Biomaterials 140 (2017) 128–137

Contents lists available at ScienceDirect

Biomaterials

journal homepage: www.elsevier.com/locate/biomaterials

Online quantitative monitoring of live cell engineered cartilage growth using diffuse fiber-optic Raman spectroscopy

Mads S. Bergholt [a, b, c, 1], Michael B. Albro [a, b, c, 1], Molly M. Stevens [a, b, c, *]

[a] Department of Materials, Imperial College London, London SW7 2AZ, United Kingdom
[b] Department of Bioengineering, Imperial College London, London SW7 2AZ, United Kingdom
[c] Institute of Biomedical Engineering, Imperial College London, London SW7 2AZ, United Kingdom

ARTICLE INFO

Article history:
Received 21 February 2017
Received in revised form
15 May 2017
Accepted 12 June 2017
Available online 14 June 2017

Keywords:
Fiber-optic Raman spectroscopy
Articular cartilage
Tissue-engineering
Live cell Raman spectroscopy
Online biomedical Raman spectroscopy

ABSTRACT

Tissue engineering (TE) has the potential to improve the outcome for patients with osteoarthritis (OA). The successful clinical translation of this technique as part of a therapy requires the ability to measure extracellular matrix (ECM) production of engineered tissues *in vitro*, in order to ensure quality control and improve the likelihood of tissue survival upon implantation. Conventional techniques for assessing the ECM content of engineered cartilage, such as biochemical assays and histological staining are inherently destructive. Raman spectroscopy, on the other hand, represents a non-invasive technique for *in situ* biochemical characterization. Here, we outline current roadblocks in translational Raman spectroscopy in TE and introduce a comprehensive workflow designed to non-destructively monitor and quantify ECM biomolecules in large (>3 mm), live cell TE constructs online. Diffuse near-infrared fiber-optic Raman spectra were measured from live cell cartilaginous TE constructs over a 56-day culturing period. We developed a multivariate curve resolution model that enabled quantitative biochemical analysis of the TE constructs. Raman spectroscopy was able to non-invasively quantify the ECM components and showed an excellent correlation with biochemical assays for measurement of collagen ($R^2 = 0.84$) and glycosaminoglycans (GAGs) ($R^2 = 0.86$). We further demonstrated the robustness of this technique for online prospective analysis of live cell TE constructs. The fiber-optic Raman spectroscopy strategy developed in this work offers the ability to non-destructively monitor construct growth online and can be adapted to a broad range of TE applications in regenerative medicine toward controlled clinical translation.

1. Introduction

Cartilage tissue engineering is a promising osteoarthritis (OA) treatment strategy that involves the generation of live replacement tissues that can be used to repair clinical cartilage defects. In a conventional approach, chondrogenic cells are encapsulated in a polymeric scaffold, which provides them with an environment to support the synthesis and elaboration of a new cartilage extracellular matrix (ECM) over time. This technique aims to generate tissues that recapitulate a robust, mechanically-functional cartilage ECM, capable of supporting physiologic mechanical loads. A key goal of cartilage tissue engineering is to implant tissues after they have developed suitable levels of the major cartilage ECM constituents (*i.e.*, glycosaminoglycans (GAGs) and collagen), allowing them to resist mechanical strains, provide a low friction articulating interface and, ultimately, exhibit long term survival in the native environment of the synovial joint. In this context, the field of cartilage tissue engineering has achieved growing success [1,2]. To date, the development of several culture strategies have allowed for the generation of engineered cartilage with ECM content and mechanical properties that match those present in native articular cartilage [3,4].

The successful clinical translation of cartilage tissue engineering requires the development of robust techniques for the assessment of tissue quality and levels of ECM content prior to implantation. The development of measurement techniques may be particularly crucial for engineered cartilage derived from human patient cell populations (autologous chondrocytes, mesenchymal stem cells [MSCs], or induced pluripotent stem cells [iPSCs]), which exhibit

* Corresponding author. Department of Materials, Imperial College London, London SW7 2AZ, United Kingdom.
E-mail address: m.stevens@imperial.ac.uk (M.M. Stevens).
[1] These authors contributed equally to the work.

http://dx.doi.org/10.1016/j.biomaterials.2017.06.015

highly variable growth rates [5]; as such, these tissues require varying *in vitro* culture durations before they have deposited a level of ECM that is suitable for implantation. The premature implantation of tissues can potentially lead to failure upon exposure to the native mechanical loading environment. The ability to perform an online monitoring of tissue growth over time, which can guide the optimal point of tissue implantation, may be essential for achieving future translational success of cartilage tissue engineering strategies.

Conventional techniques for assessing the ECM content of engineered cartilage, such as biochemical assays and histological staining, are inherently destructive. Further, the utilization of these conventional assessment techniques to monitor tissue growth over time would require the parallel fabrication of sacrificial engineered cartilage samples. Given requisite high cell densities needed to achieve sufficient ECM deposition and the inherent challenge of procuring large amounts of cells from patients, this sacrificial strategy is likely to be highly burdensome. Sample sacrificing becomes even more prohibitive when engineering large cartilage tissues for the repair of clinical sized OA defects or for replacing an entire articular cartilage surface.

