ORIGINAL MANUSCRIPT

Laser-induced transient skin disruption to enhance cutaneous drug delivery

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Keywords: Assisted delivery; Confined ablation; Diclofenac; DiD; FITC; Lidocaine; Resonant amplitude waves; Steel.

Abbreviations:

ACN: acetonitrile; CE: chemical enhancers; CM: commercial formulation; DIC: sodium diclofenac; DiD: 1,1'-dioctadecyl-3,3,3',3'-tetramethylindodicarbocyanine perchlorate; EMLA: eutectic mixture of local anesthetic; Er:YAG: erbium-doped yttrium aluminum garnet; EtOH: ethanol; FESEM: field emission scanning electron microscopy; FITC: 5-Fluoresceinisothiocyanate; HLB: hydrophilic–lipophilic balance; HPMC: hydroxypropyl methylcellulose; LID: lidocaine; LOD: lower limit of detection; LOQ: lower limit of quantification; MeOH: methanol; NaCl: sodium chloride; MW: molecular weight; Nd:YAG: neodymium-doped yttrium aluminum garnet; PBS: phosphate-buffered saline; PS: polystyrene; PW: pressure waves; RAW: resonant amplitude waves; SC: *stratum corneum*; SLS: sodium lauryl sulfate; TFA: trifluoroacetic acid.

ABSTRACT

The use of pressure waves (PW) to disrupt the *stratum corneum* (SC) temporarily is an effective strategy to increase the deposition of drug molecules into the skin. However, given the rather modest outcomes when compared with ablation-assisted drug delivery, its potential has been underestimated. Accordingly, the aim of this study was to examine the impact of Resonant Amplitude Waves (RAWs) on increasing cutaneous delivery. RAW phenomena are triggered by focusing a high-peak-power pulsed laser onto an appropriate transducer structure, under space- and time-controlled resolution.
In order to determine the optimal conditions for the generation and use of RAWs, a screening of laser parameters setting and an analysis of different geometries of the impact pattern over diverse materials used as transducers was performed, analyzing the footprint of the RAW waves in an agarose gel. The results obtained were then checked and fine-tuned using human skin samples instead of agarose. Furthermore, *ex vivo* experiments were carried out to characterize the effect of the RAWs in the cutaneous delivery of diclofenac (DIC) and lidocaine (LID) administered in the form of gels. The application of RAWs resulted in an increased delivery of DIC and LID to the skin, whose intensity was dependent on the composition of the formulation. In fact, the maximum observed for DIC and LID in short-time experiments (39.1±11.1 and 153±16 μg/cm^2, respectively) was comparable to those observed using ablation-assisted drug delivery under the same conditions.
In conclusion, the combination of RAWs with specific formulation strategies is a feasible alternative for the cutaneous delivery of drug candidates when short onset of action is required.

1. INTRODUCTION

The use of medical lasers is a valuable strategy to increase cutaneous delivery of drugs [1]. Since the first demonstration of a working laser device by Theodore Maiman *et al.* in 1960 [2], lasers have evolved at a very fast pace and have enabled many applications in a diversity of fields, ranging from industrial production to health care [3]. The latter include, in particular, their wide use in aesthetics and in clinics [4, 5]. Moreover, concerning drug delivery, there are presently two major laser-based approaches used to enhance the delivery of drugs across the skin: skin ablation and transient skin disruption. Ablation-assisted drug delivery has proven to be an effective strategy for the enhanced transcutaneous delivery of small molecules, macromolecules and biopharmaceuticals [6-9]. Nevertheless, it has not progressed as expected to the clinic, probably due to its invasiveness and aggressiveness [10].

On the other hand, disruption-assisted drug delivery mechanisms are based on the generation of transient pores by expanding the lacunar spaces within the *stratum corneum* (SC) lipid bilayers without inflicting ablation to the skin. To this end, a transducer material is irradiated with the laser and a Pressure Wave (PW) is propagated into the skin. Briefly, the corneocytes which are in contact with the transducer material expand, resulting in an area of low relative pressure (i.e. rarefaction). As a consequence of the expansion, the surrounding corneocytes are compressed, thus leading to areas with high relative pressure (i.e. compression). The intermittency between rarefactions and compressions propagate along the skin and, as a result, the organization of the lipid matrix is temporarily disrupted [11].

An early approach has relied on the PW generation by thermoelastic expansion, that is the generation of a PW in the transducer as a result of the conversion of light-energy to mechanical-energy. Surprisingly, preclinical research has only been conducted with macromolecules, usually reporting an increase on the deposition up to 50 μm depth in the skin [12-15]. An alternative mechanism for PW generation is to induce laser ablation on the transducer's surface, accompanied by the corresponding generation of PWs on its opposite face and, consequently, on the skin. The impact of this strategy on the cutaneous delivery of drugs has been evaluated both in small molecules (e.g. morphine [16] or FITC [17]) and macromolecules (e.g. 1429 Da peptide [18] or 9266 Da siRNA [17]). However, either because of the variable findings observed (between lack of statistical differences to ~14-fold maximum transdermal flux increase) or because of the lower efficiency when compared with direct skin ablation (e.g. ~4.8-fold and 23-fold transdermal flux increase of a 2190 MW peptide; PW and skin ablation, respectively [18]) this strategy has received significantly less attention than it deserves. Moreover, the aim of the PWs generated in these pioneering studies was to explore the mechanisms of indirect skin ablation enhancement, that is, to exclude the direct SC laser ablation effect. Its conclusions could therefore underestimate the importance of the irradiated material response, as well as the role that laser parameters (wavelength, pulse width, intensity, pulse repetition rate and spatial distribution on the target) play, thus masking the full potential that this technique has to offer [15, 19].

