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Applicability and safety of dual-frequency ultrasonic treatment for the transdermal delivery of drugs

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ABSTRACT

Low-frequency ultrasound presents an attractive method for transdermal drug delivery. The controlled, yet non-specific nature of enhancement broadens the range of therapeutics that can be delivered, while minimizing necessary reformulation efforts for differing compounds. Long and inconsistent treatment times, however, have partially limited the attractiveness of this method. Building on recent advances made in this area, the simultaneous use of low- and high-frequency ultrasound is explored in a physiologically relevant experimental setup to enable the translation of this treatment to testing in vivo. Dual-frequency ultrasound, utilizing 20 kHz and 1 MHz wavelengths simultaneously, was found to significantly enhance the size of localized transport regions (LTRs) in both *in vitro* and *in vivo* models while decreasing the necessary treatment time compared to 20 kHz alone. Additionally, LTRs generated by treatment with 20 kHz + 1 MHz were found to be more permeable than those generated with 20 kHz alone. This was further corroborated with pore-size estimates utilizing hindered-transport theory, in which the pores in skin treated with 20 kHz + 1 MHz were calculated to be significantly larger than the pores in skin treated with 20 kHz alone. This demonstrates for the first time that LTRs generated with 20 kHz + 1 MHz are also more permeable than those generated with 20 kHz alone, which could broaden the range of therapeutics and doses administered transdermally. With regard to safety, treatment with 20 kHz + 1 MHz both *in vitro* and *in vivo* appeared to result in no greater skin disruption than that observed in skin treated with 20 kHz alone, an FDA-approved modality. This study demonstrates that dual-frequency ultrasound is more efficient and effective than single-frequency ultrasound and is well-tolerated *in vivo*.

1. Introduction

Non-invasive transdermal drug delivery (TDD) presents an attractive method for drug administration [1,2]. In addition to the potential benefits of patient compliance associated with painless drug delivery, TDD can reduce first-pass degradation of drugs typically associated with the oral route and enables the delivery of larger molecules limited to subcutaneous injection [1,2]. Despite countless experimental investigations surrounding this route, its use clinically is largely limited to the delivery of small molecules, such as nicotine and estradiol [3]. This is due in large part to the barrier posed by the outermost layer of the skin, the *stratum corneum* (SC). While there exist several methods to overcome or permeabilize this membrane, each method has associated limitations [4]. Treatment of skin with ultrasound (US) (also known as sonophoresis), for example, has the potential to permeabilize relatively large areas, but typically requires large, bulky equipment and a power source [5]. Recently, there has been renewed research interest in sonophoresis. Two major challenges limiting greater clinical use is the portability of the equipment required and the length of the treatment required [6,7]. Studies addressing the former hurdle have focused on the use of low-profile cymbal transducer arrays that can be integrated into patches [8,9]. In addition to portability, these devices minimize the excitation voltages required, reducing power consumption [10].

With regard to the treatment times necessary, a new approach has recently been investigated to increase permeabilization efficiency, thereby decreasing the required treatment time [11]. This method employs the use of two US frequencies simultaneously. A proof-of-concept study demonstrated that the use of both low- (<60 kHz) and high-frequency (>1 MHz) US together resulted in greater transient cavitation events, as assessed by pitting data, and resulted in larger localized transport regions (LTRs) *in vitro* [11].

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While this initial study demonstrated that the simultaneous application of low- and high-frequency US could enhance cavitational activity, the experimental setup required submerging tissue in a tank to allow for both frequencies to be applied simultaneously. This setup could result in artificial enhancement in skin permeability due to the underside of the dermatomed skin layer (epidermis) being exposed to surfactant present in the coupling solution. Additionally, there has never been a mechanistic exploration of the permeability of the resulting LTRs or the safety and tolerability of this new method. Here, we build on this preliminary report of the use of dual-frequency US to first develop an experimental setup which exposes only the top surface of the skin to the coupling solution, while allowing for the simultaneous application of both 20 kHz and 1 MHz US. We then use this setup to explore LTR formation, investigate the mechanistic underpinnings of this modality, and quantify the resulting permeability of treated skin in vitro over a range of relevant treatment times. We also examine treated skin histologically to determine the level of barrier disruption as a result of the US treatment. Finally, we utilize this setup to allow for treatment *in vivo* in pigs to examine LTR formation and tolerability.

2. Experimental section

2.1. Materials

All chemicals were used as received. Sodium lauryl sulfate (SLS), 4 kDa FITC-labeled dextran, and phosphate buffered saline (PBS) were obtained from Sigma-Aldrich Company (St. Louis, MO). Lysine-fixable 3, 10, and 70 kDa dextrans labeled with Texas Red were purchased from Invitrogen (Carlsbad, CA). Allura red was obtained from TCI America (Portland, OR).

