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The effect of pulse duration, power and energy of fractional Er:YAG laser for transdermal delivery of differently sized FITC dextrans

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ABSTRACT

We studied fractional Er:YAG laser to enhance transdermal drug delivery of compounds possessing different molecular weights: FITC-dextrans (or FD) with average molecular weights of 4, 10 and 20 kDa. Vertical glass Franz diffusion cells were used to study molecular transport through dermatomed porcine skin and histological analysis of laser-treated skin was performed after treatment with different laser pulse protocols. We were comparing different pulse durations at constant or varying pulse energies. We found that the energy of the delivered pulses mostly dictates the size/depth of laser-created microchannels, while the duration of the pulses dictates the extent of thermally altered tissue. That is, tissue ablation threshold is lowered at shorter pulse durations with higher power, which is preferred as it lowers thermal effects on viable skin layers. Especially for smaller molecules, transdermal delivery is increased by increasing laser-created microchannel size, but also by making partitioning into tissue easier when less thermal damage is caused on tissue. For large molecules, molecular transport through the remainder of skin tissue becomes increasingly difficult regardless of laser pulse parameters.

1. Introduction

In the past decades the use of lasers has become customary in many areas of medicine, such as dermatology, ophthalmology, surgery, dentistry, oncology, either for diagnosis or treatment. The earliest and currently most widespread use of lasers is for various applications in dermatology, such as acne or skin hyperpigmentation treatment, scar and wrinkle reduction, tattoo, hair and birthmark removal, skin rejuvenation by resurfacing, skin cancer diagnosis and treatment, and dermal/transdermal drug delivery. The latter has become popular not only for treatments where skin is the target tissue (dermal) but also for systemic delivery (transdermal) because of its noninvasiveness, avoidance of liver or gastrointestinal metabolism and good acceptance from the patients (Zorec et al., 2013b). However, the skin's outer barrier, the stratum corneum, represents the greatest resistance to molecular transport, rendering skin practically impermeable to topically applied drugs. Transdermal drug delivery methods focus on overcoming this barrier function of the stratum corneum to increase transdermal delivery and include passive (penetration enhancers, liposomes, nanoparticles, patch technology) and active methods of enhancement (electroporation, iontophoresis, microneedles, ultrasound, laser radiation). One of these active approaches involves partial or total laser ablation of the stratum corneum, which substantially increases skin permeability even for hydrophilic molecules and large molecules such as peptides, proteins and DNA (Sklar et al., 2014).

The CO_2 (carbon dioxide) and the Er:YAG (erbium-doped yttrium aluminium garnet) laser are most frequently used lasers in transdermal drug delivery (Baron et al., 2003; Fang et al., 2004a, 2004b; Gómez et al., 2011; Hsiao et al., 2011; Huang et al., 2013; Lee et al., 2001, 2002, 2003, 2006, 2007, 2008b, 2008a, 2009; Shen et al., 2006; Wang et al., 2004; Yun et al., 2002). The most frequently used Er:YAG laser emits light in the near-infrared part of the spectrum, at 2.94 μm wavelength, characterized by exceptionally high absorption by water in biological tissue, which means that the light does not penetrate very well. This results in very small optical penetration depths and in tissue ablation with minimal thermal damage and

Abbreviations: FITC, fluorescein isothiocyanate; FD, FITC labeled dextran; FD4, FITC labeled dextran of average molecular weight 4 kDa; FD10, FITC labeled dextran of average molecular weight 10 kDa; FD20, FITC labeled dextran of average molecular weight 20 kDa.

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controlled coagulation zones, making it perfect for controlled removal of stratum corneum (Berlien and Müller, 2003). Conventional CO_2 and Er:YAG lasers were used to ablate outer skin layers with uniform beam profiles, therefore the treated surface was irradiated uniformly. However, in the past 5 years uniform beam profiles used in skin treatments including transdermal drug delivery are being replaced by the concept of fractional photothermolysis, in which the laser beam is split into smaller beams. The skin is no longer ablated homogeneously, instead only small parts of skin surface are exposed to intense laser microbeams, resulting in microscopic channels through outer skin layers, surrounded by undamaged tissue, which allows for much faster skin healing and faster restoration of skin impermeability (Farkas et al., 2010; Graber et al., 2008; Sklar et al., 2014).

Laser treatment for transdermal drug delivery enhancement takes advantage of the ablative effects of laser light on tissue. The Er:YAG laser is very effective for controlled removal of the thin dead outer layer of the skin, the stratum corneum, as it exerts its effects on tissue with minimal penetration depth and minimal thermal damage, thus sparing viable underlying layers (epidermis, dermis) from any substantial thermal damage. In addition, if laser beam is split into microbeams (fractional lasers), even larger portion of viable skin tissue is spared, which offers a very successful yet safe transdermal drug delivery enhancement method. A literature survey confirms increased interest in this modality during the last five years, with a number of studies showing effectiveness of fractional lasers, either CO_2 (Chen et al., 2012; Haak et al., 2012; Haedersdal et al., 2010, 2011; Hsiao et al., 2012; Lee et al., 2013, 2014b) or Er:YAG (Bachhav et al., 2010, 2011, 2013; Forster et al., 2010; Genina et al., 2011; Lee et al., 2010, 2011, 2014a; Oni et al., 2012; Terentyuk et al., 2012; Weiss et al., 2012; Yu et al., 2011), for transdermal delivery of various compounds. The applications include skin rejuvenation (vitamin C, hydroquinone), delivery of anesthetics (lidocaine), anti-inflammatory drugs (diclofenac), treatments for skin conditions and neoplasms (imiquimod, photosensitizers for photodynamic therapy), even vaccination, as larger molecules such as proteins or DNA can be delivered successfully. Pulses of different pulse energies, fluences and durations have been used, with differently fractioned laser beam creating microchannels of various sizes and numbers. However, we have not found any study directly comparing different pulse durations at constant or varying pulse energies, assessing the effect of different pulse shapes on skin and consequently on molecular delivery. Tissue ablation threshold is lowered at shorter pulse durations with higher power, which is preferred as it lowers thermal effects on viable skin layers. We experimented with Fotona LightWalker Er:YAG laser system (Fotona LLC, Ljubljana, Slovenia), with a fractional handpiece PS-01, able to modulate pulse duration at constant delivered energy, thereby changing the instantaneous power. The transdermal delivery after treatment with different laser protocols was assessed with FITC-labeled dextrans of different sizes.