The present work's approach entailed the generation of Resonant Amplitude Waves (RAW) via laser irradiation of selected transducer targets, as an alternative to direct skin ablation, for short-time cutaneous delivery without damaging the skin. Within this context, the specific objectives of this work were (i) to conduct a comprehensive study on the generation of RAWs through transducer irradiation, (ii) to assess the safety of the process and (iii) to evaluate its efficiency in enhancing the cutaneous delivery of commonly used drugs in dermatology.

2. MATERIALS AND METHODS

2.1. Materials

Silver (99% purity; 25µm thick) and aluminum (99% purity; 25µm thick) were obtained from Goodfellow Cambridge Ltd. (England). Steel (25µm thick) was purchased from H+S Präzisionfolien GmbH (Germany) and black polystyrene (PS; 1mm thick) from MW-Materials World (Spain). Agarose, sodium chloride (NaCl), sodium lauryl sulfate (SLS), lidocaine (LID), phosphate-buffered saline (PBS) tablets, tween 80 and trifluoroacetic acid (TFA) were provided by Sigma Aldrich (Spain). 5-Fluoresceinisothiocyanate (FITC) was received from Emp Biotech GmbH (Germany), hydroxypropyl methylcellulose (HPMC) from BASF (Spain), formaldehyde 3.7-4.0% in buffered and stabilized solution from PanReac AppliChem ITW Reagents (Spain) and OCT medium from VWR International (France). Ethanol (EtOH), methanol (MeOH) and acetonitrile (ACN) were provided by Scharlau, S.L. (Spain), sodium diclofenac (DIC) from Acofarma (Spain) and 1,1'-dioctadecyl-3,3,3',3'-tetramethylindodicarbocyanine perchlorate (DiD) from Lifetechnologies (USA). Voltadol Forte (Novartis) and EMLA (Astra Zeneca) were received from a local pharmacy. D-Squame® tape strips were generously gifted by CuDerm (USA). Ultrapure water was used to prepare all solutions.

2.2. Screening of optimal conditions for the generation of RAW *in vitro*

A Nd:YVO4 Q-Switched pulsed laser (Power Line 20E, ROFIN-SINAR laser, Munich) operating at a fundamental wavelength of 1064 nm, with a pulse width of 20 ns and a beam waist diameter of 30 µm was used to irradiate four different materials used as transducers: aluminum, silver, PS and steel. Two irradiation set-ups were studied. The laser beam was focused with a lens of 160 mm focal distance in both cases, although in the first one the laser impinged directly onto the transducer. In the second case, however, an overlay material (5 mm thick) was placed above the transducer, in order to confine the associated ablation phenomena induced by laser irradiation. Agarose gel (3% agarose in 0.1 M NaCl) [20] was placed below the transducer, as illustrated in **Figure 1.A**.

A screening protocol was conducted to identify convenient optimal laser parameters and irradiation pattern geometries for the generation of RAW in each material. In particular, the dependence of the generation of RAW on the laser diode-pump current intensity, pulse repetition frequency and total number of pulses were evaluated. The laser is equipped with a cad-like software that allows drawing and defining the laser irradiation spatial distribution per unit time.

To carry out this analysis, a square dot matrix (6x6) with distances of 500 µm between rows and columns was designed where the intensity and frequency conditions varied, keeping the number of pulses constant (**Figure 1.B**). The energy per pulse corresponding to the combination of current intensity and pulse repetition frequency is represented in **Table S1**.

After each irradiation cycle, the transducer was evaluated to define the optimal combination of current intensity, pulse repetition frequency and total number of pulses that may lead to the generation of RAW with a good balance between energy per pulse and irradiance, but without causing perforation. To do so, transducers were observed under a Nikon MM-400 Microscope (Nikon Metrology, Inc, United States) with a Nikon DS-Fi2 digital Camera attached. The microscope is equipped with a double lighting system that allows illuminating samples both from above and below. Decreasing the light intensity from the upper light source and lighting the samples from below (shown as “Top view” and “Lighting

from below" in **Figure 1.C;** respectively) enables perforations to be identified. Finally, images from the model material (agarose [21]) were also taken and the damaged area surrounding each impact was evaluated.

2.3. Comparison between the optimal conditions for the generation of RAW on human skin *ex vivo*

2.3.1 Skin preparation and RAW generation

Human skin samples from split-thickness skin grafts were collected immediately after surgery from the Department of Plastic, Aesthetic & Reconstructive Surgery, University Hospital Complex, Universidade de Santiago de Compostela. The study was approved by the Galician ethical committee (registration code 2017/103) and was conducted in accordance with the ICH and OECD guidelines [22, 23]. Skin was provided already dermatomed at a thickness of <500 µm and was kept stored at 4°C no longer than 7 days.

Skin samples were equilibrated in 0.9% NaCl solution for 30 min [24] before being exposed to RAWs. **Table 1** presents the combination of transducer, ablation mechanism and laser parameters setting evaluated. To this end, a 30X30 dot array with a separation distance of 500 µm between each element was designed. Each dot represents a laser spot, and the separation distance is measured from the center of each spot. The irradiation of the pattern needed less than 10 seconds to be completed. Skin samples not exposed to RAWs were considered as the control condition.

2.3.2 Disruption of the stratum corneum (SC): evaluation of FITC transport

Intact/RAWs exposed skin samples were mounted in standard Franz diffusion cells with the SC facing upward. The donor compartment was filled with an infinite dosing (~200 mg/cm^2) of an aqueous gel which consisted of 0.01% FITC in 5% HPMC. The receptor compartment was filled with 0.1% Tween 80 in PBS, thermostated at 37°C and stirred to prevent boundary layer effects. At the end of the experiment (30 minutes), diffusion cells were dismantled, and the residual formulation was removed from the surface. Skin samples were then frozen at -80°C.

