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A Multi-Donor Ex Vivo Platelet Activation and Growth Factor Release Study Using Electric Pulses With Durations Up to 100 Microseconds

BOGDAN NECULAES[1], ALLEN L. GARNER[2,3,4], (Senior Member, IEEE), STEVE KLOPMAN[1], CHRISTINE A. MORTON[1], AND ANDREW S. TORRES[1,5]

[1]GE Research, Niskayuna, NY 12309, USA
[2]School of Nuclear Engineering, Purdue University, West Lafayette, IN 47906, USA
[3]School of Electrical and Computer Engineering, Purdue University, West Lafayette, IN 47907, USA
[4]Department of Agricultural and Biological Engineering, Purdue University, West Lafayette, IN 47907, USA
[5]Molecular Templates, Inc., Austin, TX 78729, USA

Corresponding authors: Bogdan Neculaes (bogdan.neculaes@ge.com) and Allen L. Garner (algarner@purdue.edu)

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ABSTRACT *In vitro* platelet activation is established in various clinical settings for wound healing and tissue regeneration. After separating platelet rich plasma (PRP) from whole blood, a biochemical activator, typically bovine thrombin (BT), is applied to activate PRP, which clots the PRP and releases growth factors beneficial for wound healing. BT's drawbacks, particularly cost, availability, workflow challenges, and potential immune responses, motivated the development of *ex vivo* electrical activation of PRP using pulsed electric fields (PEFs) with durations on the order of hundreds of nanoseconds and electric field strengths from ∼10-100 kV/cm. PEFs permeabilize platelets to facilitate Ca^{2+} transport into the cells to induce platelet activation; however, membrane permeabilization does not require PEFs of such short duration and high intensity. This study demonstrates that 5-100 μs duration PEFs effectively activate platelets. Treating PRP with ten different single pulse waveforms with durations between 5 and 100 μs induces similar or higher levels of platelet derived, vascular endothelial, and epidermal growth factor release compared to BT. These results indicate the potential clinical relevance of microsecond PEFs for platelet stimulation, which may reduce the expense and device footprint for PEF mediated platelet stimulation compared to nanosecond pulse generators, facilitating technology transition for clinical and trauma applications.

INDEX TERMS Bioelectric phenomena, biological processes, biological cells, biomedical equipment.

I. INTRODUCTION

Suboptimal wound healing represents a major threat to public health and an enormous financial burden to the healthcare system. Millions of patients in the United States present every year with difficult-to-heal wounds that result in more than $25 billion spent per year on chronic wound care [1]. The major causes of these chronic skin ulcers are pressure (decubitus ulcers), venous stasis disease, and diabetes mellitus. Approximately 19-34% of diabetes patients will suffer at some point from foot ulcers with approximately 20% of moderate or severe diabetic foot ulcers requiring amputation [2]. A significant fraction of diabetic ulcers remains refractory to almost every attempted wound healing therapy. These problems are likely to increase in the near future with the aging of the population and rising rates of comorbid conditions, especially obesity.

One emerging wound healing approach uses platelet rich plasma (PRP) [3], an autologous regenerative medicine approach that may dramatically improve patients' quality of life via enhanced wound healing outcomes [3]. PRP shows efficacy not only for skin wound management via topical application, but also for internal wound management, such as tendinopathy and lateral epicondylitis, where PRP injections are preferred. Tendinopathy prevalence in the adult population is approximately 2-3.8%, with higher impact for the elderly (5-7%) [4]. Certain professional categories, such as athletes, coal miners, and spine surgeons, exhibit dramatically higher prevalence of lateral epicondylitis compared to general population [4]. Tens of millions of patients in the USA suffering from these medical conditions may benefit from PRP injection as its use becomes one of the standards

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of care. PRP use has also grown dramatically worldwide for aesthetics applications, including skin rejuvenation and hair loss treatment. Tens of millions of men and women in the USA experience hair loss and PRP injections are showing promise to alleviate this condition [5].

By separating the patient's own platelets from whole blood, activating them *ex vivo*, and placing the resulting platelet gel/activated PRP onto a wound, this evolving therapeutic approach enhances the body's natural healing processes. Platelet gel consists of concentrated platelets that are activated to release different proteins from alpha granules. The proteins, mostly growth factors, released upon platelet activation include platelet-derived growth factor (PDGF), transforming growth factor-beta (TGF-β), vascular endothelial growth factor (VEGF) and epidermal growth factor (EGF). Activated platelets, or platelet gel, can enhance wound healing [6]–[8], induce hemostasis [9], and provide antibacterial protection for wounds as they heal [10]. Wound healing with activated platelets has been explored for diabetic foot ulcers [11], hair loss [5], [12], cardiac surgery [13], oral surgery [14], and plastic surgery [15].

Bovine thrombin (BT), the current state of the art for platelet activation in wound healing workflows, can cause significant side effects, including allergic reactions, immune response, and antibody formation [16], [17]. Platelet activation using pulsed electric fields (PEFs) has been proposed as an innovative, non-biochemical alternative to BT [18]–[20]. Earlier works [18], [21] considered nanosecond PEFs (nsPEFs) essential for triggering platelet activation. The shorter duration of these nsPEFs, on the order of hundreds of nanoseconds, generally target intracellular organelles, such as the intracellular Ca^{2+} stores [18] and alpha granules in platelets that contain the growth factors to be released during activation, while additionally creating many smaller pores that are sufficiently large to allow ions into the cell [22].

