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Gelatin Nanofiber-Reinforced Alginate Gel Scaffolds for Corneal Tissue Engineering

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Abstract— **A severe shortage of donor cornea is now an international crisis in public health. Substitutes for donor tissue need to be developed to meet the increasing demand for corneal transplantation. Current attempts in designing scaffolds for corneal tissue regeneration involve utilization of expensive materials. Yet, these corneal scaffolds still lack the highly-organized fibrous structure that functions as a load-bearing component in the native tissue. This work shows that transparent nanofiber-reinforced hydrogels could be developed from cheap, non-immunogenic and readily available natural polymers to mimic the cornea's microstructure. Electrospinning was employed to produce gelatin nanofibers, which were then infiltrated with alginate hydrogels. Introducing electrospun nanofibers into hydrogels improved their mechanical properties by nearly one order of magnitude, yielding mechanically robust composites. Such nanofiber-reinforced hydrogels could serve as alternatives to donor tissue for corneal transplantation.**

I. Introduction

A severe shortage of good quality donor cornea is now an international crisis in public health. Only about 60,000 corneal transplants are performed globally each year, while 10 million people are suffering from corneal blindness [1, 2]. Furthermore, the demand for donor tissue continues to increase due to the growth of the elderly population. Meanwhile, fewer donors are eligible to donate their corneal tissue due to a popularity of the LASIK surgery [3]. Such laser treatment alters the shape and the structure of the corneas, disqualifying them for transplantation.

In the last two decades, the development of corneal substitutes has shifted towards a tissue engineering approach for long-term repair rather than replacing the damaged tissue with biocompatible artificial corneas [4]. The tissue engineering approach involves an implantation of a corneal cell-scaffold construct, also known as a tissue engineered cornea, to temporarily function as a cornea [5]. The scaffold acts as a template to promote tissue growth in three dimensions and slowly degrades over time. Eventually, it is completely replaced with new regenerated corneal tissue that integrates naturally into the host without any of the post-operative complications seen with artificial corneas.

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The success of a tissue engineered cornea is significantly dependent on the scaffold's performance. The scaffold should have similar microstructure and properties to those of the corneal extracellular matrix (ECM), which is an aqueous composite of highly-organized collagen fibrils and proteoglycans [6]. Due to its high water content of up to 80% [7], corneal ECM can be considered as a natural fiber-reinforced hydrogel. The organization of aligned uniform corneal collagen fibrils provides mechanical strength and transparency to the native cornea [8].

Hydrogels have become a material of choice for corneal scaffold fabrication because they contain similarly high water content to the corneal ECM. A variety of materials including collagen [9-11], fibrin [5] and silk [12] have been used to produce hydrogel scaffolds. However, these scaffolds still lack the highly-organized fibrous structure that is essential for scaffolds' mechanical performance and provides a spatial guidance for corneal cells to synthesize organized ECM [13]. Previous work demonstrates that fiber-reinforced hydrogels could be developed by infiltrating electrospun polycaprolactone (PCL) fibers with alginate hydrogels [14]. Although mechanical properties of the hydrogels were significantly improved with PCL fiber reinforcement, the resulting hydrogels are not suitable for corneal tissue engineering application due to their opacity.

In this work, gelatin and alginate, both of which are inexpensive and non-immunogenic natural polymers, were used to create a scaffold that mimics the corneal ECM's microstructure. Electrospun gelatin nanofibers were combined with alginate hydrogels to form nanofiber-reinforced hydrogels. Mechanical and optical properties of the resulting hydrogels were also investigated.

II. Materials and Methods

A. Production of randomly-oriented and aligned electrospun gelatin fibers

All chemicals used in this study were purchased from Sigma Aldrich, Dorset, UK. A 10 wt% gelatin solution was prepared by dissolving 10 g of gelatin from porcine skin (250 g bloom strength) in a co-solvent composed of 42 g of glacial acetic acid, 21 g of ethyl acetate and 10 g of distilled water [15]. The solution was stirred for 2 hours at room temperature and further incubated for 30 minutes at 50 °C. The solution was left to cool prior to electrospinning.