Images from the SC (xy-plane) were taken using a fluorescence microscope (Nikon Eclipse TE300) under the same analysis conditions. Corrected fluorescence was evaluated by measuring the fluorescence of each sample using the open source image processing program Fiji [25]. An intact skin sample (not in contact with FITC nor disrupted by RAWs) was considered as the blank.

Images from vertical cross-sections (xz-plane; 20 µm) were taken from the passive condition (intact skin) and from the sample disrupted by RAWs generated by confined ablation of a steel plate. Both samples were embedded in OCT medium, vertically sectioned using a Leica CM1850 UV clinical cryostat, mounted on microscope slides and examined with a confocal microscope LEICA AOBS-SP5X [26].

2.3.3 Recovery of the stratum corneum (SC)

Variations in skin temperature were evaluated on human skin samples before and after being disrupted by RAWs generated by confined ablation of a steel plate using an infrared thermometer.

At the same time, the permeabilization and recovery of the barrier function of the skin was evaluated both quantitatively and qualitatively. Regarding the quantitative evaluation, human skin samples were exposed to RAWs as described above and mounted in Franz diffusion cells. The receptor compartment

was filled with 0.1% Tween 80 in PBS and thermostated at 37°C. The donor compartment was filled with an infinite dosing (~1 g/cm^2) of an aqueous gel of 5% HPMC, with or without the addition of 2% SLS to the formulation. Intact skin (that is, not exposed to RAWs) served as the control condition. At predefined timepoints (1, 2.5, 3.5, 5, 7.5, 10, 15, 20 and 30 min), measurements of the skin resistance were conducted using a FLUKE 233 multimeter. The value at timepoint 30 was considered as 100% recovery.

The same set-up and conditions were used for the qualitative evaluation, changing only the timepoints (5, 30, 60 and 240 min). After said timepoints, a group of skin samples were cleaned and immersed in formaldehyde (4% buffered to pH 7 and stabilized with methanol) with 0.01% FITC. Stack images from the SC (xy-plane) were taken with a confocal LEICA AOBS-SP5X microscope and merged to form a maximum intensity projection.

2.3.4 Evaluation of damage on human skin

To corroborate the absence of perforation on the steel plate after confined ablation, images were taken using a Zeiss UltraPlus analytical field emission scanning electron microscope (FESEM).

Furthermore, the potential damage on human skin which may result as a consequence of four densities of laser focal spots (0, 700, 900 and 1700 dots/cm^2) was also evaluated. Human skin samples were disrupted by RAWs generated by confined ablation of a steel plate, preserved in formaldehyde (4% buffered to pH 7 and stabilized with methanol) and sent to the University Hospital Complex of Santiago de Compostela to be processed and analyzed by the Department of Pathology.

2.4. Ex vivo studies of the cutaneous delivery of drugs

2.4.1 Skin preparation and RAW generation

Human skin samples were collected, stored and processed as explained in **Section 2.3.1**. Resonant amplitude waves (RAWs) generated by confined ablation of a steel plate (details show in **Table 1**) were used on all experiments.

A group of experiments was performed using tape-stripped skin as the biological model to evaluate the impact of the SC on the passive diffusion of the molecule under investigation. The SC was removed by tape-stripping using D-squame (D-Squame® tape strips). 25 consecutive tape strips were collected from each skin sample to assure complete removal of the SC and discarded [27, 28]. The remaining skin sample (without SC) was used on the experiments (a histological evaluation of the skin samples is show in **Figure S1**).

2.4.2 Skin transport studies

The skin transport studies were conducted following a similar set-up as to the one explained in **Section 2.3.2** but with a few modifications. The formulation applied in the donor compartment was different according to the molecule and condition evaluated, thus details will be given in the respective section of each drug. All experiments were performed for 1 h.

Quantitative evaluation of the data consisted in the analysis of the delivered amounts of the drug (that is, the amounts deposited and permeated) from a biodistribution study. For this purpose, samples were taken from the receiver compartment and skin pieces were frozen and horizontally sectioned (xy-plane; 25 µm; 6 lamellae) [29]. Although the SC was also quantified, values were not included here since they were not considered as deposited into the skin, but on the skin. The extraction procedure

and the analytical method used for (i) the quantification of the drug on each lamella, (ii) the remaining skin sample and (iii) the receptor compartment will be explained in the respective section of each drug.

2.4.3 Evaluation of DIC delivery

After skin preparation, 100 mg/cm^2 of 2.3% DIC formulation (aqueous solution (AS; control), control plus 2% SLS, control plus 10% EtOH or commercial formulation (Voltadol Forte®) was applied.

The amounts of DIC permeated across and deposited into the skin were determined by UPLC-UV using a Waters Acquity® UPLC® system. Isocratic separation was performed with an Acquity UPLC BEH C18, 2.1 × 100 mm column. Briefly, the method used a mixture of 0.1% TFA-MeOH (34:66, v/v) as mobile phase, pumped at a flow rate of 0.2 mL/min. The injection volume of samples was set at 1 µL. Absorbance was measured at 277 nm. The UPLC-UV method was validated according to ICH guidelines [22]. Lower limits of detection (LOD) and quantification (LOQ) were 56 and 106 ng/mL, respectively.

The drug extraction procedure was validated by extracting DIC from skin samples previously spiked with the drug using extraction medium (H_2O-ACN (25:75, v/v)) [30]. The skin extraction procedure efficiency was 104% (n=4). All samples were centrifuged at 10000 rpm for 15 min before being analyzed by UPLC-UV with a Hettich Universal 32R centrifuge.