2. Materials and methods

2.1. Skin preparation

Dermatomed porcine ear skin was used in the study, as described previously (Zorec et al., 2013a). Briefly, the skin was obtained from local slaughterhouse immediately post-mortem (Farme Ihan LLC, Šentjur, Slovenia), before washing with steam and detergent in order not to compromise skin integrity. Skin samples were prepared within three hours post-mortem. Excess subcutaneous fat was removed, and the skin was dermatomed to 350 μm thickness (TCM 3000 BL, Nouvag®). Prepared skin samples were frozen until use, for no longer than one month.

2.2. Chemicals

Sodium chloride and fluorescein isothiocyanate labeled dextran (FITC-dextran or FD) with average MWs of 4 (FD4), 10 (FD10) and 20 kDa (FD20) were purchased from Sigma-Aldrich (St. Louis, MO, USA). With regard to molecular weights of FITC-dextrans we would like to emphasize that even though average MW is reported, there is actually a size distribution associated with each dextran. However, the manufacturer states that lower MW dextrans exhibit a more narrow range of MW distribution, while dextrans with MW greater than 10000 are more branched and have somewhat wider size distribution. Potassium chloride, di-sodium hydrogen phosphate and potassium dihydrogen phosphate were purchased from Merck (Darmstadt, Germany). PBS buffer solution was prepared in bi-distilled water (8 g NaCl, 0.2 g KCl, 1.44 g Na2HPO4, 0.24 g KH2PO4 in 1 l of water), as well as phosphate buffer solution (7.38 g NaH2PO4, 4.77 g Na2HPO4 in 1 l of water).

2.3. General experimental setup

Vertical glass Franz diffusion cells were used to study molecular transport through excised and dermatomed porcine skin. The temperature of the chamber was regulated at 37 °C by water circulation. A piece of porcine dermatomed skin was placed between two compartments right before laser treatment with the stratum corneum facing the donor compartment. The area of skin available for diffusion was 0.785 cm^2. The receiver compartment (3.1 ml) was filled with PBS (pH 7.4, 150 mM), in order to maintain constant pH and osmolarity as well as to match ion concentration to that of human body. The donor compartment contained 1 ml of FD solution (0.1 mM) in phosphate buffer (pH 6.5, 100 mM).

2.4. Laser device

Er:YAG (2940 nm, Fotona LightWalker, Fotona LLC, Ljubljana, Slovenia; see Fig. 1a) laser with a fractional handpiece PS-01 was used to pretreat porcine skin samples. Laser beam was divided into 3×3 square grid points (the side of the square measured 3.1 mm) and the diameter of each microbeam was approximately 400 μm (Fig. 1b). Fractional distribution of energy was achieved using a diffractive optical element (DOE) and the condenser lens. The duration of the pulse can be set to the nominal durations of 50 μs, 100 μs, 250 μs, 500 μs and 1000 μs. Commercial names of different duration laser pulses are: SSP (super short pulse: 50 μs), MSP (medium short pulse: 100 μs), SP (short pulse: 250 μs) LP (long pulse: 500 μs) and VLP (very long pulse: 1000 μs).

2.5. Experimental protocols

We experimented with SSP, MSP and SP pulses (50–250 μs). Output energies ranged from 80 to 380 mJ/pulse (all 9 microbeams combined). With 9 microbeams of 400 μm diameter, we achieved fluences (defined as laser energy per surface) ranging from 7.1 to 33.6 J/cm^2. In the first part of the study, we varied pulse duration while keeping pulse energy constant: shorter pulses of equal energy means higher peak power. Relative peak powers of SSP, MSP and SP pulses assuming equal energies are 1: 0.5: 0.2. At maximum energy setting of the laser that we used – 380 mJ – the peak powers of the pulses were: SSP: 7.6 kW, MSP: 3.8 kW, SP: 1.52 kW (peak powers per microbeam have to be divided by 9: SSP: 0.84 kW, MSP: 0.42 kW, SP: 0.168 kW). In the second part of the study, we kept the duration of the pulses constant and varied the delivered energy. We experimented with the shortest pulse setting, the SSP. All protocols are listed in Table 1. We performed 8 parallel repetitions for each protocol.

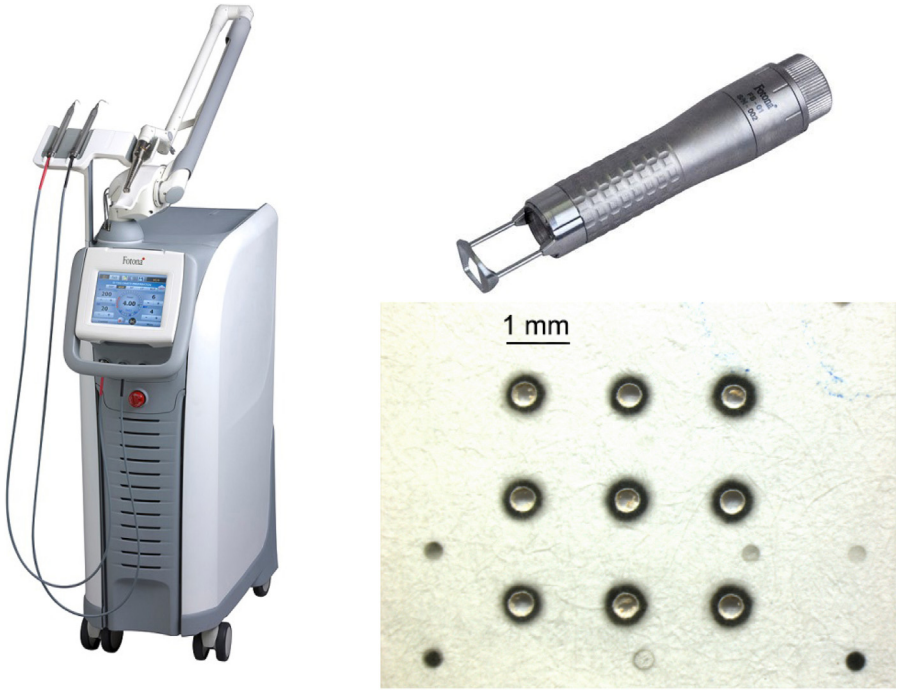

Fig. 1. a) Fotona LightWalker fractional Er:YAG laser (Fotona LLC, Ljubljana, Slovenia); b) handpiece and the grid of the fractionated laser beam: the diameter of each microbeam is approximately 400 μm, arranged into a 3 × 3 formation (the side of the square measures 3.1 mm).