However, subsequent research demonstrated that longer duration PEFs, such as 5 μs, also successfully activated platelets *ex vivo* [20]. PEF-induced platelet activation may also be tuned by modifying the coupling mechanism for pulse delivery to PRP [23] and the extracellular calcium ion (Ca^{2+}) concentration. Delivering these PEFs using capacitive coupling (high voltage electrode not directly contacting the cell suspension) permitted the tuning of bioelectric effects for other blood cell types that play critical roles in wound healing, including fibroblasts, hematopoietic progenitor cells, and mesenchymal stem cells [23], with higher viability than conductive coupling (high voltage electrode directly contacting the cell suspension) despite inducing lower membrane potentials [24]. Moreover, tuning the $CaCl_2$ concentration with capacitive coupling controls when the PRP will clot, permitting slower clotting with the same clot strength as BT [25]. Although these results with nsPEFs and 5 μs PEFs are promising, these pulse generators are often expensive and require higher voltages than pulse generators delivering longer duration PEFs. Furthermore, these longer duration PEFs would be expected to induce similar effects. These practical demands and physical behavior motivate additional studies into PRP activation protocols using PEF durations beyond the nsPEFs initially utilized.

Historically, one commonly divides PEF conditions for biological manipulation into two regimes. Conventional electroporation typically applies PEFs from hundreds of V/cm to a few kV/cm with durations above hundreds of microseconds to sufficiently charge the cell membrane (typically to transmembrane potentials on the order of 250 mV) to induce the formation of membrane pores that enable molecular transport across the cell membrane [26], [27]. Subsequent experiments explored the application of PEFs with the same overall applied energy density over durations from 10 ns to 300 ns, resulting in applied electric fields of tens of kV/cm to hundreds of kV/cm [28]–[30]. Because these PEF durations tend to be shorter than the charging time of the outer cell membrane, they do not induce pores of the same size as conventional electroporation; however, they do induce a large density of small pores that are adequate for allowing the transfer of ions across the membrane [31]–[35]. These nsPEFs may also fully charge intracellular structures such as the mitochondria or intracellular calcium stores, to facilitate intracellular manipulation [24]–[26].

While multiple applications, including electrochemotherapy [36], microorganism inactivation [37], and irreversible electroporation [38], [39], require characterizing conventional electroporation parameters and several research projects have investigated the intracellular effects of nsPEFs [28]–[35], the regime of PEF durations from 1 to 100 μs, remains ''relatively unexplored'' – ''relatively few publications involve these electric field pulses'' [40], although more recent studies have considered these parameters for cancer treatment [41]. Because of the intermediate nature of these PEF durations, appropriate tuning of PEF duration in this regime may enable a combination of intracellular and membrane level effects [40]. Similar tunability of electric field targeting was previously noted for alternating current (AC) fields by controlling the frequency with lower frequencies fully charging (and thus targeting) the membrane, higher frequencies targeting intracellular structures, and intermediate frequencies interacting with both the outer and intracellular membranes [42]. Recent studies have also demonstrated the capability to tune Ca^{2+} transport into cells and the release of intracellular Ca^{2+} stores by controlling pulse durations up to 100 μs [43], [44]. The mechanistic insights of Ca^{2+} significance for platelet activation [18] and the importance of tuning Ca^{2+} concentration in controlling clotting time and clotting strength [25] suggest the feasibility of using microsecond duration PEFs in this ''relatively unexplored region'' to activate platelets.

Therefore, this paper demonstrates the feasibility of using microsecond PEFs to activate platelets to provide a less expensive, lower voltage PEF platelet activation protocol than nsPEFs. Specifically, we compare the growth factor release from the activation of PRP prepared from whole

TABLE 1. Cell counts determined by Sysmex Xe.

Parameter	Units	Whole Blood	PRP
White blood cell	[10^3/μL]	6.22±1.88	29.08±6.89
Red blood cell	[10^6/μL]	4.18±0.57	2.61±0.82
Hemoglobin	[g/dL]	12.28±1.30	7.40±2.10
Hematocrit	[%]	38.85±4.94	24.18±7.47
Mean cell volume	[fL]	92.98±1.59	92.93±1.51
Mean cell hemoglobin	[pg]	29.48±1.51	28.65±1.24
Mean cell hemoglobin concentration	[g/dL]	31.68±1.06	30.80±0.94
Platelet count	[10^3/μL]	249±103	851±12
Neutrophils	[10^3/μL]	3.96±1.73	18.54±5.87
Lymphocytes	[10^3/μL]	1.60±0.60	7.37±2.83
Monocytes	[10^3/μL]	0.50±0.16	2.35±0.58
Eosinophils	[10^3/μL]	0.14±0.07	0.69±0.37
Basophils	[10^3/μL]	0.03±0.02	0.13±0.04
% Neutrophils	%	0.62±0.11	0.63±0.09
% Lymphocytes	%	0.27±0.09	0.26±0.08

Values reported are the average of four separate donors with uncertainty reported as standard deviation. PRP = Platelet rich plasma.

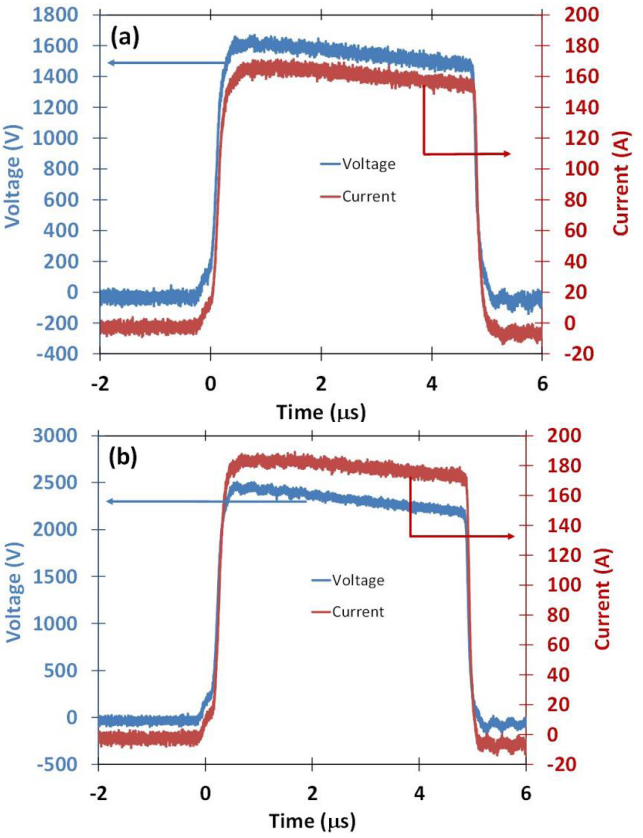

FIGURE 1. Representative voltage and current waveforms for electric pulses with ~5 μs pulse duration for conditions (a) E1 and (b) E2 across a 2 mm cuvette. The peak electric field is approximately (a) 8 kV/cm and (b) 12.5 kV/cm.