2.4.4 Evaluation of LID delivery

As in the case of DIC delivery, 100 mg/cm^2 of 2.5% LID formulation (aqueous solution (control), 2% SLS, 10% EtOH or a commercial formulation (EMLA®) was applied. The amounts of LID permeated across and deposited into the skin were determined by UPLC-UV using a Waters Acquity® UPLC® system. Gradient separation was performed with an Acquity UPLC BEH C18, 2.1 × 100 mm column. In short, the method used a mixture of (A) 0.1% TFA and (B) MeOH (100 A to 55:45 A:B, v/v) as mobile phase, pumped at a flow rate of 0.3 mL/min. The injection volume of samples was set at 5 µL. Absorbance was measured at 263 nm. The UPLC-UV method was validated according to ICH guidelines [22]. LOD and LOQ were found 115 and 373 ng/mL, respectively.

Drug extraction procedure was validated by extracting LID from skin samples previously spiked with the drug using extraction medium (H_2O-MeOH (30:70, v/v)). The skin extraction procedure efficiency was 91.9% (n=4). All samples were centrifuged at 10000 rpm for 15 min before being analyzed by UPLC-UV.

2.4.5 Evaluation of DiD transport

Approximately 100 mg/cm^2 of 68 ppm DiD in water was applied. The amounts of DiD permeated across and deposited into the skin were determined by fluorescence using a Synergy H1 microplate reader. Excitation and emission wavelengths were 649/669 nm. The analytical method was validated according to ICH guidelines [22] and LOD and LOQ were 0.176 and 0.533 ng/mL, respectively.

Drug extraction procedure was validated by extracting DiD from skin samples previously spiked with the drug using extraction medium (H_2O-EtOH (25:75, v/v)). The skin extraction procedure efficiency was 83.7% (n=3).

2.4.6 Data analysis

Data were expressed as mean ± SD. Outliers determined using the Grubbs' test were discarded. Results were compared statistically using either analysis of variance (ANOVA followed by Student Newman Keuls test) or analysis of means by Student's t-test. The level of significance was fixed at $\alpha = 0.05$.

3. RESULTS

3.1. Determination of the optimal conditions for the generation of RAW

3.1.1 Using agarose as the model material (in vitro)

A preliminary screening of laser conditions for the generation of RAW was performed using an agarose gel as model material. The optimal conditions were defined as those that may lead to the generation of RAWs without causing full perforation of the transducer. **Table 1** summarizes the results observed in different materials used as transducers (aluminum, silver, steel and PS, all commercially available in the form of thin foil or sheet) irradiated under either open air (direct), or confined under soda-lime glass.

For all the transducers evaluated, the optimal defined conditions required a much higher number of pulses when using confined ablation (**Section 2.2**) as compared to the direct ablation (~2.5-fold higher). Additionally, a much higher number of pulses was needed when PS was used as a transducer in comparison to aluminum, silver or steel (~13-fold higher).

The impact of the RAWs in the agarose gel was measured by the extension of the damaged area (**Figure 2.A**). The maximum value for the damaged area ($168\pm11\cdot10^3$ μm^2; p value < 0.0001; n=14) was obtained for steel irradiation using the confined configuration, while the minimum was obtained for confined ablation of aluminum ($2.81\pm0.76\cdot10^3$ μm^2; n=14).

3.1.2 Characterization of RAWs on human skin (ex vivo)

The impact of the RAWs generated following **Table 1** settings on human skin was evaluated according to their ability to increase the cutaneous delivery of FITC. As shown in **Figure 2.B**, significant differences were observed in the skin fluorescence (named as corrected skin fluorescence; xy-plane) upon exposure to the RAWs generated by the confined laser ablation of a steel foil transducer, as compared to the rest of conditions evaluated (p value < 0.0001; n=4). **Figure 2.C** compares the fluorescence of a human skin sample after treatment with RAWs generated by confined laser ablation on a steel transducer with the corresponding damaged areas observed in the agarose gel. (**Section 3.1.1**). A perfect match between in vitro/ex vivo experiments was noticeable. This result demonstrates the convenience of using agarose gel as a solid substrate to assess the effect of a physical technique on the skin [21, 31, 32].

Additionally, the evaluation of the cutaneous delivery of FITC by confocal microscopy (xz-plane; **Figure 2.D**) demonstrated the ability of RAWs to deliver FITC to the viable epidermis whereas, under passive administration, FITC remained retained in the SC.

Once the experimental laser parameters/transducer conditions for facilitating the transport of FITC were identified, a series of experiments were performed and aimed at evaluating their impact in terms of skin temperature, resistance and safety. Regarding the former, no significant differences were observed on the temperature of the skin before and after exposure to laser-induced RAWs (33.3 ± 0.9 and 33.7 ± 0.4 °C, before and after exposure; respectively). Alternatively, the evaluation of the skin integrity based on the resistivity values [33] indicated that the exposure to RAWs led to a temporary disruption of ~2.5 min. Interestingly, this time could be doubled to 5 min by the introduction of the surfactant sodium lauryl sulphate (SLS) in the formulation (**Figure 3.A**).

Given the easy-to-identify visual marks that the FITC penetration leaves on the skin, another experiment was conducted to define the threshold and the impact of SLS on the said temporary

disruption. An HPMC gel, containing or not SLS, was applied on RAW-impaired skin. At predefined timepoints, the gel was removed, and the skin sample was immersed in a formaldehyde solution with FITC. As observed in **Figure 3.B**, the exposure to SLS led to the extension of the RAW-pattern marks from 30 minutes to 60 minutes.

The optimum density of dots in the array used for the generation of RAWs was analyzed as a function of damage produced in the skin. **Figure 4.A** shows the FESEM images of the steel transducer after laser confined irradiation, where no perforation on the plate was observed. Furthermore, the correlation between the density of laser focal spots (dots) on the transducer and skin damage revealed no histological differences between 700 dots/cm^2 and the control condition (no dots). On the contrary, a clear damage damage (i.e. bubble formation) at the dermoepidermal junction was observed with 1700 dots/cm^2 which seems to be already initiated with 900 dots/cm^2 (**Figure 4.B**).

