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Fabrication of injectable, cellular, anisotropic collagen tissue equivalents with modular fibrillar densities

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ABSTRACT

Technological improvements in collagen gel fabrication are highly desirable as they may enable significant advances in the formation of tissue-equivalent biomaterials for regenerative medicine, three-dimensional (3D) *in vitro* tissue models, and injectable scaffolds for cell and drug delivery applications. Thus, strategies to modulate collagen gel fibrillar density and organization in the mesostructure have been pursued to fabricate collagenous matrices with extracellular matrix-like features. Herein, we introduce a robust and simple method, namely *gel aspiration-ejection* (GAE), to engineer 3D, anisotropic, cell seeded, injectable dense collagen (I-DC) gels with controllable fibrillar densities, without the use of crosslinking. GAE allows for the hybridization of collagen gels with bioactive agents for increased functionality and supports highly aligned homogenous cell seeding, thus providing I-DC gels with distinct properties when compared to isotropic DC gels of random fibrillar orientation. The hybridization of I-DC with anionic fibroin derived polypeptides resulted in the nucleation of carbonated hydroxyapatite within the aligned nanofibrillar network upon exposure to simulated body fluid, yielding a 3D, anisotropic, mineralized collagen matrix. In addition, I-DC gels accelerated the osteoblastic differentiation of seeded murine mesenchymal stem cells (m-MSCs) when exposed to osteogenic supplements, which resulted in the cell-mediated, bulk mineralization of the osteoid-like gels. In addition, and upon exposure to neuronal transdifferentiation medium, I-DC gels supported and accelerated the differentiation of m-MSCs toward neuronal cells. In conclusion, collagen GAE presents interesting opportunities in a number of fields spanning tissue engineering and regenerative medicine to drug and cell delivery.

1. Introduction

The development of new fabrication strategies to control the structure of biopolymers is a key step towards the engineering of biomimetic, functional biomaterials with extracellular matrix (ECM)-like features [1]. The nano- to macro-scale hierarchical organisation of collagen, the main fibrillar protein in the ECM, imparts specific functional and morphological cues that direct cell fate and activity. Consequently, strategies to control the structure and direct the function of three-dimensional (3D) collagen hydrogels are highly sought after, and may enable dramatic advances in the fabrication of tissue-equivalent biomaterials for regenerative medicine, *in vitro* tissue models, and injectable scaffolds for cell and drug delivery applications [2–4]. While the processing of collagen gels into densely packed nanofibrillar structures has enabled the engineering of numerous soft tissues by providing ECM-like constructs, where cells can be easily incorporated at the point of material fabrication, their *in vitro* reconstitution poses technological challenges in terms of fabricating biomimetic matrices with physiologically relevant nanofibrillar density and alignment [3,5].

A number of strategies to modulate collagen gel fibrillar density (CFD) and organization in the meso-structure have been pursued to fabricate collagenous matrices with ECM-like features. To date, four distinct methods have been reported to modulate CFD: reverse dialysis [6], evaporation [7], plastic compression (PC) [8], and continuous injection [9]. These techniques allow for the fabrication of dense collagen (DC) gels with a fibrillar density within the range of 5–25 wt%, which have been used as 3D ECM models [10,11] and to engineer several tissues, such as osteoid [12–14], dura mater [15], nucleus pulpous [16,17], skin [18,19], bladder [20], cornea [21–24], tendon [25], airways [26], and blood vessels [26]. Nevertheless, none of these methods have allowed for the formation of anisotropic DC gels [3], a feature that has been achieved in highly-hydrated collagen gels (fibrillar density < 1 wt%) through the

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application of either electric and magnetic fields [27–29], microfluidic devices [30,31], extrusion of crosslinked collagen [32], micro- and nano-topographical cues during self-assembly [33,34], or strain during fibril formation [35]. In addition, the injection of collagen gel, and not of its soluble precursor solution, has been proposed as an effective approach to exert control over locally delivered cells and drugs for therapeutic applications [36–38]. However, the injection of self-assembled collagen is limited by the weakness of the gel, lacking the strength necessary to withstand the shear-stress applied during the injection process. The use of different crosslinkers such as glutaraldehyde [32] and genipin [37] or co-agents, polyethylene glycol and NaCl [39], have increased collagen gel mechanical properties to allow for the extrusion of preformed gels through a syringe needle. However, these methods not only increase the time and the steps required to form the gels, but also reduce cell viability and biocompatibility, and may interfere with drugs and other biomacromolecules loaded into the gels. Moreover, these methods do not allow for the modulation of CFD. Indeed, the current advances in collagen gel fabrication have only addressed single, but not multiple technological challenges at the same time.