blood samples from four individual donors using BT or single PEFs with durations between 5 and 100 μs. Microsecond duration PEFs induced similar or higher levels of release of platelet derived (PDGF aa), vascular endothelial (VEGF), and epidermal (EGF) growth factors compared to BT, indicating the robust performance of microsecond duration PEFs for platelet activation. The impact of this work extends beyond elucidating the mechanisms of extended duration PEFs on platelet activation. Since it is easier and less expensive to build pulse generators that apply longer duration PEFs [45], future clinical instruments operating within this electrical parameter regime may facilitate the adoption of this PEF platelet activation protocol for various wound healing applications in the hospital and doctor's office.

II. MATERIALS AND METHODS

All whole blood and resulting samples from whole blood were handled using universal precautions. For each experiment, single units of human whole blood from four individual donors were purchased and shipped overnight at room temperature from a commercial vendor (Bioreclamation, Westbury, NY). All studies used acid citrate dextrose (ACD) as an anticoagulant. Table 1 reports the complete blood count (CBC) of these whole blood samples as the average and standard deviation of the four donors.

For a single preparation of PRP, 60 mL of whole blood was used from the unit received from Bioreclamation (Bioreclamation, Westbury, NY). The PRP was isolated using a SmartPReP2 APC +PRP preparation kit (Harvest Technologies, Belton TX). We recovered approximately 10 mL of PRP per 60 mL of whole blood for experiments (the PRP was maintained at room temperature for all tests). PRP preparation induced an approximately three-fold platelet enrichment compared to the original whole blood, as shown in Table 1. The biochemical reagents were prepared on the day of the experiment. Table 1 reports the CBC as the average and standard deviation of the PRP from the four donors prior to activation. We added BT (BioPharm Laboratories, Bluffdale, UT, catalog # 91-010) and $CaCl_2$ (Sigma Aldrich, St. Louis, MO) to achieve final concentrations of 1 U/μL and 10 mM after addition to PRP, respectively.

For the positive control samples, BT was added to 0.5 mL of PRP with $CaCl_2$ into 2 mm cuvettes (Molecular BioProducts/Thermo Scientific, Pittsburgh, PA, catalog #21-237-2). The samples were incubated at room temperature, and centrifuged at 10,000 rpm for 10 min. The supernatant was stored at -20 °C.

For the test samples, $CaCl_2$ was added to 0.5 mL of PRP in a 2 mm cuvette, treated with a single electric pulse, and incubated at room temperature. This study considered ten different waveforms with durations ranging from 5 to 100 μs for this single electric pulse. The electrically activated platelets were removed from the cuvette and centrifuged at the same speed as the control samples. Both test and control samples

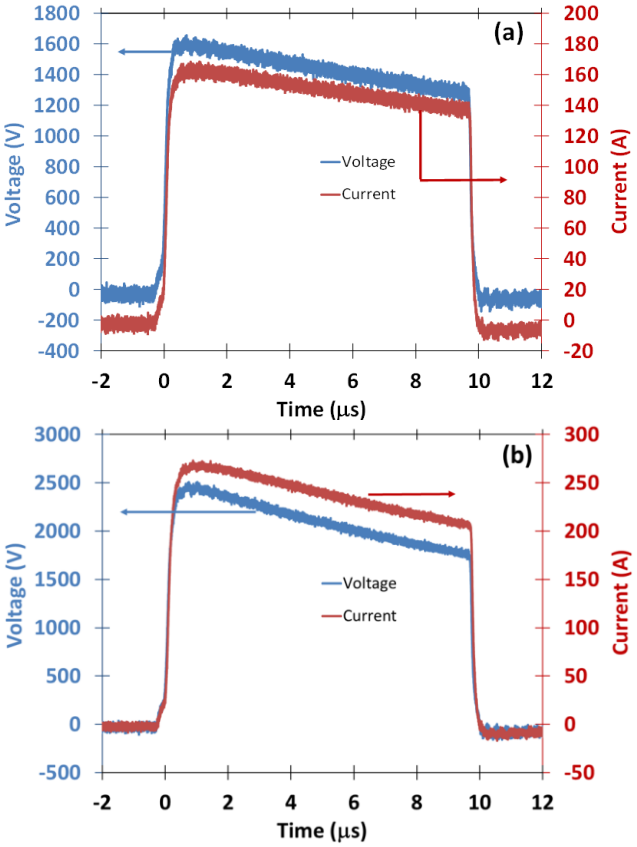

FIGURE 2. Representative voltage and current waveforms for electric pulses with ~10 μs pulse duration for conditions (a) E3 and (b) E4 across a 2 mm cuvette. The peak electric field is approximately (a) 8 kV/cm and (b) 12.5 kV/cm.