3.2. RAWs as a tool to enhance the cutaneous delivery of drugs

3.2.1 RAW-assisted delivery of diclofenac

RAW-assisted delivery of diclofenac, generated via confined ablation of a steel foil transducer (details shown in **Table 1**), resulted in higher amounts delivered compared with the passive administration for all the conditions evaluated (**Figure 5**). The amounts delivered were calculated as the sum of the quantity deposited (found in 25 to 150 μm skin layers) and permeated (found beyond 150 μm skin layers and in the receptor compartment).

RAW-assisted delivery of the control formulation (aqueous solution of sodium diclofenac) resulted in a 2.6-fold increase of the DIC delivered, as compared to the amount delivered passively. This increase was comparable to the one observed for the commercial formulation (VF). Besides, the combination of RAW and chemical enhancers resulted in a 2.5-5.5-fold increase on the delivered amounts observed when they are passively administered. Tape-stripping (that is, complete SC removal; TS) resulted in a significant increment of the delivered amounts of DIC with respect to any other condition evaluated. Finally, the combination of either SLS, EtOH or the commercial formulation with RAW resulted in significant increases in the amount delivered as compared to the passive diffusion.

3.2.2 RAW-assisted delivery of Lidocaine

All the formulations evaluated resulted in a significant increase in the delivered amount of lidocaine (deposited plus permeated) compared to the control condition. In addition, and as observed for diclofenac, RAW-assisted delivery led to an increase in the amount of lidocaine deposited and permeated across the skin under all the conditions evaluated (**Figure 6**). Surprisingly, no statistically significant differences were observed between the passive delivery of the marketed lidocaine formulation (EMLA; 52.7 ± 3.8 μg/cm^2) and the RAW-assisted delivery of LID when administered as a solution (46.9 ± 9.4, 64.2 ± 6.6 and 66.0 ± 11.0 μg/cm^2; control, SLS and EtOH, respectively). On the other hand, the application of EMLA onto the skin exposed to RAWs resulted in a high and significant increase in the amount of lidocaine crossing the SC (153 ± 16 μg/cm^2), this amount being similar to the one obtained after complete removal of the SC barrier (180 ± 12 μg/cm^2).

3.2.3 RAW-assisted delivery of DiD

Whereas for FITC, DIC or LID, the temporal impairment of the skin using RAWs resulted in an increase of the delivery of those molecules crossing the SC, DiD was found to be retained in the 50 µm deep layer of the skin (6.19 ± 1.77 and 19.1 ± 5.7 ng/cm^2; passive and RAW at 25-50 µm deep, respectively; **Figure 7**).

4. DISCUSSION

4.1. Screening of optimal conditions for RAW generation

The use of laser-induced PWs for enhancing the transport of drugs across the skin has been already evaluated by different researchers [10]. The most frequently used experimental approach (i.e. thermoelastic expansion) has made use of a 500-700 nm laser and black polystyrene as a transducer material, because of its effective conversion between light energy to mechanical energy, leading to an increase on the deposition up to 50 µm depth in the skin [12-15].

Other groups have also evaluated the impact of PWs generated through transducer ablation on the delivery of different drug molecules, resulting in disappointing results which have discouraged its use [16-18]. However, in our understanding, a comprehensive study on the generation of RAW, triggered by way of a combined, multiple laser pulse sequence, may reveal the full potential of this strategy, leading to a more effective approach than the previously explored PW-assisted delivery of drugs.

Accordingly, the first step of this work was a screening of different conditions aimed at improving the impact of RAW generated through transducer ablation in an agarose gel model substrate. The conditions were: (i) different laser parameters; (ii) direct or confined ablation using a soda-lime glass; (iii) different transducing materials (aluminum, steel, silver and black polystyrene). Both, the effect of the laser on the transducing material and that of the resulting RAWs in the agarose gel were evaluated. The absence of full perforation on the transducer was considered a key element in order to prevent skin damage. Indeed, although human skin is largely invisible to the wavelength of the laser used in our experiments (1064 nm, **Figure S2**), the previous perforation of the transducer may supply the required electrons to induce skin damage [34].

A closer look to the conditions selected following these two premises (presented in **Table 1**) revealed clear differences upon confinement or not of the laser ablation process. While the direct process relies only on the recoil momentum of the plume generated by the ablation of the transducer, the confined ablation of the transducer results in severe plume expansion restrictions, which leads to RAWs with higher amplitude and longer period [35, 36]. Under these conditions, a much lower number of pulses would be expected to completely perforate the material following confinement, but it was otherwise observed. Two effects were observed in confined ablation, the first is a decrease of the laser power after the soda-lime glass, estimated at 7.25±0.78 % of energy (n=80) due to the absorption of the glass. The other effect is the variation on the focal spots position obtained on the transducer, as compared with the dot array designed. Note that this effect is closely related to laser-transducer interaction, attenuation, and reflection of the laser beam and the RAW generated in the interaction process, although it is not the subject of study in this paper. However, the effect is shown in **Figure 4.A,** where it seems that instead of a focal spot, there are 3 or 4 spots per original array dot.

