Ultrasound Stimulation Enhances Platelet-like Particle Mediated Matrix Deformation to Improve Healing Outcomes

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Statement of Purpose: Natural platelets perform a variety of functions within the context of wound healing,[1] including their ability to bind fibrin and promote clotting following injury. Over time, platelets contract the fibrin network to induce microscopic and bulk clot collapse through a process known as clot retraction.[2] Recently developed synthetic platelet-like particles (PLPs) are capable of mimicking this ability of natural platelets to induce fibrin network collapse.[3] Unlike native platelets that actively contract fibrin matrices via their actin/myosin machinery, PLP-induced clot retraction occurs via a Brownian wrench mechanism; therefore, the kinetics of this clot retraction event are much slower than native platelet-mediated clot retraction. We aim to enhance the kinetics of PLP-mediated clot retraction to improve their performance as pro-healing therapeutics. We hypothesized that PLP-mediated clot retraction could be combined with ultrasound application in order to actively stimulate PLP motion, thus enhancing PLP-induced clot retraction at lower PLP concentrations than those required in the absence of ultrasound. In these studies, we characterize 1) how applying ultrasound to clots *in vitro* affects clot polymerization dynamics, structure and mechanical properties and 2) the effect of PLPs in combination with ultrasound on wound healing responses *in vivo*.

Materials and Methods: Ultralow crosslinked (ULC) poly(N-isopropylacrylamide) (pNIPAM) microgels co-polymerized with acrylic acid (AAc) were synthesized using precipitation polymerization. PLPs were created by conjugating ULCs to a fibrin-specific IgG antibody through EDC/NHS. Ultrasound frequency and ULC concentration studies were performed to determine the optimal parameters for maximizing PLP motion within clots. PLPs were purified and embedded in clots at the experimentally determined optimal concentration of 0.025 mg/ml in *in vitro* fibrin clots produced with 2 mg/ml fibrinogen and 0.1 units/ml thrombin. Clots were exposed to ultrasound for 24 hours at the experimentally determined optimal frequency of 1 MHz. Clot stiffness before and after ultrasound application was evaluated using atomic force microscopy (AFM) and clot structure was evaluated using cryogenic scanning electron microscopy (cryoSEM). Full-thickness dermal wounds were excised from 8-week old C57BL/6 mice, treated with 0, 0.1, or 1.0 mg/ml PLPs, and exposed to either 0 or 30 minutes of ultrasound. Wound closure was imaged for 9 days post-injury, and histological markers of wound closure (epidermal thickness and angiogenesis) were evaluated in tissues collected 9 days post-injury using Hematoxylin & Eosin (H&E) staining and immunohistochemical labeling for CD31 positively-labeled areas, respectively.

Results: PLP concentrations chosen based on optimization studies of frequency vs. concentration are lower than concentrations typically used to induce retraction; the diminished impact of PLPs alone on the network structure as determined through DiameterJ analysis of cryoSEM images reflects the use of this atypically low PLP concentration. CryoSEM analysis revealed significantly increased density and network branching after 24 hours in PLP-laden clots exposed to ultrasound relative to PLP-laden clots left without ultrasound exposure (Figure 1A). Initial studies from wounds treated with 0.1 mg/ml and 1.0 mg/ml PLPs in combination with 30 minutes of ultrasound exposure show significantly increased epidermal thickness relative to controls, indicating improved histological healing markers in these wounds 9 days post-injury (Figure 1B).

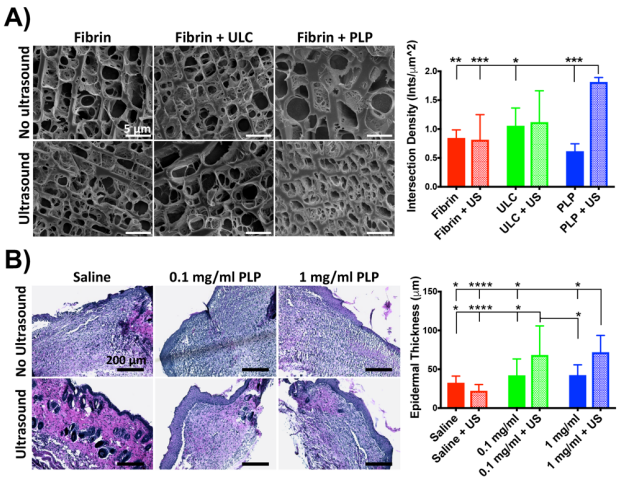

Figure 1: A) Fibrin clots containing PLPs or non-targeting ULCs were exposed to ultrasound for 24 hours, imaged using CryoSEM, and analyzed using DiameterJ in order to quantify clot structural properties. Clots exposed to a combination of PLPs and ultrasound display significantly increased density and branch points (n = 3/group). B) Wound tissue collected 9 days post-injury was stained using Hematoxylin & Eosin (H&E) in order to evaluate epidermal thickness. Wounds treated with 0.1 and 1.0 mg/ml PLPs in combination with ultrasound display increased epidermal thickness relative to controls (n = 3 slides/group). *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.

Conclusions: These results demonstrate that 1) PLPs in combination with ultrasound can enhance the overall degree of clot collapse *in vitro*, and 2) that these PLPs, in combination with ultrasound, can improve certain markers of *in vivo* wound healing in a murine full-thickness dermal injury model.

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References: **1.** Nandi, S. & Brown, A. C. Platelet-mimetic strategies for modulating the wound environment and inflammatory responses. *Exp. Biol. Med.* 241, 1138–1148 (2016). **2.** Weisel, J. W. & Litvinov, R. I. Mechanisms of fibrin polymerization and clinical implications. *Blood* 121, 1712–1719 (2013). **3.** Brown, A. C. *et al.* Ultrasoft microgels displaying emergent platelet-like behaviours. *Nat. Mater.* 13, 1108–1114 (2014).