2.6. Transdermal transport measurements

The concentration of FITC-dextrans in receiver compartment after laser treatment was measured with spectrofluorometer (Jasco, FP-6300). At each sampling time, a sample of 300 μl of receiver solution was taken for concentration measurement and replaced with fresh PBS. Concentration was calculated accordingly. We monitored the molecular delivery in the receiver solution every hour for five hours, and more frequently during the first hour.

2.7. Skin histological examination

The region of the skin exposed to the laser treatment was excised, fixed in formalin then stored in 70% ethanol until embedding in paraffin. Subsequently, 5 μm thick sections were cut in the direction perpendicular to skin layers and stained with hematoxylin and eosin. The prepared slides were observed with BX-51 microscope (Olympus, Hamburg, Germany) equipped with a digital camera DP72 (Olympus).

2.8. Data analysis

The results are expressed as the mean ± standard deviation of the mean (normality test passed in all instances). The statistical analysis of differences between various treatments was performed using Tukey test. A 0.05 level of probability was taken as the level of significance. Statistical significance between different laser pulse protocols was analyzed for the 5-h time point after the treatment.

3. Results and discussion

The effects of different lasers on the biological tissue depend on the wavelength, average power, energy, spot size and the exposure time and are dictated by tissue optical properties (they determine distribution of light inside the tissue) and thermal properties (after light energy is converted into heat, they determine its conduction in tissue). When hitting biological tissue, a part of the light is directly reflected at the surface or indirectly after scattering inside the tissue. The distribution of light inside the tissue is determined by three main processes: absorption (by various tissue-specific chromophores), scattering (deviation from straight trajectory at sites of particles and other irregularities) and refraction (change in direction of light propagation due to a change in propagation medium). Tissue reactions to light can be classified into non-thermal, thermal, ablative and optomechanical. For longer exposure times (500 ms or longer) at moderate power densities (1 W/cm^2 range), tissue is coagulated (tissue temperatures over 50 °C).

Table 1
Experimental protocols.

EXPERIMENTAL MOLECULES	LASER PROTOCOLS (pulse duration; energy of the whole beam; power of the whole beam; fluence)	
FITC-dextrans:	varying pulse duration at	SSP: 50 μs; 380 mJ; 7.6 kW; 33.6 J/cm^2
4 kDa,	constant pulse energy	MSP: 100 μs; 380 mJ; 3.8 kW; 33.6 J/cm^2
10 kDa,		SP: 250 μs; 380 mJ; 1.52 kW; 33.6 J/cm^2
20 kDa	varying pulse energy at constant pulse duration	SSP: 50 μs; 80 mJ; 1.6 kW; 7.1 J/cm^2
		SSP: 50 μs; 230 mJ; 4.6 kW; 20.3 J/cm^2
		SSP: 50 μs; 380 mJ; 7.6 kW; 33.6 J/cm^2

When exposure time is reduced to millisecond range at higher power (10 kW/cm^2 range), tissue temperature reaches 100 °C and more which causes vaporization due to local overheating. Ablative effect of laser light occur at microsecond range (the process starts after a few microseconds up to 500 μs) at even higher power output (107 W/cm^2) and turn into plasma formation and optical breakdown of tissue at the extreme end of parameter ranges (ns exposure time and over 107 W/cm^2 power density) (Berlien and Müller, 2003).

A significant advantage of the use of lasers to enhance transdermal drug delivery is the ablative effect of laser light on tissue with minimal laser beam penetration depth and consequently minimal thermal damage of viable underlying skin layers (epidermis, dermis), while ensuring maximum transdermal drug transport. For future clinical use, this means high efficacy, good safety profile and potential acceptance by the patients.

Over several years of experimentation Er:YAG laser was found effective for controlled and safe removal of the stratum corneum, leading to enhanced transdermal delivery of various molecules, even large ones such as proteins or DNA (Bachhav et al., 2013; Weiss et al., 2012; Yu et al., 2011). In addition, when fractional lasers are used, even larger portion of viable skin tissue is spared (Farkas et al., 2010; Graber et al., 2008; Sklar et al., 2014). In previous studies, pulses of different energies, fluences and durations were assessed (Bachhav et al., 2010, 2011, 2013; Forster et al., 2010; Genina et al., 2011; Lee et al., 2010, 2011, 2014a; Oni et al., 2012; Terentyuk et al., 2012; Weiss et al., 2012; Yu et al., 2011); however, we have not found any study directly comparing different pulse durations at constant or varying pulse energies. Namely, pulse energy required for the ablation of the stratum corneum is theoretically lowered at shorter pulse durations with higher power, which also lessens thermal effects on viable skin layers. Our aim was to assess how changing these parameters affects molecular delivery of the laser treated skin.

Our study consists of two parts (two types of protocols): a) protocols among which pulse duration was varied while keeping pulse energy constant; and b) protocols among which pulse duration was kept constant but their energy was varied.

3.1. Varying pulse duration at constant pulse energy

To assess the effect of pulse duration/power at constant energy on laser-enhanced transdermal penetration of permeants, we used differently sized fluorescently-labeled macromolecules: FITC-dextrans (FD): 4 kDa, 10 kDa and 20 kDa. The energy of the delivered pulses was fixed at 380 mJ (fluence: 33.6 J/cm^2). Pulses of three different parameters were used: SSP (super short pulse: 50 μs duration), MSP (medium short pulse: 100 μs duration) and SP (short pulse: 250 μs duration), with their peak powers (at 380 mJ energy) amounting to: SSP: 7.6 kW, MSP: 3.8 kW, SP: 1.52 kW.