The results obtained in the agarose gel *in vitro* were found to correlate with those observed in the *ex vivo* permeation of FITC across the skin. FITC is a small lipophilic compound (MW: 389, log P: 5.25, HLB: 7.59) commonly used in histology and also as a molecular model in transdermal delivery. The results of these experiments led us to conclude that the confined ablation of a laser beam in a steel transducer is a good strategy for the generation of RAWs. Compared with steel, PS ablation resulted in a smaller signal in agarose, the energy of the laser is higher, the time needed for the procedure is longer and, more importantly, it is potentially more dangerous. This is because, in the case that the transducer is completely perforated (i.e. laser not properly calibrated or defects on the transducer), the potential damage on the skin is higher with a laser energy resulting from 484 pulses (PS) than from 38 pulses (steel). Black PS is an extraordinary material to generate PW by thermoelastic expansion as its capacity to absorb, store and release energy is remarkable. However, for the same reasons, it is not suitable as a material to be ablated. Direct ablation of the material resulted in needle-shape perforations, while confined ablation resulted in broader perforations and melting, probably as a consequence of both the higher capacity of the PS to absorb the energy and the plume heating due to its confinement. In addition, the combination of its very low thermal conductivity (ca. 10 times lower than steel) and diffusivity (ca. 4 times lower than steel) result in heat accumulation which may cause prolonged thermal damage to the skin.

The results of the evaluation of the delivery of FITC across the skin after a short time application exposure of the formulation to the skin (30 minutes) clearly showed that the RAWs produced an alteration in the skin that facilitated the permeation of this molecular model. These results are not in line with those reported by Lee *et al.* [17], who found no effect for PWs in the skin permeation of FITC in a mouse model. Nevertheless, this could be attributed to the different type of laser (Er:YAG, 2490 nm) and transducer (black PS), as well as to the fact that the conditions reported may not have triggered the formation of RAW.

It has been previously stated that the mechanism of action of the PWs relies on the generation of transient pores within the SC lipids [11]. These transient pores are a result of the pressure changes produced by the PWs [15] without altering the skin temperature. It has been reported that this temporary disruption lasts only for a few minutes [12], although this time can be prolonged with the introduction of a chemical penetration enhancer in the formulation [37]. The results observed in the present work, reported in **Figure 3.A,** are consistent with these previous observations. Direct evaluation of the resilience of the SC after RAW administration showed, however, that the disruption of the skin could be prolonged for a longer period. As presented in **Figure 3.B**, the use of SLS as a penetration enhancer prolonged the disruption of the skin for up to 60 minutes, as noted by the ~~an element of the formulation extended the visualization of~~ RAW-pattern marks ~~(that is, the duration of the disruption of the skin) up to 60 minutes.~~ Accordingly, transdermal diffusion experiments carried out to evaluate the use of RAWs as a tool to enhance cutaneous delivery had a duration of 1 hour.

With regard to the safety of this new technique, the absence of both, complete perforation of the transducer (confirmed by FESEM in **Figure 4.A**) and skin damage (confirmed by confocal imaging in **Figure 3.B**) were observed. In addition, the effect of the number of laser spots per cm^2 on the histology of the skin was also evaluated. Thus laser spot density effects follow the same principles as those reported for fractional ablated area (number of pores per unit surface area) on the laser-mediated cutaneous drug delivery, where it is a common laser configuration associated with improved delivery and faster skin healing [10]. As observed in **Figure 4.B**, a high number of laser spots per cm^2 may lead to a phenomenon called “bubble formation” [38] where, as a consequence of a rapid bubble expansion and collapse, a clear separation can be observed between the epidermis and the dermis. However, this

effect disappeared when reducing the laser spot density in the irradiation array. Based on these results, it can be concluded that the confined ablation of a steel foil transducer using a Nd:YAG laser (1064 nm, 31A, 7kHz, 38 pulses, 23.2 mJ) with a laser spot density of 700 spots/cm^2 is satisfactory for the generation of RAWs and may be used for the cutaneous delivery of drugs without damage to the skin.

4.2. Short-time RAW-assisted cutaneous drug delivery

Once the optimal conditions for the generation of RAWs were identified, their efficacy in terms of enhancing the cutaneous delivery of different compounds was investigated, namely sodium diclofenac, lidocaine and the fluorescent marker DiD.

Sodium diclofenac is a non-steroidal anti-inflammatory drug (MW: 296, log P: 4.75, HLB: 5.81), commonly administered topically for the relief of the acute and chronic musculoskeletal pain. One reported disadvantage of the topical route of administration is the variability observed in the onset of action and effectiveness. In fact, whereas analgesic trials for acute pain (i.e. impact, strains, sprains or overuse-type injuries [39]) reported a sustained pain resolution in 74% of participants after 7 to 10 days [40, 41], analgesic trials for chronic pain (i.e. osteoarthritis and rheumatoid arthritis [42-44]) reported a limited efficacy after 2-12 weeks [45-49].

RAW-assisted delivery of sodium diclofenac resulted in an enhanced penetration into the deep layers of the skin (**Figure 5**). The combination of chemical enhancers and RAW increased up to 5.72-fold the delivered amounts when passively administered. Despite the fact that appreciable permeation was only observed with TS, the balance between the deposited and permeated amounts suggests that both, the viable epidermis and the dermis ~~do also~~ represent significant diffusional barriers [10]. Therefore, and given that sodium diclofenac treatment is based on saturating the skin in order to favor a diffusion gradient to deeper skin layers [50], the enhanced penetration observed suggests that the use of RAWs may be useful on reducing the onset of action of the treatment and, therefore, improving its effectiveness on acute and chronic pain management.

The impact of different penetration enhancers on the transport of sodium diclofenac across the skin has been extensively investigated. Kim *et al.* reported a delivery with palmitic acid and oleic acid of 1.29 and 1.61 µg/cm^{2}h (lag time of 9.67 and 9.63, respectively) after 24h [51]. Maurya and Murthy demonstrated the benefits of a 30 min skin pre-treatment with 10% oleic acid in propylene glycol, as the permeation of Voltaren® gel increased from ~1.69 to ~3.79 µg/cm^{2}h (no pretreatment and enhancer pretreatment, respectively) with a lag time of ~8h [52]. In addition, Bachhav *et al.* demonstrated the impact of laser ablation on DIC total delivery observing a 76.4-fold increase, compared to the control condition (experiment duration 24h) [53]. When comparing the data obtained in this work (permeated amount of 2.27±0.02 µg/cm^2 and delivered amount of 38.9±11.1 µg/cm^2; experiment duration 1h; **Figure 5**) with those previously reported, an overall conclusion is that, extrapolating the one-hour timepoint, the use of RAWs-leads to higher delivery than the use of chemical enhancers and that the enhancement observed with the RAWs is comparable to the one observed with ablation-assisted drug delivery.

