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Label-free mapping of microstructural organisation in self-aligning cellular collagen hydrogels using image correlation spectroscopy

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ABSTRACT

Hydrogels have emerged as promising biomaterials for regenerative medicine. Despite major advances, tissue engineers have faced challenges in studying the complex dynamics of cell-mediated hydrogel remodelling. Second harmonic generation (SHG) microscopy has been a pivotal tool for non-invasive visualization of collagen type I hydrogels. By taking into account the typical polarization SHG effect, we recently proposed an alternative image correlation spectroscopy (ICS) model to quantify characteristics of randomly oriented collagen fibrils. However, fibril alignment is an important feature in many tissues that needs to be monitored for effective assembly of anisotropic tissue constructs. Here we extended our previous approach to include the orientation distribution of fibrils in cellular hydrogels and show the power of this model in two biologically relevant applications. Using a collagen hydrogel contraction assay, we were able to capture cell-induced hydrogel modifications at the microscopic scale and link these to changes in overall gel dimensions over time. After 24 h, the collagen density was about 3 times higher than the initial density, which was of the same order as the decrease in hydrogel area. We also showed that the orientation parameters recovered from our automated ICS model match values obtained from manual measurements. Furthermore, regions axial to cellular processes aligned at least 1.5 times faster compared with adjacent zones. Being able to capture minor temporal and spatial changes in hydrogel density and collagen fibril orientation, we demonstrated the sensitivity of this extended ICS model to deconstruct a complex environment and support its potential for tissue engineering research.

Statement of significance

It is generally accepted that looking beyond bulk hydrogel composition is key in understanding the mechanisms that influence the mechanical and biological properties of artificial tissues. In this manuscript, we performed label-free non-invasive imaging and extended a robust automated analysis method to characterize the microstructural organisation of cellular hydrogel systems. We underpin the sensitivity of this technique by capturing minor changes in collagen density and fibril orientation in biologically relevant systems over time. Therefore, we believe that this method is applicable in fundamental cell-matrix research and has high-throughput potential in screening arrays of hydrogel scaffolds, making it an interesting tool for future tissue engineering research.

1. Introduction

Hydrogels are three-dimensional (3D) networks formed by natural or synthetic cross-linked polymers. Their high water-absorbing capacity and modifiable biomechanical and biochemical properties make them highly suitable carriers for different cell types [1,2]. Therefore, many efforts have been made to use hydrogels in tissue engineering applications. By combining multidisciplinary strategies based on material, life and engineering sciences, research in this field aims to restore, preserve or enhance tissue structure and function following injury or disease [3]. Because their stiffness can range from 0.1–500 kPa [4], hydrogels

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are especially appealing for regenerating soft tissues such as skin, tendons, muscles and nerves [5].

In the past decade, natural hydrogels have gained significant interest due to their native-like extracellular matrix (ECM) properties and inherent biocompatibility [1,2]. Of all natural polymers, collagen type I has received great attention in tissue engineering as it is the most abundant ECM protein in the human body. Although many tissues such as corneas, vessel walls, tendons and nerves contain collagen type I, their mechanical strength and function are related to specific alignment patterns of these fibrils [6]. In the body, collagen type I is capable of self-aggregation and crosslinking to form a tissue-specific anisotropic ECM. *In vitro* assembly, however, consistently creates a randomly oriented fibrillar hydrogel network [7].

Different methods have been probed to generate aligned collagen hydrogels including drainage [8], microfluidic channels [9] and the use of electrical gradients [10] or magnetic fields [11]. Whereas these scaffolds are organised by being subjected to external mechanical forces, a more natural approach involves uniaxial constrained cell-seeded collagen hydrogels where cell-generated tension causes self-alignment of both cells and collagen fibrils [12,13]. The use of these highly organised cellular collagen constructs for nerve repair is well studied [14–16], but little is known about the progression of these cell-induced changes in hydrogel architecture. Live imaging of the formation of aligned tissue-engineered cellular constructs will advance our understanding of the process and provide valuable new information to inform the construction of better 3D hydrogel microenvironments that mimic native ECM. Although many optical microscopy techniques can visualize individual cells in their ECM, most of them require exogenous dyes which could have phototoxic effects and perturb native cellular behavior [17]. In order to truly understand ECM remodeling by embedded cells, it is essential to continuously monitor cell-matrix interactions within the 3D construct in a label-free manner.