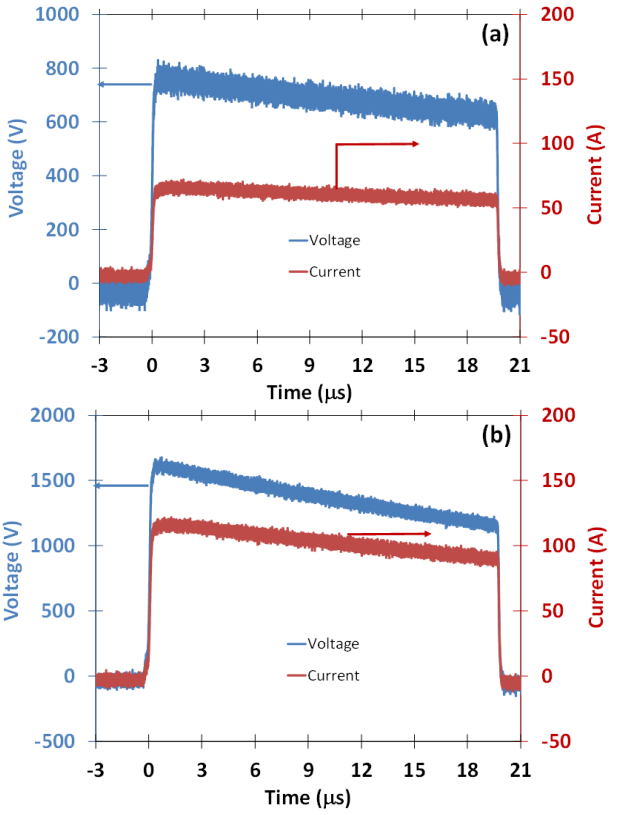

FIGURE 3. Representative voltage and current waveforms for electric pulses with ~20 μs pulse duration for conditions (a) E5 and (b) E6 across a 2 mm cuvette. The peak electric field is approximately (a) 5 kV/cm and (b) 8 kV/cm.

were incubated for 5 min prior to centrifugation. All samples underwent the same storage conditions. For comparison, we also considered PRP samples treated only with $CaCl_2$ without thrombin or PEFs.

PEF activation of PRP was performed using a specially designed instrument prototype (GE Research, Niskayuna, NY, USA), described elsewhere [45]. The output impedance of the pulse generator accounts for the specific electrical properties of PRP, which is typically more conductive than the buffers used in electroporation [45]. This custom instrument can deliver a wide range of pulse durations by modifying a Marx topology by controlling switch sequences and voltages into each capacitor stage. This device was designed to deliver electric pulses to both low conductivity buffers (e.g. standard pulsed power supplies used for electroporation) and higher conductivity solutions (e.g. blood and PRP). Details on electrical performance, including the effects of impedance mismatch and time-dependent electrical behavior, are provided elsewhere [45].

A Tektronix DPO4104 oscilloscope and a Tektronix P6015A high voltage probe were used to measure the voltage pulses applied to commercial 2 mm cuvettes filled with PRP for activation. The instrument delivers its output to two copper electrodes. The commercial electroporation cuvette is placed between these two electrodes – the two copper electrodes and the cuvette metallic walls are coupled via conductive coupling. The 2 mm cuvette filled with blood-like biological samples behaves like a resistor in parallel with a capacitor, with a resistance of approximately 10 Ω and a capacitance of approximately 1.5 μF, as measured experimentally [45]. Our instrument was designed for these type of loads.

We measured the current using a Pearson probe, model 110. Each experiment applied a single pulse from one of the ten waveforms labeled E1-E10, with E1 (Fig. 1a) and E2 (Fig. 1b) at a pulse duration of 5 μs; E3 (Fig. 2a) and E4 (Fig. 2b) at a pulse duration of 10 μs; E5 (Fig. 3a) and E6 (Fig. 3b) at a pulse duration of 20 μs; E7 (Fig. 4a) and E8 (Fig. 4b) at a pulse duration of 50 μs; and E9 (Fig. 5a) and E10 (Fig. 5b) at a pulse duration of 100 μs. Within certain limitations of the current and voltage probes, the currents and voltages measured are the ones applied to the cuvettes with PRP. We note that the impedance of the sample changes during the applied electric pulses (more noticeably in Figs. 3-5 than Figs. 1-2), which will ultimately change the transmembrane potential and the resulting electropermeabilization. The voltage decay observed in these experiments is caused by the very conductive nature of the load (PRP). Filling the cuvettes with much less conductive samples (low conductivity electroporation buffers) dramatically lowers the voltage decay [45].

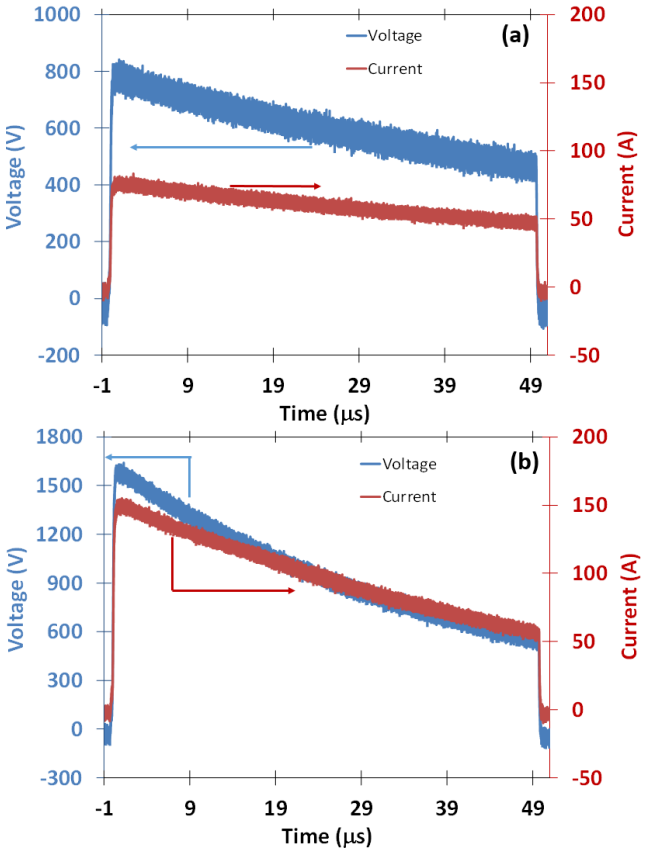

FIGURE 4. Representative voltage and current waveforms for electric pulses with ~50 μs pulse duration for conditions (a) E7 and (b) E8 across a 2 mm cuvette. The peak electric fields are approximately (a) 4 kV/cm and (b) 8 kV/cm.