Herein, we introduce an unprecedented, robust and simple method, namely *gel aspiration-ejection* (GAE) to engineer 3D, anisotropic, cell seeded, injectable, collagen gels with controllable fibrillar densities. Through the application of pressure differentials, GAE initially draws prefabricated cellular highly-hydrated collagen hydrogels into a needle, simultaneously imparting compaction and anisotropy on the gels, which are then controllably ejected to generate injectable dense collagen (I-DC) gels. The effect of GAE on I-DC nanofibrillar organization was structurally and morphologically characterised. The viability, morphology and distribution of murine derived NIH/3T3 fibroblasts and bone marrow derived mesenchymal stem cells (m-MSCs), seeded in collagen gels at the point self-assembly, and then exposed to GAE were investigated. The impact of I-DC anisotropy on both the osteoblastic and neuronal differentiation of seeded m-MSCs was compared to those seeded in isotropic DC gels produced through PC.

2. Materials and methods

2.1. Fabrication of I-DC and DC gels

GAE and PC were used to generate I-DC and DC collagen gels, respectively. A volume of 3.2 mL of rat tail tendon type I collagen (2.11 mg/mL in 0.6% acetic acid; First Link Ltd, UK) was mixed with 0.8 mL of 10 times concentrated Dulbecco Modified Eagle Medium (10× DMEM; Sigma Aldrich, Canada) and neutralized with 37 μL of 5 M NaOH (Fisher Scientific, Canada). The solution (4 mL) was then cast in a rectangular mould (18 × 43 mm^2) and incubated at 37 °C for 30 min to generate highly-hydrated collagen gels (CFD = 0.2 wt%) (Fig. 1a *i*). For I-DC production, collagen gels were perforated at one of their two small faces by a flat capillary (needle ⌀ = 0.3–0.9 mm) attached to 50 mL syringe (Fig. 1a *ii*). The piston of the syringe was then firmly pulled to create a negative pressure, necessary to aspirate the collagen gel into the capillary (Fig. 1a *iii*). By maintaining the applied negative pressure, the gel was lifted from the mould and a filter paper was placed on the opposite side of the gel (Fig. 1a *iv*) to facilitate the water expulsion from the hydrated collagen gel. The negative pressure was maintained for the time necessary to completely aspirate the gel into the capillary (t ≅ 15 min). Once the gel was completely aspirated, the capillary was removed from the 50 ml syringe and mounted on a 3 mL syringe filled with buffered solution. The gel was then ejected from the capillary by applying a gentle and constant pressure on the syringe piston to produce rod-shaped I-DC gels (Fig. 1a *v*) with CFDs ranging from 5.5 to 9.2 wt%, tunable by changing the diameter of the capillary (Fig. 1a *vi*). I-DC gels of 7.8 wt% CFD (generated with a capillary of ⌀ = 0.6 mm) were used for microstructural characterization as well as analyses of seeded cellular responses to combine the fabrication of anisotropic collagen gels of tissue-equivalent CFD together with the ease of sample fabrication.

For DC production, the rectangular-shaped highly-hydrated collagen gels were gently removed from the casting chamber and plastically compressed using 1 kPa for 5 min in combination with blotting to form rectangular sheets, as described previously [8]. The sheets (CFD = 8 wt%) were rolled along their long axis and halved to give cylindrical shaped specimens of 1.0 ± 0.1 mm diameter.

CFD values were determined as previously reported [8]. In brief, by assuming that no collagen was lost during GAE or PC, the weight percent of collagen in the scaffolds after GAE or PC was calculated by weighing the scaffolds ($n = 5$) before (W_{wet}) and after (W_{dry}) freeze drying (BenchTop K, VirTis, Canada), according to Equation (1).

$$CFD = \frac{W_{dry}}{W_{wet}} \times 100 \quad (1)$$

2.2. Immediate response of NIH/3T3 fibroblasts to I-DC fabrication

NIH/3T3 murine fibroblasts (American Type Culture Collection, USA) were used to assess the immediate cellular responses to GAE. Cells at 80% confluency were passaged and seeded at a density of 2 × 10^5 cells/mL pre gelling and I-DC and DC production. I-DC and DC gels were cultured for up to day 7 in complete medium consisting of alpha-minimal essential media (Gibco), 10% HyClone Foetal Bovine Serum, 2 mM L-glutamine (Invitrogen, USA), 100 U/ml Penicillin–Streptomycin (Invitrogen, USA) at 37 °C, 5% CO_2 and replenished every 3 days.