Fig. 2 presents cumulative amount of all FITC-dextrans in the receiver every hour for 5 h of passive diffusion after the treatment. The results in Fig. 2a (FD4), b (FD10) and c (FD20) are given in pmol/cm^2, however, please note different ranges for each panel of Fig. 2. Statistical significance ($p \leq 0.05$) between different laser treatment protocols at the 5-h time point after laser exposure is marked with an asterisk. As can be inferred from the figures, the amount of the FD in the receiver at 5 h of passive diffusion after treatment was significantly higher in the laser-treated groups when compared to control (passive diffusion only, no laser treatment), for all pulse durations and FD sizes (asterisks denoting statistical significance between laser treatment protocols and the control (passive diffusion) were left out for clarity). As untreated stratum corneum represents a significant barrier to transport of large molecules such as FD, this is not surprising. Further, as could be expected, FD

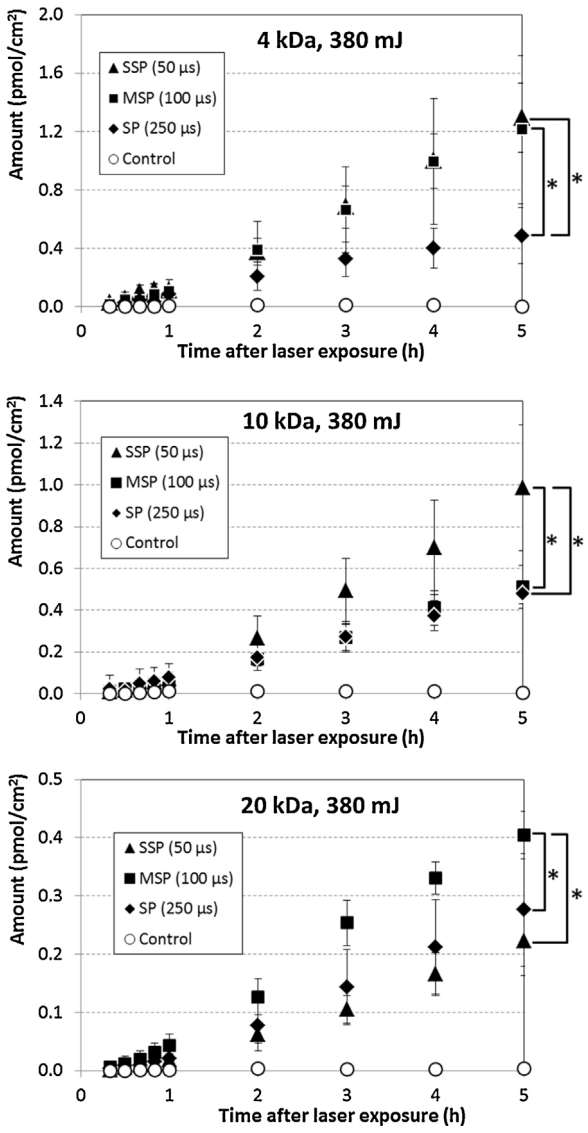

Fig. 2. Cumulative amount (in pmol/cm^2) of all FITC-dextrans (FD) in the receiver for 5 h of passive diffusion after the laser treatment: panel a) FD4; panel b) FD10; panel c) FD20. The energy of the delivered pulses (of the whole beam) was fixed at 380 mJ, while their duration/peak power differed: SSP (super short pulse: 50 μs duration, 7.6 kW power), MSP (medium short pulse: 100 μs duration, 3.8 kW power) and SP (short pulse: 250 μs duration, 1.52 kW power). Note different ranges for each panel. Statistical significance between different laser treatment protocols at the 5-h time point after laser exposure is marked with an asterisk. A 0.05 level of probability was taken as the level of significance. As all laser treatment protocols were significantly more efficient than passive diffusion, asterisks denoting statistical significance between laser treatment protocols and the control (passive diffusion) were left out for clarity.

delivery through treated skin drops with FD size (amount in the receiver: : 4 kDa > 10 kDa > 20 kDa).

However, the most interesting are the differences in FD delivery when different pulse durations/powers are used. Looking at Fig. 2a, it is evident that the amount of delivered FD4 is the lowest when the longest pulse (SP: 250 μs duration) is used, when compared to shorter pulses (MSP: 100 μs, SSP: 50 μs), even at the same delivered pulse energy. Comparison of protocols at 5 h after treatment showed no statistical significance between SSP and MSP protocols, while the efficacy of SP protocol is significantly lower than both, SSP and MSP protocols. Further, the delivery of FD10 (Fig. 2b) is the lowest when the longest two pulse protocols (SP: 250 μs and MSP: 100 μs duration) are used (no statistically

significant difference between the two protocols), and significantly higher after SSP protocol (50 μs).

This higher efficacy of shorter duration/higher power protocols may be their lower thermal influence on the tissue. Namely, type of tissue reaction as the effect of laser pulses depends on the amount and time course of the temperature rise these pulses produce. Generally, different reaction zones can be observed in the tissue after laser irradiation. The tissue is removed in the ablation (non-thermal effect) and vaporization zones (thermally removed at temperatures over 300 °C) that will be followed by carbonized tissue zone (temperatures over 150 °C), then coagulated tissue (over 60 °C) (Berlien and Müller, 2003). As permeant partitioning into thermally-altered tissue is lesser, these zones need to be as narrow as possible. When shorter pulses with higher power are used, higher ablative action is achieved, reducing unwanted thermal effects on tissue thus increasing molecular delivery trough laser-created channels.