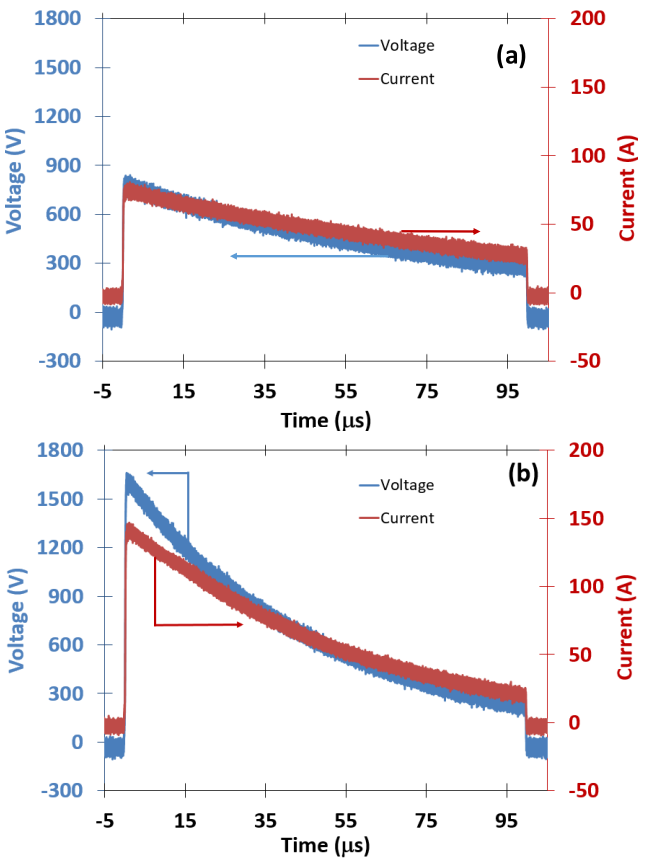

FIGURE 5. Representative voltage and current waveforms for electric pulses with ~100 μs pulse duration for conditions (a) E9 and (b) E10 across a 2 mm cuvette. The peak electric fields are (a) 4 kV/cm and (b) 8 kV/cm.

The pulse generator used to activate platelets provides flexibility in pulse amplitude, pulse duration and number of pulses [45]. We selected the pulse durations above to assess the efficacy of platelet activation for 5-100 μs duration PEFs.

We measured platelet derived growth factor (PDGF aa), epidermal growth factor (EGF), and vascular endothelial growth factor (VEGF) using the following commercial enzyme-linked immunosorbent assays (ELISAs) kits: Human/Mouse PDGF-AA Immunoassay (R&D Systems, Minneapolis, MN, catalog #DAA00B), Human EGF Immunoassay (R&D Systems, Minneapolis, MN, catalog #DEG00), and Human VEGF Immunoassay (R&D Systems, Minneapolis, MN, catalog # DVE00), respectively. The manufacturer's protocols were followed exactly for each assay without deviation. Each assay required a different sample dilution with PDGF aa samples diluted 10-fold, EGF samples 20-fold, and VEGF samples not diluted. Absorbance measurements were acquired with a standard microplate reader capable of measuring at 450 nm and a correction wavelength set at 540 nm. Standard curves for each assay were plotted for the mean absorbance values against the given standard concentrations (provided by the manufacturer). We calculated test sample concentration using these standard plots and correcting for the samples that diluted during preparation.

We normalized the results to the positive control, BT (in other words, the activation due to BT is always unity), since BT is utilized in clinical practice for platelet activation. Figure 6 and Table 2 highlight whether the PEF can generate a growth factor profile close to or higher than the clinical standard (BT). We report growth factor release as the average of these values for the four donors with error bars determined by standard deviation. We performed a two-tailed Student t-test to compare untreated PRP, $CaCl_2$, and each PEF condition to BT with statistical significance given by $p < 0.05$. Because we consider the results from four donors, we also report the results for each donor in Table 2 to demonstrate cases where a single spurious result may influence the statistical significance. We point out that the major goal of these studies is to demonstrate that microsecond PEFs induce similar growth factor release levels than BT, not that PEFs necessarily induce greater growth factor release than BT.

III. RESULTS

Growth factor release for PDGF aa, VEGF, and EGF was quantified as described above. Fig. 6 and Table 2 report the data for each growth factor as the fold difference of activation

