000 rpm. The flow rate of the solution was set to 1.5 mL/h and tip-target distance was set to 15 cm. Random PCL nanofibers were prepared from 13 wt% of PCL solution in a chloroform and methanol solvent (1:1), at a voltage of 17 kV by spinning onto a rotating mandrel having the speed of 5000 rpm. The flow rate of the solution was set to 1 mL/h and tip-target distance was 20 cm. The aligned PCL microfibers was continuously spun and nano fibers was spun intermittently. Likewise, PCL random multiscale fibers were spun separately by using the same parameters but the only difference is that the rotating mandrel speed was reduced to 1000 rpm.

2.3. Preparation of chitosan–HA(Ch-HA) Gel

Chitosan 2% (w/v) was dissolved in 1% acetic acid and this solution was neutralized to obtain hydrogel. 1% (w/v) HA was first dis-