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● *Technical Note*

USE OF HYDROXYAPATITE DOPING TO ENHANCE RESPONSIVENESS OF HEAT-INDUCIBLE GENE SWITCHES TO FOCUSED ULTRASOUND

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Abstract—Recently, we demonstrated that ultrasound-based hyperthermia can activate cells containing a heat-activated and ligand-inducible gene switch in a spatio-temporally controlled manner. These engineered cells can be incorporated into hydrogel scaffolds (*e.g.,* fibrin) for *in vivo* implantation, where ultrasound can be used to non-invasively pattern transgene expression. Due to their high water content, the acoustic attenuation of fibrin scaffolds is low. Thus, long ultrasound exposures and high acoustic intensities are needed to generate sufficient hyperthermia for gene activation. Here, we demonstrate that the attenuation of fibrin scaffolds and the resulting hyperthermia achievable with ultrasound can be increased significantly by doping the fibrin with hydroxyapatite (HA) nanopowder. The attenuation of a 1% (w/v) fibrin scaffold with 5% (w/v) HA was similar to soft tissue. Transgene activation of cells harboring the gene switch occurred at lower acoustic intensities and shorter exposures when the cells were encapsulated in HA-doped fibrin scaffolds versus undoped scaffolds. Inclusion of HA in the fibrin scaffold did not affect the viability of the encapsulated cells. (E-mail: mfabiill@umich.edu) **© 2015 World Federation for Ultrasound in Medicine & Biology.**

Key Words: **Ultrasound, Hyperthermia, Hydrogel, Fibrin, Attenuation, Gene therapy, Hydroxyapatite.**

INTRODUCTION

Ultrasound (US)-induced thermal therapies can be broadly divided into two categories based on the thermal dose. In ablative therapy, focused US is used to increase the temperature of tissue above 55°C, thus causing irreversible tissue damage *via* coagulative necrosis. In contrast, US can generate hyperthermic conditions (*i.e.,* 40–45°C), which typically do not yield direct cytotoxicity. Rather, hyperthermia can be used in various ways that are beneficial in the treatment of solid tumors including (i) increasing vascular permeability, oxygenation and perfusion (Friedl et al. 2003; Song et al. 2005); (ii) enhancing the effects of radiation therapy (Franckena et al. 2009); (iii) increasing the cytotoxicity of chemotherapeutic agents (Urano et al. 1999); and (iv) modulating the release of chemotherapeutics from temperature-sensitive liposomes (Escoffre et al. 2013; Ranjan et al. 2012).

Hyperthermia can also impact the structure and function of proteins. In response to a thermal insult, heat shock proteins (HSPs) such as HSP70B are up-regulated and act as molecular chaperones, thus stabilizing protein structure and preventing loss of protein bioactivity. Within the field of gene therapy, HSP promoters can be integrated into genetic sequences, thus enabling control of transgene expression using hyperthermia. When incorporated into cells, genetic constructs containing these heat-activated "gene switches" can be stimulated by US conditions that generate hyperthermia (*i.e.,* 43–45°C; Deckers et al. 2009). Unlike hyperthermic water baths, US-induced hyperthermia can be generated with tight spatial and temporal control with minimal effects on tissues away from the focal volume, a property that aids in potential clinical translatability. Previous studies demonstrated that US-mediated hyperthermia could regulate gene expression after heat shock gene switches were injected intradermally (Smith et al. 2002; Wilson et al. 2014), intramuscularly (Lu et al. 2009; Xu

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et al. 2004), intrarenally (Eker et al. 2011) or uptaken by the liver (Plathow et al. 2005).

Critical to the successful development of gene therapy is the development of approaches for temporal and spatial control of transgene expression. For example, regenerative growth factors must be expressed in spatially and temporally restricted patterns during angiogenesis and osteogenesis. We recently demonstrated that hyperthermia generated using focused US could thermally activate cells containing heat-activated and ligand-inducible gene switches encoding firefly luciferase (*fLuc*), vascular endothelial growth factor (VEGF), or bone morphogenetic protein 2 (BMP2; Wilson et al. 2014). These engineered cells were incorporated into a hydrogel scaffold for *in vivo* implantation, which is a commonly used strategy with the field of regenerative medicine. Hydrogel scaffolds are typically highly porous and composed of biodegradable materials that act as an adhesive substrate for cells. Due to their high water content, the acoustic attenuation of hydrogel scaffolds is much lower than soft tissue or bone. Thus, long US exposures and high acoustic intensities are required to generate sufficient hyperthermia within the hydrogel. If the hydrogel scaffold containing the heat-shock-activated cells is implanted within or adjacent to soft tissue or bone, these tissues could preferentially heat. This minimizes spatial control of transgene activation while also potentially causing heat-related damage in adjacent tissue.

