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Composite hydrogel scaffolds incorporating decellularized adipose tissue for soft tissue engineering with adipose-derived stem cells

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ABSTRACT

An injectable tissue-engineered adipose substitute that could be used to deliver adipose-derived stem cells (ASCs), filling irregular defects and stimulating natural soft tissue regeneration, would have significant value in plastic and reconstructive surgery. With this focus, the primary aim of the current study was to characterize the response of human ASCs encapsulated within three-dimensional bioscaffolds incorporating decellularized adipose tissue (DAT) as a bioactive matrix within photo-cross-linkable methacrylated glycol chitosan (MGC) or methacrylated chondroitin sulphate (MCS) delivery vehicles. Stable MGC- and MCS-based composite scaffolds were fabricated containing up to 5 wt% cryomilled DAT through initiation with long-wavelength ultraviolet light. The encapsulation strategy allows for tuning of the 3-D microenvironment and provides an effective method of cell delivery with high seeding efficiency and uniformity, which could be adapted as a minimally-invasive *in situ* approach. Through *in vitro* cell culture studies, human ASCs were assessed over 14 days in terms of viability, glycerol-3-phosphate dehydrogenase (GPDH) enzyme activity, adipogenic gene expression and intracellular lipid accumulation. In all of the composites, the DAT functioned as a cell-supportive matrix that enhanced ASC viability, retention and adipogenesis within the gels. The choice of hydrogel also influenced the cell response, with significantly higher viability and adipogenic differentiation observed in the MCS composites containing 5 wt% DAT. *In vivo* analysis in a subcutaneous Wistar rat model at 1, 4 and 12 weeks showed superior implant integration and adipogenesis in the MCS-based composites, with allogenic ASCs promoting cell infiltration, angiogenesis and ultimately, fat formation.

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1. Introduction

Adipose tissue engineering represents a promising alternative for reconstructive and cosmetic applications in plastic surgery to restore injury- or age-related soft tissue loss within the subcutaneous layer [1,2]. A common strategy is to seed three-dimensional scaffolds with regenerative cell populations, such as adipose-derived stem cells (ASCs), to create tissue substitutes that can be used to induce stable and predictable fat formation [3,4]. Ideally, the scaffold should mimic the properties of the native extracellular matrix (ECM), supporting cell adhesion and viability, and maintaining the structural integrity of the construct as it is gradually replaced with healthy adipose tissue [5].

Many studies to date have focused on designing implantable scaffolds comprised of synthetic or naturally-derived polymers that have a pre-defined shape and volume [6]. More recent efforts in this application have included the development of injectable biomaterials, including hydrogels, particulates and microcarriers, which offer a more minimally-invasive strategy for soft tissue augmentation in the clinic [7,8]. In all of these approaches, it has become apparent that many different factors can influence adipogenesis in cell-based therapies with ASCs, including complex cell-ECM and cell–cell interactions within the engineered microenvironment. These results highlight the importance of rational design of delivery vehicles for ASCs in a tissue-specific manner to enable long-term soft tissue regeneration.

As a step toward engineering adipose tissue replacements for use in plastic and reconstructive surgery, our group has been

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developing implantable and injectable biomaterials derived from decellularized adipose tissue (DAT), including 3-D scaffolds, microcarriers, and foams [9–11]. Interestingly, we have shown that the decellularized ECM of human adipose tissue provides a highly conducive milieu for fat formation both *in vitro* and *in vivo*. Other groups investigating scaffolds incorporating ECM from adipose tissue have reported similar findings [12–14], including recent work with temperature sensitive adipose-derived hydrogels [15,16]. Moreover, we have shown that the DAT can naturally induce the adipogenic differentiation of human ASCs in culture without medium supplementation with exogenous differentiation factors.