2.3. m-MSC seeding and culture

m-MSCs (C57BL/6 mice, Gibco) at 80% confluency were passaged and seeded at a density of 2 × 10^5 cells/mL pre gelling and I-DC (⌀ = 0.6 mm) and DC production. m-MSC seeded in I-DC or DC gels were cultured for up to day 21 in either complete medium, or when supplemented with either osteogenic or neuronal differentiation supplements. Osteogenic supplements consisted of 50 μg/mL ascorbic acid, 50 mM β-glycerophosphate, and 1 μM dexamethasone (all Sigma) and replenished every 3 days. For neuronal differentiation, in the first day, the medium was supplemented by 1 mM Beta-mercaptoethanol (Sigma), followed by 35 ng/mL of all-trans-retinoic acid (Sigma) for the second day. In subsequent days, 5 μM forskolin, 10 ng/mL basic fibroblast growth factor, 10 ng/mL platelet derived growth factor, and 10 ng/mL insulin-like growth factor-1 (all Sigma) were used to supplement the medium, and replenished daily.

2.4. Cell viability and morphology

Confocal laser scanning microscopy (CLSM) combined with fluorescent cell staining was used to determine cell viability and morphology. At days 1, 7, 14, and 21, transverse sections of scaffolds in 2 μM Calcein AM and 4 μM Ethidium homodimer-1 (Live/DEAD® assay, Invitrogen, USA) in phosphate buffered saline devoid of divalent cations were incubated for at 37 °C for 45 min. Excitation by an argon laser (488 nm) allowed for detection of fluorescence emission (at 515 nm) depicted as green, for live calcium-laden cells; whereas, excitation by a helium–neon laser (543 nm) allowed for emission detection at 590 nm depicted as red, binding exposed (compromised or dead cells) nuclear content. Specimen data was collected at 10× objective, with a minimum of 1 μm z-dimension slices to report maximum intensity of combined emission data. CLSM in combination with phalloidin-stained filamentous actin (F-actin; Life Technologies, USA) was used to evaluate the effect anisotropic collagen matrix on cytoskeleton alignment.

2.5. Histological sections

Gels were washed in phosphate buffered saline, fixed in 10% neutral buffered formalin overnight, dehydrated through a series of graded ethanol, embedded in paraffin and cut in transverse sections of 5 μm-thickness. von Kossa staining was performed on the trimmed sections.

2.6. Gene expression

Real-time quantitative Reverse Transcription-Polymerase Chain Reaction (RT-qPCR) was used to investigate the expression of a number of genes as indicators of either m-MSC osteoblastic (*Alp*, *Runx2* and *Opn* and *Mmp1*, *Mmp13* and *Timp1*) or neuronal (*Ina*, *Nes*, *Sca1*, *Scn10a*, and *Tubb3a*) differentiation. m-MSC seeded I-DC and DC gels were submersed in 800 μL of TRIzol (Invitrogen) reagent and total ribonucleic acid (RNA) species were extracted, as per the manufacture instructions. Messenger RNA were hybridized by deoxythymine oligomers (50 mM; Applied Biosystems) at 65 °C for 5 min prior to reverse transcription by 10 U/μL M-MLV reverse transcriptase (Life Technologies) at 37 °C for 1 h (as per manufacturer instructions with 10 mM (each) dexoribonucleic acids (Invitrogen), 0.1 M dithiothreitol (Sigma), 40 U/μL rRNasin (Promega), First Strand Buffer (Invitrogen) and adjusted with RNAse/DNAse Free H_2O (Gibco). Enzymes were inactivated by incubation at 75 °C for 15 min and resulting cDNA templates generated were diluted 20×. Optimized primers synthesized by IDT technologies at 0.1 μM amplified pooled specimen templates verifying primer efficacy. Housekeeping gene primers (*Gapdh* and *Eef2*) were amplified with each sample attesting RNA extraction efficacy prior to qPCR commencement. Cycling conditions on a BioRAD iCycler termocycler were an initial 95 °C denaturation for 6 min, followed by 35 repeats of 95 °C denaturation for 30 s, 58 °C annealing for 45 s and 72 °C extension for 1.5 min, concluding with an infinite loop of refrigeration. Results were analysed on 0.8% Agarose gels electrophoresed at 75 V for 1 h stained with 5 uL 10 mg/mL Ethidium homodimer-1 and