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# Modulation of the gene expression of annulus fibrosus-derived stem cells using poly(ether carbonate urethane)urea scaffolds of tunable elasticity

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ABSTRACT

Annulus fibrosus (AF) injuries commonly lead to substantial deterioration of the intervertebral disc (IVD). While tissue engineering has recently evolved into a promising approach for AF regeneration, it remains challenging due to the cellular, biochemical, and mechanical heterogeneity of AF tissue. In this study, we explored the use of AF-derived stem cells (AFSCs) to achieve diversified differentiation of cells for AF tissue engineering. Since the differentiation of stem cells relies significantly on the elasticity of the substrate, we synthesized a series of biodegradable poly(ether carbonate urethane)urea (PECUU) materials whose elasticity approximated that of native AF tissue. When AFSCs were cultured on electrospun PECUU fibrous scaffolds, the gene expression of collagen-I in the cells increased with the elasticity of scaffold material, whereas the expression of collagen-II and aggrecan genes showed an opposite trend. At the protein level, the content of collagen-I gradually increased with substrate elasticity, while collagen-II and GAG contents decreased. In addition, the cell traction forces (CTFs) of AFSCs gradually decreased with scaffold elasticity. Such substrate elasticity-dependent changes of AFSCs were similar to the gradual transition in the genetic, biochemical, and biomechanical characteristics of cells from inner to outer regions of native AF tissue. Together, findings from this study indicate that AFSCs, depending on the substrate elasticity, have strong tendencies to differentiate into various types of AF-like cells, thereby providing a solid foundation for the tissue engineering applications of AFSCs.

**Statement of significance**

Repairing the annulus fibrosus (AF) of intervertebral disc (IVD) is critical for the treatment of disc degeneration disease, but remains challenging due to the significant heterogeneity of AF tissue. Previously, we have identified rabbit AF-derived stem cells (AFSCs), which are AF tissue-specific and hold promise for AF regeneration. In this study, we synthesized a series of poly(ether carbonate urethane)ureas of various elasticity (or stiffness) and explored the potential of induced differentiation of AFSCs using electrospun PECUU scaffolds. This work has, for the first time, found that AFSCs are able to present different gene expression patterns simply as a result of the elasticity of scaffold material. Therefore, our findings will help supplement current knowledge of AF tissue regeneration and may benefit a diversified readership from scientific, engineering, and clinical settings whose work involves the biology and tissue engineering of IVD.



## 1. Introduction

Intervertebral disc (IVD) degeneration is the major cause of lower back pain which affects about 80% of the population and significantly contributes to healthcare expenditures [1,2]. Surgical interventions including spinal fusion and total disc arthroplasty (TDA), while effective in relieving the symptomatic pains, do not restore the spinal biomechanics and may even cause adjacent segment degeneration [3]. Instead, the emergence of tissue engineering offers a promising strategy for the treatment of disc degeneration by using engineered disc replacements which may biologically resemble native discs [4]. The annulus fibrosus (AF) is a component that confines nucleus pulposus (NP) and helps maintain physiological intradiscal pressure upon loading. The structural integrity of AF plays a critical role in the biomechanical

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properties of IVD [5–7]. Fabrication of tissue-engineered AF, therefore, appears indispensable toward successful construction of engineered IVDs.

Recently, a number of attempts have been made during the last decade to construct engineered AFs using synthetic or natural polymers as scaffold materials [8–12]. However, major challenge remains toward successful AF tissue engineering, mainly because of the tremendous complexity of AF tissue at the cellular, biochemical, microstructural, and biomechanical levels [13–15]. Anatomically, AF has an angle-ply lamellar structure of oriented fibers, which ensures stability of IVD under physiologically relevant loading. Recent effort in scaffold fabrication for AF tissue engineering has been focused on simulating such anatomical features to improve the biomechanical properties of engineered AF [11,16,17]. For example, silk-based oriented lamellar fibrous scaffolds which were able to direct the alignment of human chondrocytes and the deposited extracellular matrix (ECM) had been fabricated toward AF tissue engineering [16]. Biphasic scaffolds such as demineralized bone matrix gelatin (BMG)/poly(polycaprolactone triol malate) (PPCLM) were also used to simulate AF tissue structurally and mechanically resulting in fifty times increase in tensile strength compared to PPCLM alone, which is close to that of native rabbit AF [10]. However, such efforts rarely lead to satisfying AF replacements, partially due to the heterogeneity and gradual transition characteristics of AF tissue. A highly anisotropic tissue, AF possesses gradual transition of mechanical property along the radial direction. The elasticity of AF tissue significantly varies depending on the location. The outer AF, rich in collagen-I, has higher elastic modulus and is stiffer than inner AF. On the other hand, the inner AF, with higher level of aggrecan and collagen-II, has lower elastic modulus than outer AF. As such, the approaches to engineer AF tissue using single-phase material or single cell type to construct scaffolds will likely fail. Effective AF tissue engineering, therefore, requires recapitulating the intrinsic heterogeneity of native AF tissue from the cellular, biochemical, and biomechanical aspects.