For clinical applications in soft tissue augmentation, our aim in the current study was to develop injectable bioscaffolds that integrate the unique pro-adipogenic properties of the DAT within a customizable hydrogel delivery vehicle, to engineer composite biomaterials that have highly tunable characteristics. More specifically, methacrylated glycol chitosan (MGC) and methacrylated chondroitin sulphate (MCS) were investigated as photo-cross-linkable hydrogel carriers for the encapsulation of human ASCs within a cryomilled DAT matrix. Chitosan is a linear polysaccharide of *N*-acetyl-glucosamine and *N*-glucosamine subunits obtained from the deacetylation of chitin derived from crustaceans, which has been explored in a range of tissue-engineering applications due to its innate wound healing and antibacterial properties [17,18]. The use of chitosan as a biomaterial, however, is hampered by its poor water solubility at physiologic pH. Glycol chitosan, formed by the reaction of ethylene oxide with chitosan, is both *O* and *N*-glycolated [18], making it soluble in aqueous media from pH 2 to 12. In contrast, chondroitin sulphate is a sulphated glycosaminoglycan (GAG) that consists of repeating D-glucuronic acid and *N*-acetyl galactosamine disaccharide units. It is a highly water-soluble structural component of the human ECM that has been indicated to play important roles during embryogenesis and wound healing [19–21]. Both chitosan and chondroitin sulphate are readily chemically modifiable and can be rendered photo-cross-linkable through methacrylation to enable *in situ* gelation with low-intensity UV light under mild conditions.

To investigate the potential of our composite scaffold approach for adipose regeneration, fabrication methods were established to synthesize stable MGC- and MCS-based bioscaffolds containing 0, 3 or 5% (w/v) cryomilled DAT, with the base hydrogels formulated to have matching mechanical properties. Extensive *in vitro* cell culture experimentation was conducted to characterize the viability and adipogenic differentiation of human ASCs encapsulated within the composites. Further, the *in vivo* biocompatibility of the MGC- and MCS-based composites containing 5% DAT was assessed in a subcutaneous Wistar rat model at 1, 4 and 12 weeks, both with and without incorporation of allogenic rat ASCs.

2. Materials and methods

2.1. Materials

Chondroitin sulphate (weight average molecular weight of approximately 15 kDa) was generously donated by Stellar Pharmaceuticals Inc. (London, ON, Canada). Unless otherwise stated, all chemicals were purchased from Sigma–Aldrich Canada Ltd. (Oakville, ON, Canada) and used as received.

2.2. Adipose tissue procurement and processing

Adipose tissue samples were collected from female patients undergoing elective breast reduction or abdominoplasty surgery at the Kingston General Hospital or Hotel Dieu Hospital in Kingston, ON, Canada. The samples were transported to the lab on ice in sterile, cation-free phosphate buffered saline (D-PBS; Thermo Scientific HyClone, Fisher Scientific, Oakville, ON, Canada), supplemented with 20 mg/mL bovine serum albumin (BSA). The donor age, weight and height were recorded. Within 2 h, the tissues were processed for ASC isolation or decellularization, using published methods [9]. This study was reviewed and approved by the research ethics board at Queen's University (REB# CHEM-002-07).

2.2.1. Cryo-milling of decellularized adipose tissue

Following decellularization, the DAT was snap frozen in liquid nitrogen, lyophilized, and finely minced with sharp scissors. The minced DAT was then cryomilled using a laboratory ball mill (Sartorius Stedim Mikro-Dismembrator, Goettingen, Germany) at 2500 rpm for 2 min. The milled DAT was stored at 4 °C until further use. Immediately prior to composite scaffold fabrication, the DAT was sterilized by exposure to UV light for 60 min.

2.3. Polymer purification and reaction

The chemical compositions of all glycol chitosan (GC) and chondroitin sulphate (CS) derivatives were assessed by ^{1}H NMR spectroscopy using a Bruker Avance-600 Ultrashield spectrometer with a 5 mm TBI S3 probe and data collection with Bruker's XWIN NMR software. The samples were prepared by dissolving the polymer overnight in deuterium oxide at a concentration of 20 mg/mL and adjusted to pH 10 using 1 M sodium hydroxide. All GC-based polymers were analyzed at 90 °C and all CS-based polymers were analyzed at room temperature.