To obtain randomly-oriented electrospun gelatin fibers, a grounded copper plate wrapped with aluminum foil was used as a collector. While a grounded 5 cm-diameter mandrel rotating at 3100 RPM was used to collect aligned gelatin fibers. The gelatin solution was loaded into a 20 mL plastic syringe and pumped through a blunt 18 G needle at 0.003 mL min^{-1}. The needle was horizontally 10 cm away from the collector. An applied voltage of 12 kV was applied between the needle and the collector. The electrospun mats were collected after 10 hours and were dried in a desiccator for 24 hours prior to further investigation.

B. Production of electrospun gelatin fiber-alginate hydrogels

A 3 wt% of sodium alginate solution was prepared by dissolving 3 g of alginic acid sodium salt in 97 g of distilled water for 15 minutes at 50 °C. A 200 mM of calcium chloride ($CaCl_2$) aqueous solution was also prepared. Gelatin mats were submerged into a beaker containing the alginate solution for 20 minutes to allow infiltration of alginate solution. The products were then rinsed 2-3 times in distilled water to remove alginate on the surfaces. Next, they were placed into a beaker containing $CaCl_2$ solution for 12 hours. Excess $CaCl_2$ was removed by rinsing the final products 2-3 times in distilled water. The resulting hydrogels were stored in distilled water until use. All processes were implemented at room temperature. In addition, homogeneous alginate hydrogels were also produced by slowly pouring alginate solution onto filter paper soaked in $CaCl_2$ solution. After 12 hours, the alginate hydrogels were rinsed 2-3 times and were stored in distilled water until use.

C. Mechanical characterization

Mechanical properties of electrospun gelatin mats and hydrogels were characterized by uniaxial tensile testing. All testing samples had a gage size of 20 x 5 mm. The samples were gripped with custom-built stainless steel grips. The tensile tests were performed with a universal testing machine (model 5544, Instron, Canton, MA) with 5N load cell. The samples were stretched at a rate of 0.05 mm s^{-1} until failure. A minimum of $n = 6$ were used for each material.

D. Scanning Electron Microscopy & Confocal Microscopy

The morphology of fibers in the electrospun mats was characterized by scanning electron microscopy (SEM, Carl Zeiss, Cambridge, UK) at an accelerating voltage of 15 kV. Prior to the process, the samples were coated with a thin layer of gold to produce a conductive surface. The diameter of the fibers were analyzed from SEM images using ImageJ. To observe morphology of gelatin fibers in the hydrogel, the hydrogels were dried in a desiccator for 48 hours and were broken into fragments before coating with a thin layer of gold. Confocal microscopy was also used to observe gelatin fibers in the composite hydrogels. Thirty milligrams of rhodamine 6G was added into the gelatin solution before electrospinning to obtain fluorescent electrospun gelatin fibers. The fluorescent electrospun gelatin mats were infiltrated with alginate gel as above. Images of gelatin fibers in the hydrogels were obtained with an FV1000 Olympus upright confocal microscope (Olympus, Japan).

E. Light transmission measurement

Percentage of light transmission through hydrogels and porcine cornea were measured with a UV-Vis spectrophotometer (Ultrospec 2100, Amersham Bioscience, UK) across the visible wavelengths (400-700 nm).

III. RESULTS

A. Microstructure of gelatin fiber-alginate hydrogels

The electrospun gelatin mats shrank and formed into transparent and soft films as soon as they contacted alginate solution (Figure 1 a). These films became rigid hydrogels after crosslinking in $CaCl_2$ solution (Figure 1 b). The resulting hydrogels were transparent regardless whether randomly-oriented or aligned gelatin mats were used. Thicknesses of the hydrogels varied from 0.35 - 0.5 mm, depending on the thicknesses of gelatin mats.

SEM image shows that the gelatin fibers were continuous and uniform (Figure 1 c). Electrospun gelatin fibers obtained from the static collector were randomly-oriented with an