In this work, we demonstrate that the acoustic attenuation of a hydrogel scaffold (*i.e.,* fibrin) can be increased to the level of soft tissue by doping the scaffold with hydroxyapatite (HA; $Ca_{10}(PO_4)_6(OH)_2$) nanoparticles. HA is the mineral component of bone and aids in both angiogenesis and osteogenesis. HA has been previously incorporated into hydrogels to inhibit cell-mediated compaction, decrease cell migration and influence viral delivery (Kidd et al. 2012; Liu and Williams 2010). Fibrin was chosen as the hydrogel due to its high biocompatibility and extensive use in tissue engineering studies. These studies demonstrate that heat-shock-mediated transgene activation in HA-doped fibrin gels can occur at shorter US exposures and lower acoustic intensities than previously reported (Wilson et al. 2014).

MATERIALS AND METHODS

Acellular scaffold preparation

Fibrin scaffolds with 0.5%, 1%, or 2% w/v clottable protein were prepared by combining bovine fibrinogen (76% protein, 92% clottable protein; Sigma-Aldrich, St. Louis, MO, USA) dissolved in Dulbecco's modified Eagle medium (DMEM) with bovine thrombin (2 U/mL; Thrombin-JMI, King Pharmaceuticals, Bristol, TN, USA) and bovine lung aprotinin (0.1 U/mL; Sigma-Aldrich). HA-doped fibrin scaffolds were prepared by suspending HA nanopowder (<200 nm particle size, >97% purity; Sigma-Aldrich) in a 1:3 (v/v) solution of fetal bovine serum (FBS; Atlanta Biologicals, Flowery Branch, GA, USA) and DMEM. The mixture was sonicated on ice for 60 s using a sonicator (model 450, Branson, Danbury, CT, USA) to disperse the HA aggregates. The HA mixture was combined with bovine fibrinogen dissolved in DMEM, bovine lung aprotinin, and bovine thrombin such that the final concentrations of fibrinogen, aprotinin, and thrombin in the resulting scaffold were 1% (w/v) clottable protein, 0.05 U/mL, and 2 U/mL, respectively. All solutions were degassed under vacuum before polymerization.

For the undoped and HA-doped scaffolds, the fibrin mixture was allowed to polymerize inside custom sample molds made from polyvinyl chloride (PVC) pipe. Each mold consisted of a length of PVC pipe (inner diameter: 34 mm, height: ~10 mm or 20 mm) sealed on both ends with Tegaderm membrane (3M, St. Paul, MN, USA). The scaffolds polymerized for 30 min at room temperature before use.

Measurement of scaffold acoustic properties

The speed of sound and attenuation of the acellular scaffolds were measured using a broadband pulse technique in an experimental setup similar to Selfridge (1985). A single-element US transducer (3.5 MHz, diameter: 19.1 mm, f-number: 2; Olympus, Waltham, MA, USA) was positioned in a temperature controlled (*i.e.,* 24.5 ± 0.5°C), degassed water tank such that the focus was perpendicular to the surface of a brass reflector plate. Each scaffold composition was prepared in triplicate at two nominal thicknesses. The thickness of each scaffold was measured using digital calipers and the scaffolds were imaged with B-mode US (10L probe operating at 6.3 MHz; Logiq 9, GE Healthcare, Milwaukee, WI, USA). All scaffold samples were allowed to thermally equilibrate within the tank for at least 1 h before acoustic measurements. For each sample pair, the thinner sample was first carefully placed on the brass plate, such that the transducer position was not disturbed and the beam profile interrogated the scaffold. The transducer was excited using a pulser/receiver (5073 PR, Olympus) and the resulting reflection was digitized using an oscilloscope (HDO4934, Teledyne LeCroy, Chestnut Ridge, NY, USA). To account for insertion loss, the process was repeated using the thicker scaffold sample. The speed of sound and attenuation of olive oil, presented similarly to the scaffolds, was also measured as a control due to the availability of published values (Benedito et al. 2002; Chanamai and McClements 1998; Treeby et al. 2009).

The acquired radiofrequency (RF) waveforms were analyzed in MATLAB (Mathworks, Natick, MA, USA).