The trends of enhancement efficiency for FD4 and FD10 seem to suggest that, in general, shorter pulses (higher peak pulse power) are more efficient than longer pulses (lower peak pulse power): SSP (super short pulse: 50 μs) > MSP (medium short pulse: 100 μs) > SP (short pulse: 250 μs), however, these trends are not always statistically significant. Interestingly, for the delivery of the smallest molecule – FD4 – SSP and MSP pulses were equally efficient, while for the larger FD10, the shortest pulses (SSP) were more efficient than both, SP (the longest), as well as MSP (medium); the difference was statistically significant. The reason for this difference is probably easier partitioning of the smaller molecule (FD4) through the thermally carbonated and coagulated parts of the laser-created microchannels, compared to the larger FD10. At 50 μs pulse duration (SSP) this thermally modified part is very thin and does not hinder molecular delivery. Conversely, when longer, 100 μs pulses (MSP) are used, this coagulated zone is deeper. We postulate that this probably means less obstruction for the penetration of the smaller molecule (FD4) and more for the larger FD10

Looking at Fig. 2c, this "the shorter, the better" trend cannot be observed for the largest molecule, FD20. While there is no statistically significant difference between SSP and SP, the medium duration – MSP – pulses seem to be the most efficient for FD20 delivery (some statistically significant improvement over both, SSP and SP). However, we could find no logical explanation for this somewhat higher efficiency of MSP protocol. The overall low delivery of FD20 can be attributed to the large molecular size of FD20 that makes partitioning into intact or thermally modified tissue equally difficult.

As the shortest duration/highest power pulse parameter setting at constant delivered energy seems to be the best pick for the range of molecular sizes we experimented with, we chose SSP (50 μs duration) pulses for the next part of our study, where we assessed the effect of different pulse energies on molecular delivery.

3.2. Varying pulse energy at constant pulse duration

In this second part of the study we used super short pulses (SSP; 50 μs) of three different laser output energies: 80, 230 and 380 mJ/pulse (corresponding to 1.6, 4.6 and 7.6 kW peak powers, respectively). Fig. 3 presents cumulative amount of all FITC-dextrans in the receiver for 5 h of passive diffusion after the treatment. The concentrations in Fig. 3a (FD4), b (FD10) and c (FD20) are given in pmol/cm^2 (please note different ranges for each panel). Statistical significance ($p \leq 0.05$) between different laser treatment protocols at the 5-h time point after laser exposure is marked with an asterisk. Again, molecular delivery was significantly higher in the laser-treated groups when compared to control (passive diffusion only, no laser treatment), for all pulse

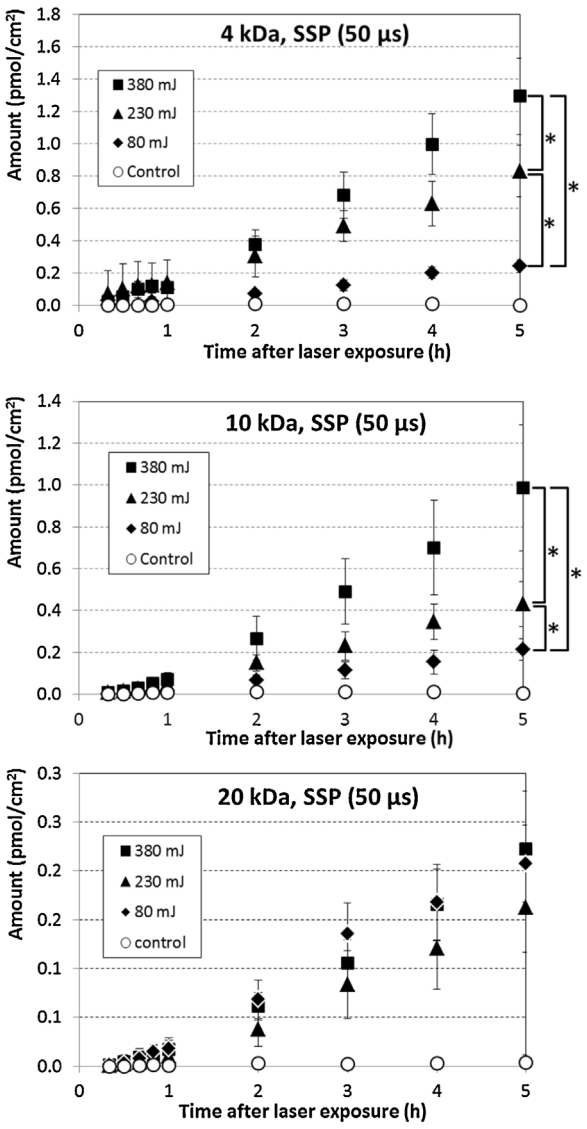

Fig. 3. Cumulative amount (in pmol/cm^2) of all FITC-dextrans (FD) in the receiver for 5 h of passive diffusion after the laser treatment: panel a) FD4; panel b) FD10; panel c) FD20. The duration of the delivered pulses was fixed at 50 μs duration, while their energy/peak power (of the whole beam) differed. Three settings were used: 80 mJ, 230 mJ, 380 mJ (corresponding to 1.6, 4.6 and 7.6 kW, respectively). Note different ranges for each panel. Statistical significance between different laser treatment protocols at the 5-h time point after laser exposure is marked with an asterisk. A 0.05 level of probability was taken as the level of significance. As all laser treatment protocols were significantly more efficient than passive diffusion, asterisks denoting statistical significance between laser treatment protocols and the control (passive diffusion) were left out for clarity.

energies and FD sizes (asterisks denoting statistical significance between laser treatment protocols and the control (passive diffusion) were left out for clarity). Looking at Fig. 3a and Fig. 3b, there seems to be a prevailing trend (the differences are statistically significant, see asterisks in the graphs): the higher the energy, the larger the molecular delivery, which is not surprising. However, increased delivery with increased pulse energy seems to be more proportional for the smaller molecule, FD4 than the larger one, FD10. In other words, when the energy of the delivered pulse is increased from 80 to 230–380 mJ, the delivery of FD4 from the donor into the receiver solution is increased by approximately the same amount. This proportionality is lesser for FD10 where larger enhancement in molecular delivery is observed when the energy is increased from 230 to 380 mJ then when energy is increased from

80 to 230 mJ. For the largest molecule – FD20–increasing pulse energy from 80 to 230–380 mJ has no effect on the delivery as there are no statistically significant differences among the protocols, or even a noticeable trend.