Lidocaine was also chosen as a model drug for assessing the value of the RAWs because it is a molecule commonly used either as a topical anesthetic (alleviating superficial skin acute pain) and for the local treatment of peripheral neuropathic pain [54]. Lidocaine presents similar physicochemical properties than DIC but a lower lipophilicity (MW: 234, log P: 2.44, HLB: 11.1). Given its moderate lipophilicity, it has ~~previously~~ been hypothesized that the SC represents the main diffusional barrier for its~~the~~

transcutaneous delivery ~~of lidocaine~~ [55]. Despite the fact that marketed composition containing penetration enhancers (EMLA®) was reported to increase anesthesia up to 500 µm depth into the skin [56], several authors claim that, specially before injections, minimal benefit is observed compared with control, as pain was still experienced in many of the subjects [57, 58]. Consequently, new alternatives (i.e. concomitant use with other interventions) have been investigated to relieve pain and provide comfort to the patient [59].

In this study, the use of RAWs resulted in an enhanced penetration of lidocaine (**Figure 6**). The combination of RAWs with either SLS or EtOH was not, however, as successful as expected. The strategies described to facilitate the transcutaneous delivery of drugs can be divided in two groups: those which disrupt the structure of the skin (increasing the drug diffusion through the skin) and those which increase the drug partitioning into the skin [60, 61]. Given that the transdermal flux (that is, the amount of a drug that is able to diffuse across the skin per unit area and unit time) is calculated based on the diffusion and partitioning of the drug, an improvement on any of both strategies will increase the cutaneous drug transport [62]. While RAW, SLS and EtOH increase the diffusion of the drug, the commercial formulation evaluated (EMLA) improves drug partitioning into the skin [63]. As a consequence, the amounts of Lidocaine delivered as a result of the combination of RAWs with EMLA may reduce pre-injection pain discomfort.

For a better understanding of the results obtained, these were compared with other enhancement techniques used to increase the transdermal delivery of LID. Some examples for passive strategies are the results obtained by Chun *et al.* or by Lee *et al.*, where transdermal fluxes of 16.1 µg/cm^{2}h (experiment duration 24h) [64] and ~57.6 µg/cm^{2}h (no lag time, experiment duration 25h) [65] were reported. Additionally, among the active strategies, Bachhav *et al.* reported the impact of laser ablation on human skin, which led to the delivery of ~3000 µg/cm^2 (experiment duration 24h) [55]. By comparing these data with those reported in **Figure 6** (permeated amount of 56.9±1.4 µg/cm^2 and delivered amount of 153±16 µg/cm^2), it maybe concluded that, extrapolating the one-hour timepoint, the RAW-assisted delivery of LID results more effective than the use of chemical enhancers and as effective as ablation-assisted drug delivery.

Finally, a model compound used to assess the value of RAWs was the fluorescent marker DiD (MW: 1052, log P: 10-12, HLB: 1). The DiD molecule is larger and more lipophilic than diclofenac or lidocaine. The impact of these properties in its transcutaneous delivery is shown in **Figure 7**. Namely, the use of RAWs did not allow the diffusion of this molecule beyond 50 µm.

Overall, these results indicate that RAW-assisted delivery maybe a useful approach for overcoming the SC barrier and that the extent of the transcutaneous penetration is largely influenced by the physicochemical properties of the molecules and the components of the formulation.

5. CONCLUSIONS

Pressure waves (PW) were originally conceived as an alternative to ablation-assisted drug delivery. However, the modest outcomes observed for this technique ~~recorded~~ may have discouraged and neglected this technique. Here, after characterization of the optimal conditions for the generation of resonant amplitude waves (RAWs), their efficacy to enhance the cutaneous delivery of commonly used drugs in dermatology has been evaluated. Among the different experimental conditions explored, the confined ablation of a steel transducer under specific laser settings resulted in the generation of RAWs

thatwhich led to a temporary and safe disruption of the skin. This disruption was translated into an enhanced penetration of different compounds, i.e. sodium diclofenac, lidocaine and DiD. The extension of the penetration was not only affected by the RAWs, but also by the physicochemical properties of the drug/marker and the co-administration of chemical enhancers.

Despite the fact that RAWs are on an early stage of development, the reported results open a vast array of possibilities and applications. In terms of basic science, we have demonstrated RAWs to be promising to promote efficient and safe transdermal drug delivery, although the best results are yet expected when properly combined with specific formulation strategies. Besides, the applied research of this technique has been focused on the treatment of musculoskeletal pain or on the reduction of pre-injection pain discomfort, albeit the treatment of other diseases (i.e. vaccine delivery or cutaneous cancers) may also benefit from the RAWs.

In brief, lLaser technology is an effective strategy to increase the passage of drug molecules through the skin, however, whilebut, whereas classic ablative lasers rely on the formation of micropores on the skin, RAWs provide a non-invasive procedure that avoids skin damage on the patients. We envisage that its translation to the clinic will follow a similar structure than the one observed for the P.L.E.A.S.E. laser (i.e. ER:YAG laser; ablative), the Dotscan 10600 (i.e. CO2 laser; ablative) or the LL Dermal device (i.e. Q-switched Nd:YAG laser; PW); where the device is operated by a trained professional that ensures its correct manipulation and the safety of the patient.