2.3.1. Purification and methacrylation of glycol chitosan

The GC (weight average molecular weight of approximately 120 kDa, from Wako Chemical USA Inc., Richmond, VA, USA) was purified using an established protocol [22]. Briefly, the polymer was dissolved in deionized water, filtered, and dialyzed against distilled water using 50 kDa dialysis tubing (Spectrum Laboratories Inc., Rancho Dominguez, CA, USA). The purified GC was frozen and lyophilized. Subsequently, the GC was methacrylated using glycidyl methacrylate via an established protocol [22]. Purified GC was dissolved in deionized water to obtain a 2% (w/v) solution that was adjusted to pH 9.0 using 1 M sodium hydroxide. Glycidyl methacrylate at 3% (v/v) was added to achieve an initial molar ratio of glycidyl methacrylate to free amine (per mol glycol chitosan residue) of 0.1. The solution was allowed to react for 24 h before the reaction was neutralized (pH 7) with 1 M hydrochloric acid. The methacrylated glycol chitosan (MGC) solution was dialyzed twice against deionized water with 12 kDa dialysis tubing (Fisher Scientific, Oakville, Canada) for 2 h. The purified MGC was frozen and lyophilized.

2.3.2. Methacrylation of chondroitin sulphate

The methods for the methacrylation of the CS using methacrylate anhydride were adapted from the approach described by Li et al. [23]. CS was dissolved at 0.2 g/mL in 1.0 M sodium phosphate. A 1:1 blend of methacrylic anhydride and dimethylsulfoxide (DMSO) was prepared and added to the dissolved CS in a volumetric ratio of 0.06:1. After 2 h incubation at room temperature, the pH was adjusted to pH 10 with 5 M sodium hydroxide, and then allowed to react for an additional 22 h. The solution was then dialyzed against deionized water for 12 h with 3.5 kDa dialysis tubing (Fisher Scientific, Hanover Park, IL, USA), and the purified methacrylated chondroitin sulphate (MCS) solution was frozen and lyophilized.

2.3.3. Hydrogel characterization

MGC and MCS hydrogels were fabricated via photo-polymerization based on previously-established methods [22]. To prepare hydrogels with varying Young's moduli, the prepolymer concentration in solution was adjusted prior to photo-cross-linking, with the objective of obtaining MGC and MCS concentrations yielding hydrogels with similar moduli. Specifically, MGC hydrogels were made from MGC solution concentrations of 1.5, 2, 2.5 and 3% (w/v) and compared against MCS hydrogels prepared from MCS solution concentrations of 8, 10 and 15% (w/v) (4 replicate samples (n) with 3 measurements of each sample (N)). A 5 mg/mL solution of Irgacure 2959 (2-hydroxy-4′-(2-hydroxyethoxy)-2-methylpropiophenone) photoinitiator in deionized water was added to the prepolymer solution to achieve a final concentration of 0.05% (w/v). The gels (100 μL) were fabricated in a 16-well chamber glass slide (Nalgene Nunc International, Rochester, New York) and photo-cross-linked with long-wavelength ultraviolet light (320–390 nm, EXFO Lite, EFOS Corporation, Mississauga, Canada) at an intensity of 10 mW/cm^2 for 3 min, to form hydrogels with a diameter of 7 mm and a height of 3 mm.

The indentation method described in the study by Hayes et al. was used to measure the Young's modulus of fully hydrated gels in deionized water ($n = 4$, $N = 3$) [24]. A TA XT plus texture analyzer (Texture Technologies Corp., New York) equipped with flat-ended cylindrical indenters (3 and 7 mm diameter) was used to apply force at a rate of 0.05 mm/s over a distance of 0.5 mm. The Poisson ratio and the Young's modulus for each gel were calculated using equations derived from Hayes et al., as described previously [24].

2.4. Fabrication of composite hydrogel scaffolds

Composite MGC-DAT and MCS-DAT hydrogels were fabricated via photo-polymerization, using methods adapted from the previous section [22]. Based on the initial mechanical characterization studies of the hydrogels, the MGC was dissolved at a concentration of 1.5% (w/v) and the MCS at a concentration of 10% (w/v) for all subsequent studies. The Irgacure 2959 photoinitiator solution (5 mg/mL) was sterile syringe filtered and added to achieve a final concentration of 0.05% (w/v). Prepolymer solutions were prepared containing 0, 3 or 5% (w/v) milled DAT and mixed by gentle pipetting. 100 μL of each solution was transferred into a 16-well

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