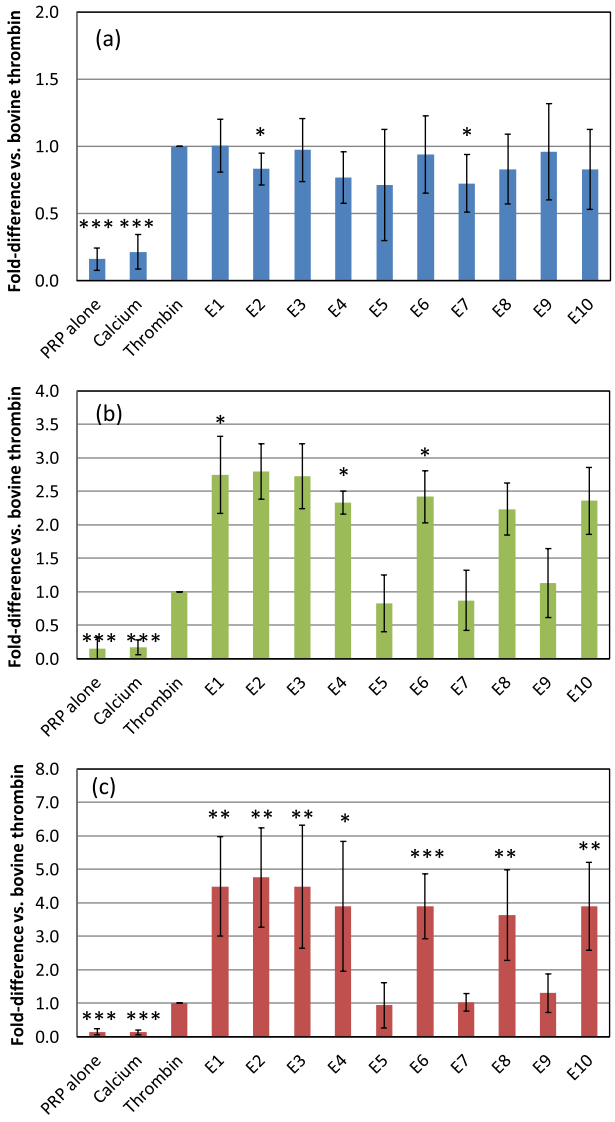

FIGURE 6. Growth factor release normalized to bovine thrombin (BT) mediated release for untreated platelet rich plasma (PRP), PRP exposed to ions with no pulsed electric field (PEF) treatment (Calcium), bovine thrombin (Thrombin), and PEFs E1-10 with typical waveforms shown in Figs. 1-5. (a) Platelet derived growth factor (PDGF-aa), (b) vascular endothelial growth factor (VEGF), and (c) epidermal growth factor (EGF). Data is reported as the average of three measurements from four donors with the error bars determined by standard deviation. Statistically significant differences with BT are denoted by * ($p < 0.05$), ** ($p < 0.01$), and *** ($p < 0.001$).

compared to the BT activation, including the growth factor release for the negative controls (termed "PRP"), PRP treated with $CaCl_2$ only (termed "Calcium"), positive control with BT activated PRP (termed "BT"), and the growth factor release for PEFs E1-E10 defined above. For PDGF aa, VEGF, and EGF, untreated PRP and $CaCl_2$ differed statistically from BT based on a two-tailed Student t-test ($p < 0.001$). All PEF conditions induce either statistically significant greater ($p < 0.05$) or the same level of PDGF aa, VEGF, and EGF than BT.

TABLE 2. Experimental Data for Growth Factor Release Normalized to Bovine Thrombin (BT) for Platelet Rich Plasma (PRP), Calcium, and Pulsed Electric Field Conditions E1-10.

Condition	PDGF-aa	VEGF	EGF
PRP	0.26	0.46	0.052
	0.14	0.11	0.097
	0.058	0.11	0.190
	0.18	0.13	0.24
Ca^{2+}	0.21	0.15	0.18
	0.38	0.40	0.04
	0.19	0.27	0.20
	0.072	0.20	0.09
BT	1.00	1.01	1.00
	1.00	1.00	1.00
	1.00	1.00	1.00
	1.00	1.00	1.00
E1	1.29	1.01	5.35
	0.97	2.35	3.03
	0.90	2.01	6.13
	0.86	1.52	3.45
E2	0.85	0.94	6.58
	0.98	1.92	3.10
	0.80	1.30	5.18
	0.70	1.55	4.17
E3	1.07	0.97	4.76
	1.13	1.98	2.54
	1.06	1.97	6.88
	0.62	1.43	3.74
E4	0.96	1.04	5.82
	0.83	1.33	1.25
	0.77	1.45	4.67
	0.51	1.36	3.86
E5	0.93	0.97	1.76
	1.18	1.78	1.14
	0.35	0.83	0.17
	0.38	1.07	0.70
E6	1.09	1.06	5.01
	1.12	2.02	2.68
	1.04	1.56	4.19
	0.51	1.53	3.72
E7	0.79	0.65	1.29
	1.00	1.68	0.80
	0.54	0.85	0.79
	0.56	0.93	1.19
E8	1.14	1.06	5.23
	0.95	1.92	2.10
	0.66	1.14	4.16
	0.57	1.26	3.05
E9	1.22	0.94	2.1
	1.31	2.10	1.29
	0.71	1.14	0.75
	0.59	1.22	1.06
E10	1.06	1.03	5.11
	1.09	2.17	2.79
	0.67	1.34	4.94
	0.49	1.26	2.71

IV. DISCUSSION

This study evaluated growth factor release for platelet activation via electric stimulation for PEFs with durations from 5 to 100 μs to develop a cost effective, external biochemical agent-free method for electrical platelet activation.

While initial work considered nsPEFs as essential for platelet activation [18], the experimental results reported here demonstrate the effectiveness of much longer duration PEFs for these workflows, potentially highlighting a cost effective PEF-based alternative to BT for platelet activation. This experiment used BT, the typical clinical activator used today in topical wound healing workflows, as a positive control. Clinicians may evaluate *in vitro* potency of PEF activation by comparing the growth factor release with that due to BT. We previously evaluated BT and platelet lysis in terms of growth factor release [49] and future work will include also freeze/thaw cycles in comparisons for growth factor release with BT and PEF. Nanosecond PEF induced growth factor release [49] directionally mirrors the experimental trends presented here.

Similar to a previous pilot study utilizing 5 μs PEFs for platelet activation [20], we observed that 5-100 μs PEFs of appropriate electric field intensity can induce similar or greater growth factor release than BT, making these PEFs reasonable choices for applications that typically use BT to induce growth factor release and platelet activation. These results are important for demonstrating the utility of microsecond PEFs in platelet activation and growth factor release protocols because previous *in vitro* studies showed that higher VEGF and EGF released by activation with PEFs [20] correlated with higher proliferation results in cell culture assays for human dermal fibroblasts and human dermal microvascular cells. These results highlight the potential for tuning growth factor release by adjusting PEF parameters, with potential clinical impact. VEGF regulates vasculogenesis and angiogenesis, and is a potent inducer of vascular permeability [46]. EGF stimulates the proliferation of various cell types, such as fibroblasts and epithelial cells [47]. Recombinant EGF, already a therapeutic product sold commercially, has demonstrated clinical promise, such as in treating diabetic foot ulcers [48].