As the size of the laser-created channels in skin increases with amount of delivered energy, larger molecular delivery after high-energy pulses was expected. However, the results seem to suggest that molecular delivery is not simply proportional with pathway size. Instead, different pulse energy thresholds (and with that channel sizes) are required for differently sized molecules to achieve optimal delivery through the laser-created channels. This is particularly evident from the results for the largest molecule, FD20 (Fig. 3c) where, although the delivery through laser-treated skin is enhanced and the permeant is able to pass through laser-created microchannels, the size of these delivery pathways has no effect on the magnitude of delivery enhancement.

For easier assessment of how laser pulse parameter changes affect transdermal delivery of differently sized FITC-dextrans, their cumulative amounts in the receiver for 5 h of passive diffusion after the treatment for all pulse protocols are compiled again in Fig. 4 (panel a: FD4, panel b: FD10, panel c: FD20). Changing the duration of the pulse (at constant energy) and with that the extent of thermally modified tissue seems to have larger effect than varying the size of the microchannels (by changing pulse energy) for transdermal delivery of the smallest molecule, FD4 (Fig. 4a). Namely, increasing pulse duration from 100 to 250 μs at 380 mJ pulse energy lowers FD4 transdermal delivery more than reducing pulse energy from 380 to 230 mJ at the shortest pulse duration – 50 μs. Further, for FD10, three out of five pulse protocols lead to practically equal molecular delivery: 250 μs/380 mJ, 100 μs/380 mJ and 50 μs/230 mJ. The most efficient protocol for FD10 delivery seems to be the 50 μs/380 mJ combination, while the least efficient is 50 μs/80 mJ (statistically significant differences). Lastly, for the largest molecule, FD20, the results of all five pulse protocols are very close to one another, with no discernable trend. The only protocol statistically different from the rest is 100 μs/380 mJ combination; again, we could find no logical explanation for this seemingly higher efficiency of this protocol.

In summary, changes in two characteristics of the laser-created microchannels seem to become noticeable when changing pulse parameters: i) the size of the created microchannels, for which the energy of the delivered pulses is the most important parameter, and ii) the extent of thermally-altered tissue that can – at constant energy – be controlled predominantly by differing pulse duration/power combination. For smaller molecules, both seem to affect the outcome of transdermal delivery enhancement significantly. Namely, transdermal delivery of the permeant is increased by increasing laser-created microchannel size, but also by making partitioning into tissue easier when less thermal damage is caused on tissue. Increasing molecular size, it becomes more difficult to separate the effects of these two mechanisms. For large molecules, transport through skin tissue becomes increasingly difficult regardless of the properties of the laser-created microchannels through the outermost skin layers. However, it needs to be emphasized that this holds true for the ranges of parameters we experimented with. Namely, as each molecule we experimented with exhibits different changes in transdermal delivery going from one experimental protocol to the next, an optimal pulse protocol is probably different for each molecule, and optimal protocols for each molecule may lie outside of parameter ranges we experimented with. In other words, by changing pulse parameters we may also be able to see changes in transdermal delivery of the largest molecule, FD20, however, optimal parameter ranges for this molecule may be different than the ones we experimented with.

Further, the ranges of the delivered amount of FD4 vs. FD10 are very similar. Specifically, the amount of delivered FD4 is higher

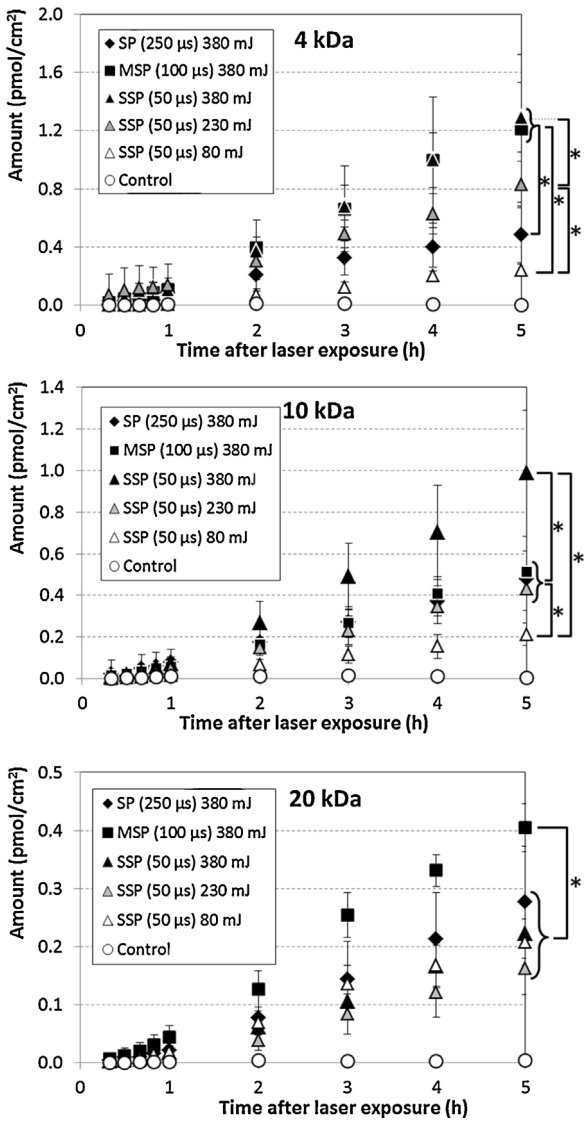

Fig. 4. Cumulative amounts (in pmol/cm^2) of all FITC-dextrans (FD) in the receiver for 5 h of passive diffusion after the laser treatment: panel a) FD4; panel b) FD10; panel c) FD20. All pulse protocols – (various duration/constant energy + various energy/constant duration protocols) are compiled in a single graph for each FD. Note different ranges for each panel. Statistical significance between different laser treatment protocols at the 5-h time point after laser exposure is marked with an asterisk. A 0.05 level of probability was taken as the level of significance. As all laser treatment protocols were significantly more efficient than passive diffusion, asterisks denoting statistical significance between laser treatment protocols and the control (passive diffusion) were left out for clarity.

