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Controlled heparin conjugation on electrospun poly(ε-caprolactone)/gelatin fibers for morphology-dependent protein delivery and enhanced cellular affinity

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ABSTRACT

Electrospun fibrous scaffolds have now been shown to possess great potential for tissue engineering applications, owing to their unique mimicry of natural extracellular matrix structure. In this study, poly(ε-caprolactone) and gelatin were electrospun to fabricate tissue-engineered scaffolds with three different fiber morphologies (1.0 μm, 3.0 μm and co-electrospun containing both 1.0 and 3.0 μm diameter fibers). Subsequently, these scaffolds were conjugated with heparin to immobilize a bioactive molecule by electrostatic interactions. This study determined the quantity of heparin conjugation on the scaffolds and that the crosslinking time and the fiber morphologies govern the extent of heparin conjugation on the fibers. In order to evaluate the release capacity of the heparin-conjugated scaffolds, lysozyme was used as a model protein for conjugation. The heparin-conjugated scaffolds provided high loading efficiency and cumulative release of lysozyme with a relatively linear relationship. In addition, the release kinetics was significantly dependent on heparin conjugation and fiber morphology. This fundamental investigation into how fiber morphology and crosslinking protocols can affect the heparin binding ability of electrospun fibers is crucial for predicting the delivery of many different types of bioactive molecules from an electrospun scaffold for tissue engineering applications.

1. Introduction

Electrospinning technology provides the production of either randomly oriented or aligned fibers with dimensions on the scale of nanometers to micrometers from natural and/or synthetic polymers, and has gained much attention as a potential method for generating scaffolds for tissue engineering and drug delivery [1]. Electrospun scaffolds with a high surface-area-to-volume ratio are well suited for tissue engineering applications because their fibrous structure is very similar to the structure of native extracellular matrix (ECM) [2]. Electrospun scaffolds have been used in a number of different applications, including the engineering of blood vessels [3], tendons and ligaments [4], bone [5] and cartilage [6]. Currently, there has been increasing interest in developing a "smart biomaterial", which would be able to provide not only physical templates for cell accommodation, but also microenvironments that could control cell fate [7]. Such scaffolds would be expected to encourage proliferation and differentiation of cells seeded onto them in vitro as well as enhance the physiological responses needed for tissue regeneration in vivo [1].

Recent research has presented numerous techniques for preparing electrospun fibers and functionalizing them by incorporating diverse bioactive molecules such as growth factors and cytokines. These methods include physical adsorption of bioactive molecules, emulsion electrospinning, coaxial electrospinning and surface modification of the electrospun fibers, followed by chemical conjugation (formation of a covalent bond) of the desired molecule onto the surface of the fibers [1,8]. Physical adsorption is the simplest way to load bioactive molecules onto electrospun fibers and can be achieved by dipping a scaffold into an aqueous, protein-containing solution. However, this method is of limited use, because it often results in uncontrolled release patterns, including an intense initial burst release of the adsorbed moiety [9]. In emulsion electrospinning, bioactive molecules are blended with polymers dissolved in organic solvents prior to electrospinning. The resultant fibers obtained from this method may provide more sustained release kinetics than physical adsorption, because the bioactive molecules are randomly dispersed within emulsion electrospun fibers rather than exposed on the surface of the fibers. However, the bioactivity of these molecules can easily be lost during the preparation of the polymer–protein solution and/or during the electrospinning process, both of which can cause conformational changes to proteins that damage their function. Coaxial electrospinning is a modified version of traditional electrospinning techniques. In this method, two different solutions are fed into the system and are electrospun coaxially and simultaneously to generate a composite "core–shell" structure in which the desired bioactive molecules are entrapped in the core

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of the scaffold. The release kinetics for the bioactive factor is thus controlled by the shell or barrier layer of fibers. Although the bioactivity of the proteins released from this type of scaffold is still controversial [10], the core–shell structure of electrospun fibers appears to reduce the initial burst release efficiently and delivers molecules in a more sustained manner compared with emulsion electrospinning, because the shell layer plays a role in retarding the diffusive release of bioactive molecules from the core [10].

In contrast to the above-mentioned methods, surface modification of electrospun fibers can be achieved by plasma treatment and chemical hydrolysis of biodegradable aliphatic polyesters, which results in the generation of oxygen-based functional groups such as carboxyl, carbonyl and hydroxyl groups, and these can be used for further immobilization of bioactive molecules that could enhance cellular interactions such as cell adhesion and proliferation [11,12]. In addition, the direct chemical immobilization of bioactive molecules onto scaffolds has been favored because a covalent bond between proteins and the fiber surface is stable enough to last for an extended incubation period. However, this direct protein immobilization onto the surface of polymeric biomaterials is still a matter of debate in terms of the stability or activity of bioactive molecules.

Recently, heparin has been used as a linker molecule between growth factors and polymeric biomaterials. Heparin is a highly sulfated glycosaminoglycan, which possesses strong binding affinity (electrostatic interactions) to a variety of growth factors including fibroblast growth factor (FGF), transforming growth factor-β, vascular endothelial growth factor (VEGF), and platelet-derived growth factor-BB [13–16]. The specific interaction of heparin-binding growth factors with heparin is considered to be essential for storage, release and protection of the growth factor from heat, pH and enzymatic degradation [14]. For this reason, researchers have devised a number of ways to conjugate heparin on diverse types of scaffolds and protein delivery carriers. To date, heparin has been used to functionalize biomaterials for local delivery of growth factors via porous scaffolds [17], hydrogels [18], electrospun fibers [19] and nanoparticles [13].