Collagen type I fibrils are capable of generating two types of intrinsic optical signals: autofluorescence and second harmonic generation (SHG) [18,19]. Both processes can be induced by femto-second pulsed laser light but the resulting signals differ in wavelength and intensity. The frequency-doubled SHG signal has a much higher signal-to-noise ratio compared to the Stokes-shifted autofluorescence signals [20]. Yielding high contrast and submicron resolution images on a non-invasive basis, SHG microscopy holds great promise in the field of biomedical imaging [21] and tissue engineering [22,23].

When studying collagen fibril organisation, extraction of quantitative information from these SHG images is not trivial. Often, time consuming manual data extraction is used which might suffer from subjective interpretation. To overcome this possible bias, image correlation spectroscopy (ICS) has been used to predict bulk mechanical properties of collagen hydrogels in an automated and objective manner. By calculating the autocorrelation function (ACF) of a fibrous SHG image, quantitative parameters such as pore size, collagen density, fibril length, thickness and orientation can be extracted [24–27]. Recently, we proposed an alternative ICS model for random fibril orientation which included SHG-specific polarization effects to obtain a more accurate ACF amplitude recovery as shown by simulations and experimental data on a collagen type I hydrogel dilution series [28].

In the current work, we expand our previous approach and describe the extension towards the characterization of cellular self-aligning collagen hydrogels designed for tissue engineering. The power of the extension is demonstrated in two relevant applications. First, we perform a collagen hydrogel contraction assay to evaluate cell-mediated hydrogel changes over time at the macroscopic and microscopic level. We explore the sensitivity of the extended ICS model by using two different but related cell types that can exhibit minor differences in contractile capacity, namely human dental pulp stem cells (hDPSCs) and their glial differentiated derivatives (d-hDPSCs) [15]. Secondly, we examine whether this model can be used to estimate fibril orientation in cellular hydrogels. To test the accuracy of the estimated orientation parameters, we implement a validation experiment in which manually obtained and automated (ICS) outcomes are compared. Finally, we apply the ICS model to quantify and map collagen fibril organisation in a self-aligning d-hDPSCs containing hydrogel for neural tissue engineering.

2. Materials and methods

2.1. Materials and products

All products were purchased from Sigma–Aldrich (Bornem, Belgium) unless stated otherwise.

2.2. Cell culture

Human third molars were collected from donors (15–20 years of age) undergoing extraction for orthodontic or therapeutic reasons at Ziekenhuis Oost-Limburg, Genk, Belgium. The medical ethical committee of Ziekenhuis Oost-Limburg approved this study on February 3rd 2014 and written informed consent from all donors, or from legal guardians in case of under-aged donors, was obtained. hDPSCs were isolated, cultured and differentiated towards Schwann-like cells (d-hDPSCs) as described by Martens et al. [15].

2.3. Aligned cellular hydrogels

A tethered cell-seeded collagen gel was prepared according to methods described previously [15,29–31] with some modifications. Briefly, gels were prepared on ice by mixing 1 volume of 10x MEM with 8 volumes of type I rat tail collagen (2 mg/ml in 0.6% acetic acid; First Link, Wolverhampton, UK). The pH of the mixture was neutralized dropwise using 1 M sodium hydroxide, after which 1 volume of d-hDPSCs suspension was added to give a final seeding density of 10^6 cells/ml. The resulting mixture was cast within an ice-cold rectangular stainless steel mould (37 mm × 27 mm × 4.5 mm) and tethered at each end through the use of a porous mesh. The gels were allowed to set for 15 min at 37 °C, transferred to a #1.5 glass bottom petri dish and subsequently immersed with standard culture medium. From this moment on (t = 0 h after casting), tethered gels with initial dimensions of 16 mm × 6.5 mm × 4.5 mm were kept at 37 °C in a humidified atmosphere containing 5% CO_2 in a cell culture incubator or in the microscope stage incubator for imaging at 0, 4, 8 and 32 h after casting. The orientation of the 3D construct is referred to as x for the long axis of gel, y for the shorter axis and z for the height. The mould was always positioned such that the x-axis of the hydrogel was parallel to the polarization of the incident light, which is always along the x-axis of the image in this paper. Since alignment occurs in the direction of tension generated by the cellular gel contraction being resisted by the tethering bars, elongated cells and aligned collagen fibrils along the x-axis are expected in the acquired images. Per hydrogel, 6 cells were randomly selected in the central part of the tethered system and around each cell, 3 defined regions near the cellular processes (axial, diagonal and parallel with respect to cellular processes) (Fig. 3a) were imaged. Differentiated hDPSCs from 4 different donors were used to carry out 4 independent experiments (n = 4).