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Controlled release of basic fibroblast growth factor for angiogenesis using acoustically-responsive scaffolds

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ABSTRACT

The clinical translation of pro-angiogenic growth factors for treatment of vascular disease has remained a challenge due to safety and efficacy concerns. Various approaches have been used to design spatiotemporally-controlled delivery systems for growth factors in order to recapitulate aspects of endogenous signaling and thus assist in translation. We have developed acoustically-responsive scaffolds (ARSs), which are fibrin scaffolds doped with a payload-containing, sonosensitive emulsion. Payload release can be controlled non-invasively and in an on-demand manner using focused, megahertz-range ultrasound (US). In this study, we investigate the *in vitro* and *in vivo* release from ARSs containing basic fibroblast growth factor (bFGF) encapsulated in monodispersed emulsions. Emulsions were generated in a two-step process utilizing a microfluidic device with a flow focusing geometry. At 2.5 MHz, controlled release of bFGF was observed for US pressures above 2.2 ± 0.2 MPa peak rarefactional pressure. Superthreshold US yielded a 12.6-fold increase in bFGF release *in vitro*. The bioactivity of the released bFGF was also characterized. When implanted subcutaneously in mice, ARSs exposed to superthreshold US displayed up to 3.3-fold and 1.7-fold greater perfusion and blood vessel density, respectively, than ARSs without US exposure. Scaffold degradation was not impacted by US. These results highlight the utility of ARSs in both basic and applied studies of therapeutic angiogenesis.
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1. Introduction

Exogenous, pro-angiogenic growth factors can stimulate blood vessel formation and restore perfusion in preclinical models of cardiovascular disease [1–5]. However, the clinical use of pro-angiogenic growth factors for treating conditions, such as coronary artery and peripheral artery diseases, has been disappointing [6–12]. Retrospective analysis of these studies revealed many potential shortcomings in the preclinical to clinical transition of therapeutic angiogenesis. One crucial reason is related to the presentation of the growth factor including variables such as the administration route, dose, and duration of treatment [13–15]. In human studies, basic fibroblast growth factor (bFGF) [6–8] or genes encoding for acidic FGF [16–18], vascular endothelial growth factor (VEGF) [9,10,19], or hepatocyte growth factor (HGF) [11,12] were administered using intravascular or intramuscular injections. Growth factors administered using these routes have short *in vivo* half-lives, slow tissue penetration, and the tendency to cause systemic side effects (e.g., nephrotoxicity, edema formation) [6,20].

The paradigm of acellular (i.e., inductive) tissue engineering has been to incorporate angiogenic growth factors within a hydrogel scaffold, which is then implanted at or adjacent to the site of intended vascularization. Growth factor release from the scaffold is dependent on factors such as the growth factor-scaffold affinity as well as the rates of enzymatic and cellular degradation of the scaffold [21]. This approach can extend the *in vivo* half life of the growth factor [22], localize its actions to the site of implantation [23], and promote cellular processes involved in angiogenesis [24]. Despite these advantages over bolus injections, conventional

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hydrogels do not enable spatiotemporal control of growth factor release. In contrast, endogenous growth factors are expressed in spatially- and temporally-regulated patterns during angiogenesis. Taking VEGF-A as an example, the spatial gradient of the growth factor impacts the directionality of blood vessel growth while differences in temporal gradients influence vessel density [25,26].

Several approaches have been used to impart spatiotemporally-controlled release from hydrogels. By altering material properties, temporally-controlled release (e.g., burst, sustained, or delayed) of bFGF, VEGF, and platelet derived growth factor (PDGF) has been achieved with collagen [27], alginate [28,29], and poly(lactide-co-glycolide) (PLG) [30] based scaffolds. Anisotropic (e.g., bi-layer) scaffolds composed of collagen or PLG enable spatially-controlled delivery of bFGF, VEGF, and PDGF [27,31]. By definition, however, these *a priori* approaches do not provide the ability to modulate the spatiotemporal gradients or released dose of growth factor once the scaffold is fabricated and implanted *in vivo*. This is potentially problematic when trying to personalize pro-angiogenic growth factor therapy based on the patient response during treatment. Thus, a scaffold where growth factor delivery can be actively modulated could facilitate the understanding of how variations in growth factor presentation impact angiogenesis.

We have developed fibrin-based hydrogels where the delivery of payloads such as growth factors can be controlled non-invasively and in an on-demand manner using focused ultrasound (US) [32,33]. These acoustically-responsive scaffolds (ARSs) contain a fibrin matrix doped with micron-sized, sonosensitive emulsions. Payloads, which are contained within the perfluorocarbon (PFC) emulsion, are released from the ARS through a non-thermal mechanism termed acoustic droplet vaporization (ADV) [34,35]. Megahertz-range US is used to generate ADV, which causes vaporization of the PFC phase within the emulsion and expulsion of the encapsulated payload [36,38,39].

Previously, we demonstrated that release from the ARS is a threshold-based phenomenon that is dependent on characteristics of the emulsion, scaffold, and US [39]. Additionally, we showed in an *in vivo* proof-of-concept study that US can modulate release of a surrogate payload (i.e., dextran) from an ARS [32,33]. In the current study, we focus on the *in vitro* and *in vivo* delivery of bFGF using ARSs (Fig. 1). bFGF-loaded emulsions were generated using a microfluidic device, which yielded monodispersed particles having more consistent release kinetics than the heterogeneous particles used in our prior work [32,33,39]. We characterized the *in vitro* release of bFGF from ARSs, including bioactivity of the released bFGF and we evaluated the angiogenic response of subcutaneously-implanted ARSs. Overall, as will be shown, ARSs yield a robust angiogenic response that is controlled by focused non-invasive US.