To date, various techniques have been applied for online monitoring (i.e. nondestructive tissue monitoring during culture) including microdialysis [6], magnetic resonance imaging [7] and micro-computed tomography (microCT) [8]. These modalities are however either invasive, require expensive equipment, and/or do not provide specific molecular information about the tissue engineered constructs. Interestingly, near-infrared (NIR) and mid-infrared (MIR) spectroscopy have been applied to quantify collagen, GAG, and water [9–12]. These monitoring techniques are associated with several limitations: infrared spectroscopy is associated with limited photon penetration due to water absorption in the wavelength range (>1100 nm) and has inherently less molecular specificity [13]. Alternatively, Raman spectroscopy is a highly promising inelastic light scattering technique that can probe the vibrational modes of molecular bonds in tissue samples. This technique can be used to interrogate the biochemical composition (*i.e.*, biochemical conformation of proteins, carbohydrates, lipids, nucleic acids, etc.) of tissues with high molecular specificity [14]. For instance, Raman spectroscopy has previously been performed on commercially available engineered oral mucosa, scaffolds, and tissue pellets [15–18]. Raman spectroscopy can be performed in aqueous environments and may hold key advantages over NIR spectroscopy in its high biomolecular specificity and probing of highly hydrated tissues, such as articular cartilage [19,20]. We have recently demonstrated the ability of 532 nm Raman microspectroscopic imaging to quantify the relative distribution of the major ECM constituents (GAG and collagen) in devitalized tissues [20]. Visible laser excitation, however, poorly penetrates tissue. Since articular cartilage represents a thin avascular tissue layer (~3 mm thickness) that is highly transparent in the tissue optical window (*i.e.*, 700–1100 nm) [21], it could represent an excellent tissue for diffusely probing the bulk biochemical composition using NIR laser excitation.

Here we develop a novel diffuse fiber-optic Raman spectroscopy strategy and introduce a comprehensive workflow designed to monitor and quantify ECM biomolecules in live cell tissue engineered cartilage constructs online. In this study, we explore the potential utility of this methodology by assessing: 1) the potential deleterious effect of Raman spectral acquisitions on construct viability and growth, 2) the ability of Raman spectral acquisitions to quantify the concentrations of ECM constituents in constructs (via multivariate curve resolution analysis) and quantify the spectral similarity between engineered and native cartilage tissues (via principle component analysis), and 3) the ability of fiber-optic Raman spectroscopy to monitor long term, live tissue growth over a 6 week culture period. The presented strategy could potentially allow us to define tissue specifications to identify engineered cartilage that is suitable for implantation, in order to achieve future translational success of cartilage tissue engineering.

2. Materials and methods

2.1. Tissue samples: native and engineered cartilage

Immature articular cartilage cylindrical explants were harvested from the femoral condyles of 4-week old calf knee joints obtained from a local abattoir (N = 3 animals). Explants were extracted via biopsy punch and the superficial and deep zones of the tissue were excised, yielding cylindrical explants of predominantly middle zone cartilage (⌀4 × 2 mm). Explants were stored dry at −20 °C for up to two weeks until testing. Tissue engineered cartilage constructs were prepared from primary bovine articular chondrocytes obtained from 4-week old bovine calf carpometacarpal joints (N = 5 animals per study). Tissue was enzymatically digested for 15 h in 1.5 mg/mL of type-IV collagenase (Invitrogen) and isolated cells were encapsulated in 2% (w/v) type VII agarose at a nominal density of 30 × 10^6 cells/mL and configured as ⌀4 × 2 mm tissue engineered (TE) construct disks, as described previously [22]. These relatively small constructs were utilized for this investigation to mitigate the introduction of spatial heterogeneities, as supported by histological characterizations in prior investigations on this TE system [23]. Constructs were cultured for up to 56 days in a chondrogenic media formulation, consisting of high glucose DMEM supplemented with 100 nM dexamethasone, 100 μg/mL sodium pyruvate, 50 μg/mL L-proline, 1% ITS + premix (6.25 μg/mL insulin, 6.25 μg/mL human holotransferrin, 6.25 ng/mL selenium), 1% PS/AM antibiotic antimycotic, and 173 μM ascorbate-2-phosphate. Media was supplemented with 10 ng/mL TGF-β3 or maintained TGF-β free.

2.2. Mechanical testing and biochemical assays

The equilibrium compressive Young's modulus of TE constructs was determined using a custom-made testing apparatus consisting of a stainless steel loading platen and an inline load cell (500 g; Honeywell) mounted to a micrometer-actuated vertical translating stage (Newport Spectra Physics). While immersed in phosphate buffered saline (PBS), TE constructs were subjected to a 10% axial compressive strain and allowed to undergo stress relaxation until equilibrium (reached at 20 min). The subsequent reaction force was recorded and used with sample geometry for modulus calculations. A two-way ANOVA was performed ($\alpha = 0.05$ and statistical significance set at $p < 0.05$) to assess the effect of culture duration and TGF-beta supplementation on the Young's modulus of TE constructs. A Tukey's HSD post-hoc test was run to examine differences between the groups.

Following Raman analysis and mechanical testing, TE constructs were analyzed for their biochemical contents. Each construct was digested via proteinase-K (0.5 mg/mL; 56 °C for 16 h) and subsequently processed for its GAG and collagen contents, via the dimethylmethaline blue [24] and orthohydroxyproline [25] assays, respectively.

2.3. Live cell Raman spectroscopy instrumentation

The integrated fiber-optic Raman spectroscopy and culture system capable of live cell spectral acquisition is shown in Fig. 1A. The in-house built Raman system consists of an NIR diode laser ($\lambda_{ex} = 785$ nm) (maximum output: 500 mW, B&W TEK Inc.), and a