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Acoustic droplet–hydrogel composites for spatial and temporal control of growth factor delivery and scaffold stiffness

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ABSTRACT

Wound healing is regulated by temporally and spatially restricted patterns of growth factor signaling, but there are few delivery vehicles capable of the "on-demand" release necessary for recapitulating these patterns. Recently we described a perfluorocarbon double emulsion that selectively releases a protein payload upon exposure to ultrasound through a process known as acoustic droplet vaporization (ADV). In this study, we describe a delivery system composed of fibrin hydrogels doped with growth factor-loaded double emulsion for applications in tissue regeneration. Release of immunoreactive basic fibroblast growth factor (bFGF) from the composites increased up to 5-fold following ADV and delayed release was achieved by delaying exposure to ultrasound. Releasates of ultrasound-treated materials significantly increased the proliferation of endothelial cells compared to sham controls, indicating that the released bFGF was bioactive. ADV also triggered changes in the ultrastructure and mechanical properties of the fibrin as bubble formation and consolidation of the fibrin in ultrasound-treated composites were accompanied by up to a 22-fold increase in shear stiffness. ADV did not reduce the viability of cells suspended in composite scaffolds. These results demonstrate that an acoustic droplet–hydrogel composite could have broad utility in promoting wound healing through on-demand control of growth factor release and/or scaffold architecture.

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1. Introduction

Scaffolds, typically fabricated with porous structures and composed of biodegradable materials, are frequently used in regenerative medicine as an adhesive substrate for the attachment of cells and/or the encapsulation of inductive proteins (e.g. growth factors) [1–3]. As a critical component of the local, cellular microenvironment, growth factors can affect the migration, survival, proliferation and differentiation of cells. In most cases, the release of growth factors, either chemically conjugated to the scaffold material [4,5] or physically contained within particulates [6–8], is a process dominated by molecular diffusion and material degradation. The ability to modulate growth factor release from these passive systems is severely limited, especially after in vivo implantation of the scaffold.

Non-invasive control of protein release from a scaffold could improve the efficacy and safety of growth factor-based therapies. Additionally, such control could facilitate the generation of distinct spatial and/or temporal profiles of growth factor availability within the scaffold, thus more closely mimicking the patterns of growth factor expression observed during endogenous wound healing [9,10]. Various stimuli such as pH [11] and proteases [12], as well as energy-based stimuli such as magnetism [13,14], electricity [15], light [16], and temperature [17], have been used to trigger the release of therapeutic agents. However, the ability to translate these externally modulated systems to the clinic is limited by the inability to focus the triggering stimulus or interact with deep tissue implants.

In addition to responding to chemical signals such as growth factors, cells also respond to the mechanical properties of their microenvironment [18]. Within a scaffold, physical properties such as elasticity, pore size, and degradation rate affect cellular processes [19,20], and on-demand perturbation of those properties

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would provide new avenues by which to "actively" regulate cell behavior in an engineered tissue. Although a recent study demonstrated the externally controlled modification of ferrogel architecture post-implantation [14], most modifications to scaffold architecture are done prior to cell seeding and scaffold implantation [21,22] due to the use of chemicals or processing techniques that are not biocompatible [2].

One externally applied stimulus capable of inducing "on-demand" release of growth factors and scaffold architecture modification is ultrasound. Using both thermal and non-thermal mechanisms, ultrasound has been used extensively to facilitate the regeneration of both soft tissue and bone [23–25]. Ultrasound is an attractive stimulus for interacting with scaffolds since it can be applied non-invasively, focused with sub-millimeter precision, and delivered in a spatio-temporally controlled manner to sites deep within the body. The ultrasound-based mechanism used in the presented studies is termed acoustic droplet vaporization (ADV), whereby an emulsion (i.e. surfactant-stabilized, liquid droplets) is converted into gas bubbles upon exposure to ultrasound beyond a threshold pressure amplitude [26,27]. ADV has been studied as a release mechanism for therapeutic agents, primarily with emulsions designed to be delivered intravascularly [28–35]. Acoustically sensitive emulsions that undergo ADV are composed of a perfluorocarbon (PFC) liquid such as perfluoropentane (PFP, C_5F_{12}, 29 °C boiling point). At normal body temperature (i.e. 37 °C), micron-sized PFP emulsions do not vaporize due to the increase in internal (i.e. Laplace) pressure, and hence boiling point elevation, of PFP when formulated as droplets [36,37]. During ADV, the PFC liquid is converted into a gas in a microsecond timeframe [38] via a non-thermal mechanism [39]. PFCs, which have been used in medical applications as blood substitutes [40] and ultrasound contrast agents [41], are inert and biocompatible, with exhalation being the main route of excretion for low molecular weight PFCs, such as PFP, that are intravascularly administered [42]. Due to their extreme hydrophobicity and lipophobicity [43], PFCs are poor solvents for therapeutic agents such as growth factors. Therefore, for water-soluble agents such as growth factors, a double emulsion of the form water-in-PFC-in-water (W_1/PFC/W_2) is used to contain growth factor within the W_1 phase [32,34,44,45]. The PFC within the double emulsion serves multiple functions. First, the PFC – which is exceedingly hydrophobic [43] – acts as a diffusion barrier for the hydrophilic payload contained within the W_1 phase droplets. Second, during ADV, the PFC undergoes a liquid-to-gas phase transition, thus dispersing the W_1 phase droplets and facilitating release of the encapsulated growth factor.