Cell proliferation results for nsPEFs [19] and microsecond PEF platelet [20] activation are also promising. For example, a prior study [49] compared the proliferation of serum-starved human epithelial cells in response to the supernatant from PEF, BT, or TRAP (thrombin receptor activating peptide)-activated PRP to that observed with the supernatant of vehicle-treated PRP (i.e., platelet-poor plasma). Releasate from PEF-activated PRP, but not BT-activated or TRAP-activated PRP, significantly increased cell proliferation relative to the vehicle control. Furthermore, previous studies showed that PEFs did not release growth factors by mechanically fracturing the platelets and that PEFs induce a different platelet activation mechanism than BT, which mediates significant effects on the platelet cytoskeleton [50]. Of particular relevance, TEM showed that fibrinogen/fibrin fibers were associated with PEF-activated platelets, suggesting an effect on the adhesion receptor GPIIb-IIIA [50], which plays an important role in mediating platelet activation [51] due to its involvement in platelet Ca^{2+} channel activation [52].

Because nsPEFs induce intracellular Ca^{2+} release from intracellular calcium stores and nanopore formation that facilitates Ca^{2+} transport into the cell [53],[54] by electrophoresis or diffusion during or after the PEFs, respectively [55], it was initially hypothesized that nsPEFs were specifically required for platelet activation due to their unique ability to induce these phenomena [18]. The importance of Ca^{2+} transport into the cells was further illustrated by our recent study demonstrating that changing extracellular Ca^{2+} concentration during PEF-induced platelet activation could control clotting strength and time to clotting [25].

The present study demonstrates platelet activation in the ''relatively unexplored'' microsecond pulse duration region [40], indicating that nsPEFs are not necessary for platelet activation (see our previous research [19] and others in the bioelectrics community [18]) and that microsecond PEFs may be inducing changes in Ca^{2+} transport into platelets to trigger activation. In fact, recent studies have demonstrated that microsecond duration PEFs can also impact intracellular Ca^{2+} concentration [43], [44]. Reference [43] showed that both nanosecond and microsecond PEFs could increase intracellular concentration, with nsPEFs inducing Ca^{2+} release from the endoplasmic reticulum and microsecond PEFs causing the influx of extracellular Ca^{2+} through electropermeabilization; both mechanisms required extracellular Ca^{2+}, which also explains the importance of extracellular Ca^{2+} concentration in our earlier platelet activation study [25]. Reference [44] showed that applying a single 100 μs pulse induced cytosolic Ca^{2+} peaks in two different cell types without extracellular Ca^{2+}, indicating that the release must come from the endoplasmic reticulum. Thus, microsecond PEFs up to 100 μs effectively control intracellular Ca^{2+} concentration through both plasma membrane electropermeabilization and intracellular targeting, with our work here suggesting the potential for these long pulses to impact Ca^{2+} dynamics in platelets, if the activation mechanism is the same as previously proposed for nsPEFs [18]. Future research may further investigate the mechanisms for platelet activation for additional pulse durations and intensities, most notably field strength, duration, and number of PEFs, and also tuning Ca^{2+} concentrations to optimize PEF parameters and platelet activation. Also, a future topic of interest may be the ''electrical'' dose in platelet activation. An initial study observed that changing the number of identical PEFs did not change growth factor release from platelets [19]; however, a comprehensive study involving dose changes with pulse duration, electric field, and number of electric pulses remains incomplete and will ultimately be required for parameter optimization.

Our results demonstrate platelet activation with PEF durations up to 100 μs - the longest duration electric pulse used so far for *ex vivo* activation of PRP. This indicates that these PEFs most likely increase the necessary intracellular release and extracellular transport of calcium release for platelet activation that also occur for nsPEFs, which is consistent with the latest biophysical insights on microsecond duration PEF

interactions with cells [43], [44]. Practically, this observation is critical for commercialization and technology transition to the clinic since microsecond duration pulse generators are less complicated technologically to construct, less expensive, and require lower voltages. For instance, a typical commercial BTX MicroPulser Electroporator costs ∼$2600, while nanosecond pulse generators capable of generating sufficient voltage to induce similar effects would cost on the order of five to ten times more depending on parameter flexibility, voltage, and repetition rate requirements. The requirement of lower voltages for microsecond PEFs is especially important for transitioning this technology to the clinic since the high voltages required for nsPEF systems (often at least 10 kV) at least require extra scrutiny from safety personnel and may require special electrical power connections not required typically for hospital electronics. In this study, we have achieved platelet activation for applied voltages as low as 800 V (cf. Fig. 3a, 4a and 5a), corresponding to an electric field of 4 kV/cm; prior studies required 10 kV/cm at shorter pulse durations to achieve platelet activation [56]. Future work will investigate potential electrochemical reactions between the electrodes and PRP, especially at very long pulse widths. Additionally, a comprehensive study on clotting time as a function of these microsecond pulse durations, much as done previously for examining the behavior of clotting time and clotting strength as a function of $CaCl_2$ concentration [25], will provide additional valuable information concerning the applicability and tunability of this technique for internal applications.