The mechanical property of the substrate plays an important role in cellular behaviors. To date, numerous studies have shown that the elasticity of the matrix effectively directs the lineage specification of stem cells in either 2D or 3D environments [18–24]. For instance, human BMSCs were effectively differentiated into bone, muscle or neuronal lineages when they were cultured on stiff, medium or soft substrates, the stiffness of which was close to that of corresponding native tissues [18]. Recently, it has been shown that AF cells showed distinct expression of Col$\alpha$1, Col2$\alpha$1, and aggrecan mRNAs when cultured on hydrogels of different elastic modulus [25]. Hence, the elasticity of the scaffold is an important factor in AF tissue engineering. Considering the region-specific feature in the stiffness of AF tissue, an ideal scaffold for AF tissue engineering, therefore, should not only mimic the anatomical criss-cross features of native AF tissue, but should also have similar region-specific mechanical characteristics to induce differentiation of stem cells into appropriate cell types and secrete ECM similar to native AF tissue.

To date, AF cells or bone marrow mesenchymal stem cells (BMSCs) have been used in the majority of AF tissue engineering studies [8–11,26–28]. Recently, AF-derived stem cells (AFSCs) have been identified in humans and rabbits [29,30]. Being AF tissue specific, AFSCs preferentially differentiate into various types of resident cells in native AF tissue and therefore are an ideal cell source for AF tissue engineering. In this study, we aimed to test whether AFSCs respond to substrate elasticity and may be differentiated into AF tissue using substrates whose elasticity is comparable to AF tissue. While being overwhelmingly used in current studies [3,11], popular bulk-degradation biopolymers such as PLGA and PCL are not ideal scaffold materials for AF tissue engineering because their mechanical property markedly deteriorates upon degradation *in vitro* or *in vivo* [31–33]. Instead, poly(trimethylene carbonate) (PTMC)-based copolymers, being capable of surface degradation, may retain their mechanical property during degradation [31,34]. Such polymers also degrade much more slowly, which may favor matrix production and remodeling in engineered AF constructs.

Importantly, the distinctions in matrix composition of various AF regions are resulted from different types of cells, which produce specific types of ECM corresponding to the zone where they reside [15]. Therefore, it is of interest to check whether AFSCs can be differentiated into various cells corresponding to the cell types in native AF tissue upon culturing on substrates of different elasticity and consequently, produce matching ECM. To this end, we synthesized a series of biodegradable poly(ether carbonate urethane) ureas (PECUUs) of different elastic moduli which approximates the elastic modulus of native AF tissue [15,31]. We then prepared fibrous PECUU scaffolds which mimicked the fibrous feature of AF tissue using electrospinning technique for AFSC culture. The effect of substrate elasticity on the growth, gene expression, and biochemical and biomechanical characteristics of AFSCs was studied.

## 2. Materials and methods

### 2.1. Materials

Pluronic L-31 (PEO-PPO-PEO, mol. wt. ~1100, Aldrich) was dried by azeotropic distillation from toluene. Trimethylcarbonate (TMC, Jinan Daigang) was vacuum dried with $P_2O_5$ before use. 1,6-Diisocynatohexane (HDI, 99+%, ACROS), 1,4-diamine-butane (Putrescine, 99%, ACROS), and stannous octoate (96%, Alfa Aesar) were used as received. Toluene and N,N-dimethylformamide (DMF), purchased from Chinasun Specialty Products, were dried over $CaH_2$ and vacuum distilled before use. Hexafluoroisopropanol (HFIP, >99%, Hete Chemical Technology) was used as received.

### 2.2. Synthesis of poly(ether carbonate urethane)-ureas (PECUUs)

The PECUUs were synthesized by a three-step polymerization in "one pot" according to a previous study (see Scheme 1 in Ref. [35]) [31]. In brief, PEO-PPO-PEO (5.5 g, 5 mmol), stannous octoate (506.2 mg, 1.25 mmol), TMC (by calculation) and toluene (70 mL) were added to a 500 mL three-necked reaction flask under nitrogen atmosphere. The flask was sealed and placed into an oil bath at 110 °C. After 24 h, 1 ml of the resulting polymer solution was collected for $^1$H-NMR characterization. Copolymers with various PTMC length were synthesized by adjusting the feed ratios of TMC to PEO-PEO-PPO. Following the first step, the resulting polymer solution was diluted with toluene to 15 wt%, and then 15 wt% HDI (10 mmol) solution in toluene was added. The reaction was carried out at 75 °C for 4 h under continuous stirring. After the solution was cooled to room temperature, 1 wt% putrescine solution in DMF (10 mmol) was added dropwise and the reaction was continued overnight. The final product was purified by precipitation in ethanol/water (v/v, 3/7) twice, filtered, and vacuum dried.

### 2.3. Polymer characterization

$^1$H-NMR spectra were recorded on a nuclear magnetic resonance system (INOVA 400 MHz, Varian) using deuterated chloroform ($CDCl_3$) as solvent. The chemical shifts were calibrated against solvent signal of $CDCl_3$. ATR-FTIR spectra were recorded on a FTIR spectrometer (FTIR 6700, Nicolet). Differential scanning

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