In this study, lysozyme (14.3 kDa) was selected as a model protein to determine how the heparin-conjugated and unconjugated scaffolds bound and released lysozyme in vitro. Because lysozyme has strong binding affinity (isoelectric point 10.7) for heparin via electrostatic interactions [21,22], it was expected that lysozyme would interact with the heparin moieties on the heparin-conjugated electrospun PCL/gelatin fibers.

The objectives of the present study were (1) to control heparin conjugation on electrospun PCL/gelatin scaffolds with variations in crosslinking time and fiber morphology, (2) to evaluate their effects on the conjugated amount of heparin, (3) to obtain controlled release kinetics of lysozyme (a model protein) that had been securely coupled to the heparin moiety on conjugated PCL/gelatin fibers, and (4) to investigate the influence of heparin on cellular adhesion and proliferation on these electrospun scaffolds. Blend electrospun PCL/gelatin fibers were designed to exploit the reactive amine groups exposed on the surface of the fibers for heparin conjugation. Heparin conjugation was induced via a crosslinker, resulting in amide bonds between the PCL/gelatin fibers and the heparin molecules. Two governing parameters (crosslinking time and fiber morphology) were varied in order to determine how they would affect the number of available amine groups and, consequently, the amount of heparin that could be conjugated to each scaffold. In addition, the in vitro release kinetics of lysozyme and cellular adhesion to the scaffold were estimated to determine the effect of heparin conjugation on cumulative lysozyme release and cell adhesion and proliferation on the functionalized scaffolds, respectively.

2. Materials and methods

2.1. Electrospinning of PCL/gelatin fibers

The electrospinning system consisted of a syringe pump, a high-voltage generator (Spellman High Voltage, Hauppauge, NY, USA), and a collecting mandrel (41.1 mm in diameter). A positive charge of 12 kV was applied to the tip of the syringe needle, and the distance between the syringe needle and the grounded collector was 15 cm. The PCL/gelatin blend solution was ejected via a 20-gauge blunt needle at a constant flow rate of 2 ml h^{-1} for the 10% solution and 5 ml h^{-1} for the 15% solution. Electrospun fibers were collected on the cylindrical mandrel with a rotation rate of ~1000 rpm, and the fibers were kept under a vacuum desiccator until needed. The polymer blend of poly(ε-caprolactone) (PCL; intrinsic viscosity = 1.77 dL g^{-1}, Lactel Absorbable Polymers, Pelham, AL, USA) and gelatin (from porcine skin, Sigma Chemical Co., St. Louis, MO, USA) was prepared as follows. A 1:1 weight ratio of PCL and gelatin was dissolved in 1,1,1,3,3,3-hexafluoro-2-propanol to make both a 10% (w/v) and a 15% (w/v) solution. Each of the solutions (10% and 15%) was first electrospun individually to fabricate scaffolds with different fiber morphologies (small and large fiber diameters). In order to obtain scaffolds with a combination of small and large diameter fibers, both solutions (10% and 15%) were co-electrospun using two separate syringe pumps. These two pumps were placed to face each other, while a collecting mandrel was positioned between them. All chemical reagents were obtained from Sigma Chemical Co. unless stated otherwise.

Electrospun PCL/gelatin scaffolds were cut into dimensions 1×1 cm^2 and crosslinked using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC; Thermo Scientific, Waltham, MA, USA) and *N*-hydroxysuccinimide (NHS). Crosslinking was performed for different lengths of time (15, 30, 60, 120 and 240 min) using 25 mM EDC/10 mM NHS dissolved in ethanol. The crosslinking process was terminated by washing the scaffolds three times with 0.1 M Na_2HPO_4 and then three times with distilled water.

For the analysis of fiber morphology, the electrospun PCL/gelatin fibers were sputter-coated with gold (Hummer™ 6.2, Anatech Ltd, Denver, NC, USA) and observed using scanning electron microscopy (SEM; S-2260 N, Hitachi Co. Ltd, Japan). The diameter distribution of the electrospun fibers at each electrospinning condition was determined by selecting 20 fibers at random from three images to measure, and the result was expressed as the mean ± standard deviation ($n = 20$) with the aid of Image J software (NIH, Bethesda, MD, USA).

2.2. Determination of free amines on PCL/gelatin fibers and fiber crosslinking

The amount of residual amine groups remaining on the electrospun PCL/gelatin scaffolds was determined using 2,4,6-trinitrobenzene sulfonic acid (TNBSA; Thermo Scientific) according to the manufacturer's instructions. Briefly, 0.25 ml of 0.01% (w/v) TNBSA, prepared using 0.1 M sodium bicarbonate (pH 8.5), was added to electrospun PCL/gelatin scaffolds, and this mixture was incubated at 37 °C for 2 h with gentle agitation. Following incubation, a mixture of 10% sodium dodecyl sulfate (SDS, 0.25 ml) and 1 N HCl (0.125 ml) was added to each sample, and the absorbance at 335 nm was measured using a spectrophotometer (SpectraMax M5; Molecular Devices, Sunnyvale, CA, USA). The relationship between the absorbance and the concentration of free amine groups was calculated using a calibration curve obtained with a series of diluted gelatin solutions. The final concentration of amine groups was normalized to the dry weight of each sample ($n = 3$). The