2. Materials and methods

2.1. Preparation and characterization of the double emulsion

Double emulsions with a water-in-PFC-in-water (W_1/PFC/W_2) structure were prepared as previously described [32,38]. Briefly, a triblock fluorosurfactant, consisting of Krytox 157FSH (CAS# 51798-33-5, DuPont, Wilmington, DE, USA) and polyethylene glycol (MW: 1000, CAS#: 24991-53-5, Alfa Aesar, Ward Hill, MA USA), was dissolved at 2% (w/w) in 1 g of perfluorohexane (PFH, C_6F_{14}, boiling point: 56 °C, CAS#: 355-42-0, Strem Chemicals, Newburyport, MA USA). The PFC solution was combined at 2:1 (v/v) with a W_1 phase containing 1 mg/mL basic fibroblast growth factor (bFGF, Cat#: GF003AF, EMD Millipore, Temecula, CA USA), 7.5 mg/mL bovine serum albumin (Sigma-Aldrich, St. Louis, MO USA), and 7.5 μg/mL heparin (Cat #: 375095, Calbiochem, San Diego, CA, USA) in phosphate-buffered saline (PBS, Life Technologies, Grand Island, NY USA). The PFC and W_1 phases were sonicated (Q55, QSonica, Newton, CT USA) for 30 s while on ice. The resulting primary emulsion, with a water-in-PFC (W_1/PFC) structure and mean diameter of approximately 2 μm [40], was pumped at 0.5 μL/min into the inner channel of a quartz microfluidic chip (Cat#: 3200146, junction: 14 × 17 μm, hydrophilic coating, Dolomite, Royston, United Kingdom) using a syringe pump (KDS-410, kd Scientific, Holliston, MA USA). Simultaneously, 50 mg/mL Pluronic F68 (CAS# 9003-11-6, Sigma-Aldrich) in PBS was pumped at 2.5 μL/min into the outer channels of the chip using a second syringe pump (78-0388, kd Scientific).

Blank or fluorescently-labeled emulsions were prepared as described above with either PBS or 0.1 mg/mL Alexa Fluor 488-labeled dextran (MW: 10 kDa, Life Technologies) as the W_1 phases, respectively. Emulsions were characterized with a Coulter Counter (Multisizer 4, Beckman Coulter, Brea, CA USA) in the range of 1–30 μm. To confirm emulsion morphology, the dextran-loaded emulsion was imaged using an inverted confocal microscope (SP5X, Leica Microsystems, Inc., Buffalo Grove, IL USA) at the University of Michigan Microscopy & Image Analysis Laboratory. The encapsulation efficiency of bFGF in the emulsion was determined by first allowing the emulsion to settle from the supernatant, which contained non-encapsulated bFGF. Next, an aliquot of the emulsion pellet was broken, as done previously [32], and the bFGF concentration was measured using an enzyme-linked immunosorbent assay (ELISA) (Cat#: DY233, R&D Systems, Minneapolis, MN USA). The encapsulation efficiency was determined by comparing the measured bFGF concentration with the theoretical concentration initially loaded into the emulsion. For all experiments, ARSs were prepared using emulsion pellet, which minimized the carryover of non-encapsulated bFGF.

2.2. Fabrication and characterization of the ARS

ARSs were prepared using 10 mg/mL clottable protein by first combining bovine fibrinogen (Sigma-Aldrich) dissolved in degassed (36% O_2 saturation) Dulbecco's modified Eagle's medium (DMEM, Life Technologies), with 10% (v/v) bovine thrombin (20 U/mL, Thrombin-JMI, King Pharmaceuticals, Bristol, TN, USA), 0.025 U/mL aprotinin (Sigma-Aldrich), and 1% (v/v) emulsion. ARSs used for *in vivo* experiments contained 0.125 mg/mL Alexa Fluor 647-labeled fibrinogen (Cat#: F35200, Molecular Probes, Eugene, OR USA) for non-invasive monitoring of fibrin degradation.

The ADV and inertial cavitation (IC) thresholds of the ARSs were determined using previously described methods [39]. Briefly, 0.5 mL ARSs were cast in 24-well Bioflex plates (Flexcell International, Burlington, NC, USA) by aliquoting the ARS mixture into each well and allowing it to polymerize for 30 min at room temperature. The ARSs were exposed to focused US generated by a calibrated, single-element transducer (2.5 MHz, H108, f-number = 0.83, focal length = 50 mm, Sonic Concepts, Inc., Bothell, WA USA) in the range of 0–8.0 MPa peak rarefactional pressure. The complete acoustic setup is described in Section 2.3. A calibrated hydrophone (HGL-0085, dynamic range = 1–50 MHz, Onda, Sunnyvale, CA USA) was placed 6 cm away from the focus of the transducer to detect backscattered acoustic signals generated in the ARS during the US exposure. The radiofrequency signals collected with the hydrophone and digitized by an oscilloscope (sampling rate = 100 MHz) were analyzed in MATLAB (The MathWorks, Natick, MA, USA) using the fast Fourier transform. The ADV threshold was determined by analyzing the fundamental frequency since bubbles formed in the ARS due to ADV significantly increase the scattered, fundamental signal [41,42]. The ADV threshold was defined as the lowest acoustic pressure at which the increase in fundamental signal was observed. The IC threshold was computed using the broadband

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