than the amount of FD10, but the difference is much smaller than one might expect. As surprising as this may seem, this result agrees well with data published by Lee at al. (Lee et al., 2011). The authors also used fractional Er:YAG laser to deliver (among other molecules) FD4, FD10 and FD20. Although absolute values of the delivered amounts are approximately an order of magnitude higher in this study when compared to our results (which is not surprising, because the skin was treated with more passes and more microchannels were created than in our study), we can observe very similar differences in molecular delivery between differently sized FITC-dextrans. Namely, the molecular fluxes for FD4, FD10 and FD20 published by Lee at al. (Lee et al., 2011) are 17.8, 12.6 and 1.4 pmol/cm^2/h, respectively, which shows similar differences in delivery between differently sized molecules. This seems to suggest that, for these ranges of laser pulse parameters,

molecular delivery enhancement is not linear with molecular size but drops drastically for molecules larger than approximately 10 kDa.

3.3. Histology

The region of the skin exposed to the laser treatment was examined histologically to observe laser-created microchannels through upper skin layers for all experimental protocols listed in Table 1. Skin was cut in the direction perpendicular to skin layers and sections cut through the middle of microchannels were observed with a microscope. Micrographs of skin after 5 different laser pulse protocols are shown in Fig. 5.

The effect that increasing pulse duration (hence decreasing pulse peak powers at constant beam energy) has on skin can be seen when comparing panels a) b) and c) on Fig. 5. As evidenced by FITC-dextran delivery results presented in section 3.1 (Fig. 2), the shortest pulse creates the most favorable microchannels for molecular delivery (panel a) on Fig. 5). First, the created pathways go as deep as 150 micrometers into the dermis, and second, the tissue indeed seems to be removed through laser pulse ablative action as there are no visible zones of thermally altered tissue and no residual tissue fragments. Further, the increase in pulse duration (Fig. 5 panels b) and c)) somewhat decreases the channel depth but the more important differences are probably i) the presence of harder top layer residue (epidermis with stratum corneum) that may obstruct molecular delivery (Bachhav et al., 2013), and ii) deeper thermally altered zones in the remaining tissue, both due to the shift from ablative towards thermal action of laser pulses. Although different thermally modified tissue zones (carbonized and coagulated) cannot be discerned in histology micrographs (Fig. 5), more modified epidermal and dermal tissue can be observed for longer pulse protocols (darker borders of laser-created microchannels). However, there seems to be less difference between the histology slides presented on panels b) and c) (corresponding to pulse durations 100 μs and 250 μs), compared to the shortest pulse protocol (panel a). This implies that the thermally modified zones of the laser created microchannels may not change proportionally with pulse duration but instead may be a sort of a threshold response.

Further, using the shortest duration pulses while decreasing laser energy from 380 to 230 and on to 80 mJ/pulse notably decreases tissue ablation depth as is apparent from Fig. 5: panels a), d) and e). At 230 mJ/pulse energy, the laser-created pathway traverses epidermis and ends in the very top part of the dermis, while 80 mJ pulse is only able to ablate the top part of the epidermis, which seems to support our assumption that changing pulse energy predominantly affects the depth of the laser created microchannels. This seems to correspond well with the skin ablation depths reported in the literature, using fractional Er:YAG lasers. Lee et al. (Lee et al., 2010, 2014a) state that the ablation depth equals 4 μm per J/cm^2 fluence which agrees well with ablation depths that can be inferred from Fig. 5. Namely, at 7.1 J/cm^2 fluence, we were just able to ablate the stratum corneum into the top layer of epidermis (panel e)), while at 33.6 J/cm^2 fluence we achieved ablation depths between 120 and 150 μm (as estimated from panels a), b) and c)). Further, Forster et al. (Forster et al., 2010) report ablation of only stratum corneum when using low fluences