2.2. Preparation of skin samples and ultrasonic treatment

All procedures were approved by the Massachusetts Institute of Technology Committee on Animal Care. Porcine skin was used because of its physiological similarity to human skin [12]. Skin was procured by Research 87 (Boylston, MA). The preparation and storage of skin samples followed previously published protocols [13]. Briefly, back and flank skin was dissected from female Yorkshire pigs within an hour of euthanization. This skin was sectioned into 25-mm wide strips and the subcutaneous fat was removed using a razor blade. The skin was then stored at −85 °C for up to six months. Prior to use in experiments, skin was thawed for 20 min in PBS and the hair was trimmed using surgical scissors. The skin was then dermatomed to 700 μm using an electric reciprocating dermatome (Zimmer Orthopedic Surgical Products, Dover, Ohio), and cut into 25 × 25-mm samples. The skin samples were then mounted in a custom diffusion cell to enable simultaneous sonication of the skin with both 20 kHz and 1 MHz US. Specifically, high vacuum grease (Dow Corning, Midland, MI) was applied to the flange of the custom-built top (FineLine Prototyping, Raleigh, NC) and 15-mm inner-diameter diffusion cell receiver chamber (PermeGear, Hellertown, PA). The skin was applied to the custom-designed top and then sandwiched between the top and receiver chamber. The top and bottom chambers were then clamped together and the receiver chamber was filled with PBS (12 mL) and a small volume of PBS was added to the custom-designed top to keep the skin hydrated until treatment.

Skin samples were treated in a similar fashion to previously reported methods [11,14,15]. Specifically, a 20 kHz horn (Sonics and Materials, Inc. Model VCX 500, Newtown, CT) and a 1 MHz horn (Therasound 3 Series, Richmar Corporation, Chattanooga, TN) were employed. The 20 kHz US horn was positioned 3 mm above the skin surface and operated at an intensity of 8 W/cm^2 (by calorimetry) and a 50% duty cycle (1 s on, 1 s off). The 1 MHz horn was placed on the side of the 20 kHz horn, approximately 5 cm from the leading edge of the skin. The 1 MHz horn was programmed to operate at a power of 2.0 W/cm^2 continuously. The custom diffusion top was filled with 300 mL of a solution of 1 wt.% SLS and 0.04 wt.% Allura red dye in PBS. The treatment times tested ranged between 4 and 8 min, which are within the range of commonly-tested treatment times in the literature [16,17]. Thermal effects were determined to be negligible as the temperature increase of the coupling solution was less than 2 °C even at the longest treatment time considered. Prior work has shown that an increase of at least 10 °C is necessary to appreciably permeabilize the skin [18]. Treatments utilizing 20 kHz US alone were used as a control throughout the study. The use of 1 MHz US alone was not tested because it has been established in the literature that high-frequency US (≥1 MHz) at typical intensities, such as those used here, do not generate transient cavitation [1]. As a result, there would not be any quantifiable LTR formation and the resulting flux of 4 kDa dextran would not be significantly different than that observed in native skin [18].

2.3. Skin resistivity measurements

Electrical resistivity has previously been shown to be an accurate measure of skin perturbation [15,19]. The resistivity across skin samples was determined using a method similar to previously published protocols [17,20]. Specifically, a 10 Hz sinusoidal wave with root-mean-square voltage of 100 mV was employed (Hewlett Packard Model 33120A, Palo Alto, CA). This signal was then applied across the skin using two Ag/AgCl electrodes (In Vivo Metric, Healdsburg, CA). The resulting current was measured using a multimeter (Fluke, Model 87 V, Everett, WA) and the resistance calculated using Ohm's Law. Finally, the resistivity was found by multiplying the resistance by the area of the skin exposed (the diffusion cells have an exposure area of 1.76 cm^2). In all measurements, the background resistance (resistance of PBS in the diffusion cell without skin) was accounted for by subtracting it from the resistance observed when the skin was mounted in the diffusion cell. All skin samples were ensured to have a starting resistivity of at least 35 k Ω·cm^2, otherwise, the skin sample was considered damaged and discarded [19].

2.4. Quantification of LTR area

Immediately after the US treatment, the coupling solution was discarded and the diffusion top and skin were washed thoroughly with PBS to remove any remaining coupling solution. The diffusion cell was then disassembled, the skin removed and placed on a paper towel, and blotted dry to prepare it for imaging. The skin was imaged using a digital camera (Panasonic DMC-ZS7, 12.1 megapixels, shutter speed 1/2000 s) positioned approximately 10 cm above the skin. The resulting image was then cropped making sure that only the portion of the skin exposed to US was captured (1.5 cm × 1.5 cm) and processed using ImageJ 1.46r (National Institutes of Health, Bethesda, MD). Specifically, the blue channel of the LTR image was isolated because it has been shown to give the best contrast between LTRs and non-LTRs [21]. The image was then inverted, and the average pixel intensity of non-LTRs was measured. This pixel intensity was subtracted from the entire image to account for variations in lighting at the time of imaging. The image was then re-inverted. The threshold was adjusted using the default value found by ImageJ. Finally, the LTR area was quantified using the "Analyze Particles" function.

2.5. Steady-state dextran permeability

Once the skin samples were imaged to quantify LTR size, they were remounted in clean diffusion cells using standard glass tops (PermeGear, Hellertown, PA), and filled with PBS. A magnetic stir bar was added to the receiver chamber. The donor chambers were filled with 1.5 mL of 0.1 wt.% 4 kDa dextran in PBS. The receiver chambers were magnetically stirred at 500 rpm. To ensure that the lag-phase was overcome, the receiver chambers were sampled between hours 20 and 48. For each sample, a 200 μL aliquot was taken and replaced