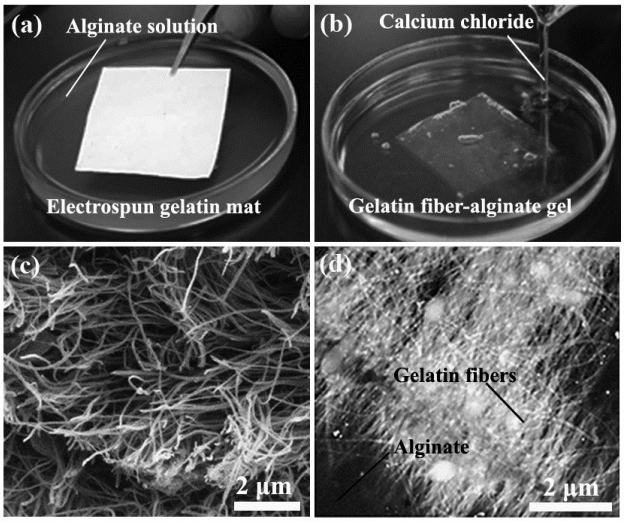

Figure 1. (a), (b) Production of a gelatin fiber-alginate hydrogel. (c) SEM of the cross-section of an electrospun gelatin mat, (d) Confocal image of gelatin fibers in the alginate hydrogel produced from aligned gelatin mat (the image was obtained at 10 μm depth from the surface)

average diameter of 130 ± 19 nm. While, the fibers obtained from the mandrel were aligned in the rotating direction with an average diameter of 67 ± 7 nm. Confocal imaging of the hydrogels produced from aligned gelatin mats showed that the gelatin fibers remained aligned in the hydrogels (Figure 1 d). These results confirmed that electrospun gelatin fibers

were successfully introduced into the alginate hydrogels, forming nanofibrous structures.

B. *Mechanical properties of gelatin fiber-alginate hydrogels*

The tensile elastic modulus and elongation at break of electrospun gelatin mats and gelatin fiber-alginate hydrogels are shown in Table I. Fiber alignment significantly improved the modulus of electrospun gelatin mats under parallel loading. In contrast, fiber alignment resulted in less stiff electrospun mats under perpendicular loading (data is not shown). Brittle fractures occurred at small percentages of elongation for both types of electrospun gelatin mats.

TABLE I. A COMPARISON OF MECHANICAL PROPERTIES OF DIFFERENT FIBER-REINFORCED HYDROGELS

Materials	Mechanical properties	
	Tensile elastic modulus (MPa)	***Elongation at break (%)***
Electrospun gelatin mats (random fibers)	27.69 ± 2.52	6 ± 1
Electrospun gelatin mats (aligned fibers)	133.87 ± 20.80*	8 ± 2*
Electrospun PCL mats (random fibers) [14]	1.15 ± 0.22	230 ± 130
Alginate hydrogels	0.078 ± 0.019	32 ± 7
Gelatin fiber-alginate gels (random fibers)	0.45 ± 0.10	64 ± 14
Gelatin fiber-alginate gels (aligned fibers)	0.50 ± 0.11*	63 ± 9*
PCL fiber-alginate gels (random fibers) [14]	0.56 ± 0.30	130 ± 65

* Loading in the direction of gelatin fibers

Nearly one order of magnitude improvements in tensile elastic modulus of alginate hydrogels were obtained by introducing either randomly-oriented or aligned electrospun gelatin fibers into the hydrogels. Reinforcement with aligned gelatin fibers resulted in slightly stiffer and stronger hydrogels than reinforcement with randomly-oriented fibers, providing that the load was parallel to the fibers. In addition, fiber-reinforced hydrogels also fractured at larger strains than homogenous alginate hydrogels.

C. *Light transmission of gelatin fiber-alginate hydrogels*

Figure 2 a - c compares transparency of homogeneous alginate hydrogels and gelatin fiber-alginate hydrogels to porcine cornea. It is obvious that gelatin fiber-alginate hydrogels are transparent as the letter A can be clearly seen through. In contrast, the previously developed PCL fiber-alginate hydrogels are not transparent (Figure 2 d). Quantitative measurements show that slightly greater proportion of light can pass through the developed gelatin fiber-alginate hydrogels than the porcine cornea (Figure 2 e).