In the presented in vitro studies, droplet–hydrogel composites – consisting of a fibrin matrix doped with a PFC double emulsion – were generated. These novel composites provide a uniquely bioactive platform that enables the noninvasive regulation, both spatially and temporally, of biochemical and mechanical stimuli relevant to tissue regeneration. Fibrin was chosen for the hydrogel because it has been used extensively in tissue engineering studies, polymerizes rapidly under mild conditions, and is approved by the US Food and Drug Administration (FDA) for clinical use. The experiments focus on characterizing several formulations of the composites before and after ADV in terms of (1) morphological and mechanical properties, (2) the release of bioactive, basic fibroblast growth factor (bFGF) contained within the W_1 phase, (3) enzymatic and cell-based fibrinolysis of the scaffold and (4) viability of cells co-encapsulated in the composite scaffold. These composites may be useful in tissue regeneration where bFGF has been shown to induce angiogenic [46,47] or osteogenic [48,49] responses.

2. Materials and methods

2.1. Emulsion preparation and characterization

The double emulsion was prepared by modifying a previously published method [32]. The primary emulsion (W_1/PFC) was formed by dissolving Krytox 157 FSL (CAS# 51798-33-5, DuPont, Wilmington, DE, USA), a perfluoroether with carboxylic acid functionality and Krytox 157 FSL-polyethylene glycol copolymer in PFP (CAS# 678-26-2, Strem Chemicals, Inc., Newburyport, MA, USA) at concentrations of 0.5% (w/w) and 1.0% (w/w), respectively. Krytox, including its derivatives and copolymers, has been used to stabilize emulsions in in vitro studies with mammalian cells and *Caenorhabditis elegans* [50] as well as in in vivo studies with chicken embryos [45] and rats [34]. The PFP phase was then combined with an aqueous solution of bFGF, reconstituted at 50 μg ml^{-1} in phosphate buffered saline (PBS) containing 1% (w/v) bovine serum albumin (BSA) and 10 μg ml^{-1} heparin, at a volumetric ratio of 2.1:1. Heparin was included because it has been shown to protect bFGF from degradation [51]. The phases were emulsified, while in an ice bath, using the microtip accessory of a sonicator (model 450, 20 kHz, Branson, Danbury, CT, USA) operating at 125 W cm^{-2} for 30 s in continuous mode. The resulting primary emulsion was added drop-wise at a 1:2 volumetric ratio to a 10 mg ml^{-1} solution of Poloxamer 188 (Sigma–Aldrich, St Louis, MO, USA), dissolved in PBS containing 1% (w/v) BSA and 10 μg ml^{-1} heparin, which was in an ice bath and being stirred at 1100 rpm for 10 min. To minimize carryover of non-emulsified bFGF, the double emulsion was washed by allowing the emulsion to settle, removing the supernatant, and adding fresh PBS with 1% BSA and 10 μg ml^{-1} heparin. The concentration of bFGF in the supernatant was assessed using an enzyme-linked immunosorbent assay (ELISA) (DY233, R&D Systems, Inc., Minneapolis, MN, USA). The emulsion was sized using a Coulter counter (Multisizer 3, Beckman Coulter Inc., Brea, CA, USA). Except for the bFGF release experiments, sham double emulsions were used, which did not contain bFGF in the W_1 phase. To assess the double emulsion structure, fluorescein sodium salt (Sigma–Aldrich) was dissolved in the W_1 phase. The resulting emulsion was diluted in PBS and mounted on a microscope slide in a cover-well imaging chamber (Electron Microscopy Sciences, Hatfield, PA). Confocal fluorescent images of the droplets were taken using an inverted SP5X microscope with a 63× objective (Leica, Wetzlar, Germany). For all experiments, the emulsion was used "as is" without any further purification or size separation.

2.2. Hydrogel fabrication

Fibrin gels and droplet–hydrogel composites with either 5 or 10 mg ml^{-1} clottable protein were prepared by combining bovine fibrinogen (Sigma–Aldrich) dissolved in Dulbecco's modified Eagle medium (DMEM), with bovine thrombin (2 U ml^{-1}, Thrombin-JMI, King Pharmaceuticals, Bristol, TN, USA), and 0%, 1% or 5% (v/v) of the double emulsion. All solutions besides the emulsion were degassed under vacuum prior to polymerization. Gels were allowed to polymerize for 30 min at room temperature prior to use. For cell culture and bFGF release studies, 0.5 ml gels (final dimensions: 16 mm diameter, 2 mm height) were cast in wells of 24-well culture plates (Fisher Scientific, Pittsburgh, PA, USA). For mechanical testing, 2.5 ml gels (final dimensions: 36 mm diameter, 2 mm height) were cast in six-well HT Bioflex plates (Flexcell International Co., Hillsborough, NC, USA). In some experiments the gels were doped with Alexa Fluor 647 (AF647)-fibrinogen (Invitrogen, Grand Island, NY, USA). A summary of the composite hydrogel formulations can be seen in Table 1.

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