This suggests that further *in vivo* studies of microsecond PEFs are the next step for developing a flexible, cost-effective, and safe PRP-activation protocol. Initial *in vivo* wound healing studies using nsPEF activated PRP have demonstrated positive results [57], [58]. BT activated PRP and nsPEF activated PRP both inactivated *A. aumannii*, *P. aeruginosa*, and *S. aureus* in broth dilution assays, *ex vivo* assays of swine skin acquired post-mortem, and *in vivo* assays of male Sprague Dawley rats [57]. Another study showed that nsPEF activated PRP induced more endothelial cells, collagen, and cells containing VEGF in ischemic hindlimb wounds in New Zealand White rabbits compared to saline-treatment [58]. Reperfusion of blood in large skin flap wounds in these rabbits was also higher due to nsPEF PRP treatment than saline [58]. The results presented here and elsewhere [23], [25] suggest the potential for tuning activated PRP growth factor profile and time to clot by leveraging the electrical pulse coupling method (capacitive or conductive), Ca^{2+} concentration, and/or PEF duration. This could ultimately provide a cost-effective protocol for activating platelets without requiring external biochemical agents, such as BT. It should be noted that recent research proposed *ex vivo* electrical activation of platelets beyond autologous wound healing applications, specifically for cell culture media production [59]. We are currently in the process of selecting a specific wound healing protocol with electrically activated PRP for testing in a human pilot trial.

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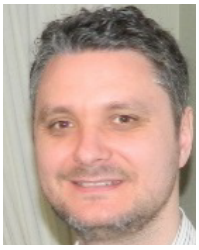

BOGDAN NECULAES received the B.S. and M.S. degrees in physics from the Alexandru Ioan Cuza University of Iasi, Romania, and the M.S. and Ph.D. degrees in nuclear engineering and radiological sciences from the University of Michigan. He is currently a Principal Scientist and the Business Portfolio Leader at GE Research, Niskayuna, NY, USA, and a Scientific Advisor at Santiste Medical, a regenerative medicine company. He led one of the largest X-ray source programs at GE Research in the recent decades that delivered, with funding from GE and NIH, the first in the world distributed X-ray source for Inverse Geometry Computed Tomography. He has been leading the first successful X-ray source demonstrations with Carbon Nano Tube cold cathodes at GE. He has initiated and chaired the Bio-Electromagnetics Initiative at GE Global Research, a multidisciplinary research platform focused on creating novel electromagnetic means for manipulation of biological samples. His teams demonstrated new means for cell permeabilization using infrared lasers to enable exogenous molecule delivery, novel gene delivery methods *in vitro* using magnetic nanoparticles and magnetic fields, and innovative *ex vivo* platelet activation techniques with pulse electric fields for autologous wound healing applications. He has published numerous peer-reviewed articles in prestigious peer-reviewed journals while being awarded 23 patents. His current research interests include physics-based applications in medical imaging, bioelectrics, biomedical devices, and additive manufacturing for medical implants.

ALLEN L. GARNER (Senior Member, IEEE) received the B.S. degree (Hons.) in nuclear engineering from the University of Illinois at Urbana–Champaign, in 1996, the M.S.E. and Ph.D. degrees in nuclear engineering from the University of Michigan, Ann Arbor, in 1997 and 2006, respectively, and the M.S. degree in electrical engineering from Old Dominion University, Norfolk, VA, USA, in 2003.

He was an active duty Naval Officer from December 1997 to December 2003, serving onboard the USS Pasadena (SSN 752) and an Instructor of the Prospective Nuclear Engineering Officer course at Submarine Training Facility. From 2006 to 2012, he was an Electromagnetic Physicist at GE Global Research Center, Niskayuna, NY, USA. In August 2012, he joined the School of Nuclear Engineering, Purdue University, West Lafayette, IN, where he is currently an Associate Professor and the Undergraduate Program Chair. He is currently a Captain with the Navy Reserves assigned as the Commanding Officer of the ONR S&T 102 unit in Washington, DC, USA. His research interests include electron emission, gas breakdown, high-power microwaves, and biomedical applications of pulsed power and plasmas.

Prof.Prof. Garner is a member of the IEEE International Power Modulator and High Voltage Conference (IPMHVC) Executive Committee and the Dielectrics and Electrical Insulation Society Administrative Committee. He received the 2016 IEEE Nuclear and Plasma Sciences Early Achievement Award, two Meritorious Service Medals, a Navy and Marine Corps Commendation Medal, and five Navy and Marine Corps Achievement Medals. He served as the Techncial Program Chair for the 2016 IEEE IPMHVC and the Treasurer for the 2018 IEEE IPMHVC. He is a licensed Professional Engineer in Michigan.

STEVE KLOPMAN received the Associate's degree in electrical engineering technology. He has been working at GE Global Research as an Electronics Technician for 11 years. Most of his projects cover X-ray-based medical imaging, MRI, and bioelectrics. He has also contributed to projects for aviation and oil and gas applications. His current research interests include electronic hardware design, building, and testing.

CHRISTINE A. MORTON received the B.A. degree in biology from The Sage Colleges-Russell Sage College, Troy, NY, USA, in 1990. From 1990 to 2004, she was a Research Associate at the Albany Medical Center, Albany, NY, USA. From 2004 to 2010, she was with the Department of Biological Sciences, Rennselaer Polytechnic Institute. She joined GE Research, Niskayuna, NY, USA, in 2010, where she is currently a Lead Scientist.

ANDREW S. TORRES received the B.A. degree in music from Trinity University, San Antonio, TX, USA, in 1994, and the M.S. and Ph.D. degrees in chemistry from the Northwestern University, Evanston, IL, USA, in 1998 and 2002, respectively.

He was a Lead Scientist and a Senior Scientist at the GE Global Research Center, Niskayuna, NY, USA, from 2002 to 2010 and from 2010 to 2015, respectively, and the Technology Development Manager at GE Ventures, from 2015 to 2016. In January, 2016, he was the Manager of the Process Sciences Operations and Logistics Group, Regeneron Pharmaceuticals, where he was promoted to Senior Manager and Group Lead, in November 2017. In April 2019, he joined Molecular Templates, Austin, TX, USA, where he is currently a Senior Manager.