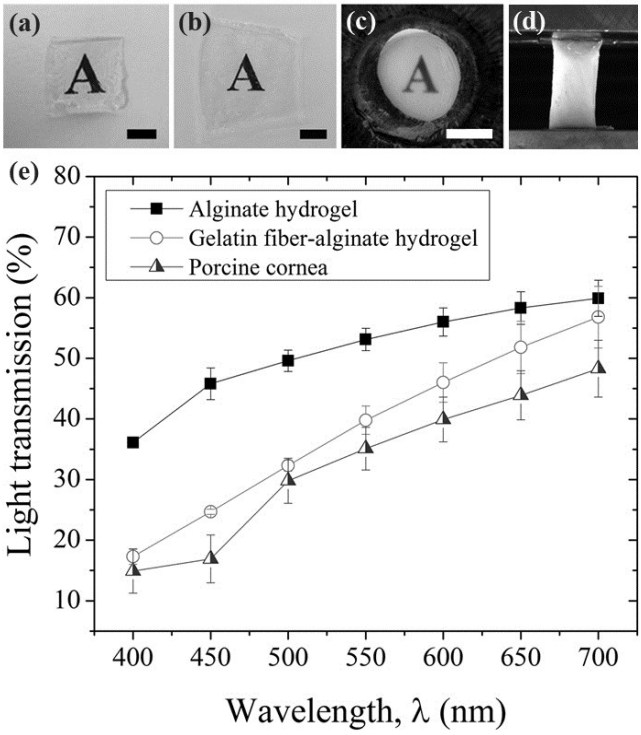

Figure 2. Transparency of different materials: (a) alginate hydrogel without gelatin fibers, (b) alginate hydrogel with gelatin fibers, (c) porcine cornea. Paper with a letter 'A' was placed underneath all materials to compare their transparency. Scale bars are equal to 1 cm. (d) PCL fiber-alginate hydrogel [14] (e) Percentage of light transmission

IV. DISCUSSION

In recent years, there have been a few attempts to produce fiber-reinforced hydrogels for tissue engineering applications [14, 16]. However, these fiber-reinforced hydrogels are not transparent and thus they are not suitable for corneal tissue engineering applications. In contrast, in this work, infiltrating the electrospun gelatin fibers with alginate solution resulted in transparent fiber-reinforced hydrogels. The gelatin fibers could be introduced into the hydrogels due to their hydrophilicity. When electrospun gelatin mats contacted the alginate solution, gelatin fibers attracted water molecules to accumulate around them. Since the water molecules also bound to alginate chains, gelatin fibers were therefore surrounded with a matrix of alginate chains. In the presence of Ca^{2+} ions, these alginate chains became crosslinked and formed into gel, trapping the gelatin fibers inside its structure.

Even though electrospun gelatin mats are much stiffer than electrospun PCL mats, reinforcing the alginate hydrogels with PCL fibers results in slightly stiffer composite hydrogels than ones reinforced with gelatin fibers. Due to their hydrophobicity, the electrospun PCL mats can only attract small amount of alginate solution. The resulting composites therefore contain a small volume fraction of compliant alginate hydrogel. In contrast, electrospun gelatin mats are very hydrophilic and therefore absorb significant

volume of alginate solution. The resulting gelatin fiber-alginate hydrogels are thus contain a large volume fraction of alginate hydrogel, resulting in less stiff composite hydrogels than PCL fiber-alginate hydrogels.

Not only do the developed gelatin fibre-reinforced alginate hydrogels have an elastic modulus (E = 0.45 - 0.5 MPa) very close to that of the native cornea (E = 0.579 - 4.9 MPa) [6, 17-18], they are also optically transparent. One possible explanation for their transparency is that gelatin fibers are uniform in diameter and are relatively small (67 ± 7 nm) compared to the wavelength of visible light (400 - 700 nm), minimizing the amount of light scattering from the materials [19]. In contrast, PCL fibers are relatively large and less uniform (786 ± 528 nm). The significant amount of light scattering from the composite hydrogels results in opaque materials.

Fiber orientation also affects the performances of the resulting hydrogels. Hydrogels that consist of aligned fibers are slightly stiffer and stronger than ones reinforced with randomly-oriented fibers providing that the applied load is parallel to the fibers. However, the hydrogels containing aligned gelatin fibers are only stiff along the fiber axis but very compliant in the transverse direction. Hydrogels that contain biaxially aligned fibers should be developed to yield biaxially stiff scaffolds similar to the native cornea.

V. Conclusion

This work shows that mechanically robust transparent hydrogels can be simply fabricated from two inexpensive natural polymers: gelatin and alginate. Electrospun gelatin nanofibers were successfully introduced into hydrogels for the first time, enhancing mechanical properties of the hydrogels by approximately one order of magnitude. These novel nanofibrous composites have a great promise as scaffolds for corneal transplantation when the donor tissue is not available.

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