6. ACKNOWLEDGEMENTS

We would like to acknowledge the University of Santiago de Compostela, the Xunta de Galicia, and the Fondo Europeo de Desenvolvemento Rexional for financial support. SR acknowledges Dr. Verena Santer for providing valuable comments. We would like to thank the Department of Pathological Anatomy of the University Hospital Complex of Santiago de Compostela for the histological evaluation of the skin samples.

7. DECLARATION OF INTEREST

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

8. FUNDING

This work has received financial support from the Consellería de Cultura, Educación e Ordenación Universitaria (Competitive Reference Groups; Ref: ED431C 2017/09), Program for Development of a Strategic Grouping in Materials – AeMAT (Ref: ED431E2018/08) and Potencial Growth group (Ref: ED431B2017/64), Gobierno de Aragón "Construyendo Europa desde Aragón" (Ref: T54_17R), Centro

singular de investigación de Galicia (accreditation 2016-2019; Ref: ED431G/05) and the European Regional Development Fund (ERDF).

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FIGURE CAPTIONS

Figure 1. (**A**) Scheme of the set-up used for the screening of the different laser conditions for RAW generation: direct laser irradiation on different transducers (aluminum, silver, PS and steel) or using a soda-lime glass as ablation confining medium, (**B**) laser diode pump current intensity vs. laser frequency test algorithm on a 6x6 dot matrix with a constant number of pulses, and (**C**) damage caused on the transducer as observed in an optical microscope under normal (top view) and inverse illumination (perforation) as well as on the agarose (normal illumination).

a. laser; b. transducer; c. agarose; d. soda-lime glass.

Figure 2. Evaluation of the RAW generated according to the optimal conditions selected from the preliminary screening evaluation (**Table 1**). (**A**) Dimension of the damaged area observed on the agarose (xy-plane, n=14). (**B**) Quantitative evaluation of the deposition of FITC (xy-plane, n=4). (**C**) Qualitative comparison between the results observed in panels **A** and **B** for the conditions *a* and *i*. (**D**) Qualitative evaluation of the enhancement by condition *i* (xz-plane, exposure of the formulation to the skin was 30 minutes). * significant differences (p value between 0.01 to 0.05). **** extremely significant differences (p value < 0.0001).

a. passive (no RAWs); b. aluminum (direct ablation); c. aluminum (confined ablation); d. silver (direct ablation); e. silver (confined ablation); f. black polystyrene (direct ablation); g. black polystyrene (confined ablation); h. steel (direct ablation); i. steel (confined ablation).

Figure 3. Evaluation of the resilience of the skin after RAW administration: (**A**) skin resistance (n=3) and (**B**) qualitative evaluation by means of confocal images (xy-plane). Circular dotted lines highlight RAW-pattern marks on the skin. * significant differences (p value between 0.01 to 0.05).

Figure 4. Evaluation of the skin disruption caused by the RAW generated using laser confined irradiation of a steel transducer. (**A**) FESEM image of the steel transducer exposed to laser confined ablation (magnification x100 and x3000). No perforation is observed in any of the irradiated zones. (**B**) Histological evaluation of the skin after being irradiated using patterns with different laser focal spot densities (xz-plane; hematoxylin and eosin stain; magnification x20). Lower right squares represent the different focal spot densities in an xy-plane. Arrows point at histological damage.

a. control (no RAWs); b. 700 dots/cm^2; c. 900 dots/cm^2; d. 1700 dots/cm^2.

Figure 5. Comparison of the amounts of diclofenac delivered after application for 60 min of 4 different formulations either by passive delivery (P), RAW-assisted delivery and through tape-stripped skin (TS) (n≥3). The amounts delivered were calculated as the sum of the quantity deposited (found in 25 to 150

µm skin layers) and permeated (found beyond 150 µm skin layers and in the receptor compartment). Sodium diclofenac was administered in form of aqueous solution (control), control plus 2% sodium lauryl sulfate (SLS), control plus 10% ethanol (EtOH) and commercial formulation (Voltadol Forte)). The horizontal dotted line represents LOQ. * significant differences (p value between 0.01 to 0.05). ** very significant differences (p value between 0.001 to 0.01). **** extremely significant differences (p value < 0.0001).

Figure 6. Comparison of the amounts of lidocaine delivered after application for 60 min of 4 different formulations either by passive delivery (P), RAW-assisted delivery and through tape-stripped skin (TS) (n≥3). The amounts delivered were calculated as the sum of the quantity deposited (found in 25 to 150 µm skin layers) and permeated (found beyond 150 µm skin layers and in the receptor compartment). Lidocaine was administered as an aqueous solution (control), control plus 2% sodium lauryl sulfate (SLS), control plus 10% ethanol (EtOH) and commercial formulation (EMLA)). The horizontal dotted line represents LOQ. ** very significant differences (p value between 0.001 to 0.01). *** extremely significant differences (p value between 0.0001 to 0.001). **** extremely significant differences (p value < 0.0001).

Figure 7. Comparison of 1,1'-dioctadecyl-3,3,3',3'-tetramethylindodicarbocyanine perchlorate (DiD) biodistribution after application for 60 min, after passive and RAW-assisted delivery (n=4). The horizontal dotted line represents LOQ. ** very significant differences (p value between 0.001 to 0.01).

TABLE CAPTIONS

Table 1. Laser parameters setting conditions selected from the screening of four different transducers. The conditions were selected based on their capacity to produce an apparent alteration of the agarose gel used as a model substrate, without causing the perforation of the transducer. In the confined condition, a soda-lime glass from a local supplier was used as confining medium.

SUPPLEMENTAL FILES

Figure S1. Histological comparison between (**A**) control skin and (**B**) tape-stripped skin.

Figure S2. Effect of the wavelength of light emitted on laser-tissue interaction. Modified from [10].

Table S1. Energy per pulse (mJ) corresponding to each laser parameter settings of **Figure 1.B**.