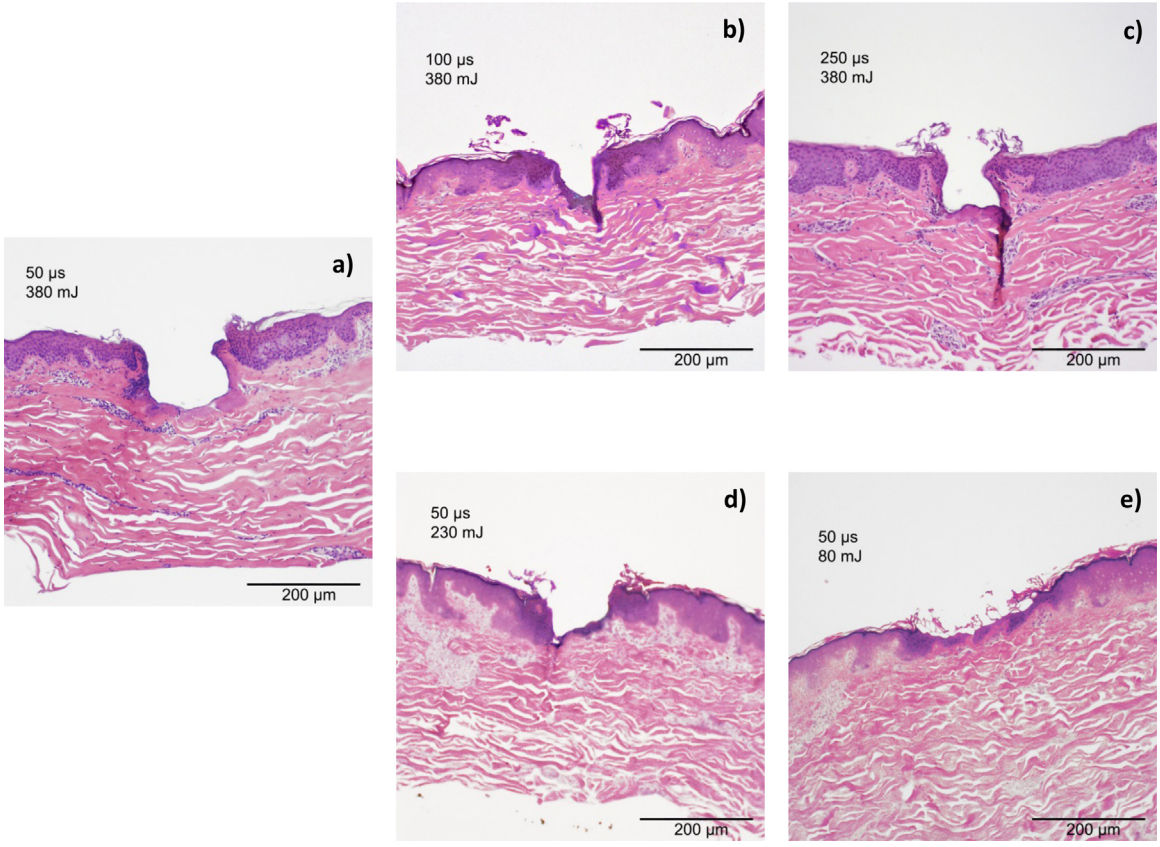

Fig. 5. Micrographs of skin sections cut in the direction perpendicular to skin surface, through laser-created microchannels after different pulse protocols: a) b) and c) The energy of the delivered pulses was fixed at 380 mJ (fluence: 33.6 J/cm^2), pulse durations were varied: a) 50 μs, b) 100 μs and c) 250 μs, with their peak powers (at 380 mJ energy) amounting to: SSP: 7.6 kW, MSP: 3.8 kW, SP: 1.52 kW, respectively. Panels a) d) and e) Pulse duration was the same: 50 μs, laser output energy was varied: a) 380 mJ/pulse (fluence: 33.6 J/cm^2), b) 230 mJ/pulse (fluence: 20.3 J/cm^2), c) 80 mJ/pulse (fluence: 7.1 J/cm^2), corresponding to 7.6, 4.6 and 1.6 kW peak powers, respectively.

(4–8 J/cm^2), epidermis when using fluences of 12 and 24 J/cm^2, and microchannels reaching the dermis at fluences higher than 48 J/cm^2. These findings match ablation depths seen in Fig. 5, especially for 7.1 J/cm^2 (panel e)) and also for 20.3 J/cm^2 (panel d)). However, at higher fluences, our ablation depths were somewhat larger: between 120 and 150 μm at 33.6 J/cm^2 fluence (panels a), b) and c)), while mean depths in Forster et al. (Forster et al., 2010) were 75.6 and 89.3 μm at 48 J/cm^2 and 144 J/cm^2 fluence, respectively. Similarly, a series of articles by Bachhav et al. (Bachhav et al., 2010, 2011, 2013) confirm better agreement with our experiments at lower fluences than at higher fluence values. That is, fluence of 90 J/cm^2 was needed to reach 150 μm depth in their experiments, as opposed to 33.6 J/cm^2 fluence to achieve depths between 120 and 150 μm in our study.

The reason for this discrepancy with the published data at higher fluences may be due to the difference in the used pulse duration. Namely, when comparing different pulse energies (fluences), pulse duration in our study was shorter – 50 μs – than for example in Forster et al. (Forster et al., 2010) or Bachhav et al. (Bachhav et al., 2010, 2011, 2013), where pulse duration was 350 or 400 μs. In other words, as can be inferred from the comparison of panels a), b) and c) in Fig. 5, even with the same pulse energy (fluence) between the three protocols, the shortest pulses seem to produce the deepest microchannels. Another possible reason for this discrepancy may be an overestimation of the diameter of our fractional handpiece PS-01 microbeams, which we rounded up to the maximum of 400 μm (see Fig. 1b). The actual diameter is somewhat smaller and, accordingly, the fluence of our laser pulses may be higher, which brings our results even closer to the findings of other researchers. This seems to be confirmed by the diameter of the created microchannels seen in Fig. 5 that range from approximately 100 μm to 300 μm. However, these numbers cannot be directly translated into the laser microbeam diameter as the actual area of skin altered by the microbeam depends on the laser pulse parameters.

This response of tissue in terms of microchannel depth (Fig. 5 panels a), d) and e)) is also much more proportional to laser pulse energy than tissue response to changes in pulse duration (Fig. 5 panels a), b) and c)), which agrees well with molecular delivery results in Figs. 2 and 3. Namely, the delivery of FD4 and FD10 always increases when laser pulse energy is increased from 80 to 230–380 mJ (Fig. 3). On the other hand, molecular delivery results in Fig. 2 indicate that changing laser pulse duration causes a threshold effect in the delivery of the smallest two molecules, similar to the effects on microchannel properties shown in Fig. 5. As stated before, for the largest molecule, FD20, transport through skin tissue is low and fairly independent of changes in pulse parameters and the properties of the laser-created microchannels. In summary, both, molecular delivery as well as microchannel properties display more proportionality when pulse energy is varied than when laser pulse duration is varied.

The effect of microchannel density and depth (controlled by fluence) was investigated by different researchers for various compounds. The delivery of diclofenac (Bachhav et al., 2011) and two therapeutic antibodies (Antithymocyte globulin and Basiliximab) (Yu et al., 2011) was found to increase when either microchannel number or depth (laser fluence) was increased. Another study by Bachhav et al. (Bachhav et al., 2013) showed increased delivery of cytochrome c and FITC-BSA with increased microchannel number, however, only the delivery of FITC-BSA increased with increasing fluence, on the other hand, increasing microchannel depth had no effect on cytochrome c delivery. Another study by the same authors (Bachhav et al., 2010) reveals similar findings for lidocaine delivery (no dependence on fluence, increased delivery with increasing microchannel density). However, Oni et al. (Oni et al., 2012) do report increased lidocaine delivery with increasing fluence, up to the microchannel depth of 250 μm. These results suggest that molecular transport through laser-created microchannels depends strongly on permeant's physicochemical properties (weight, partition coefficient, ionization), not only on the properties of the transport pathways. In our study, we only examined the effect of molecular weight.

Further, as evident from micrographs in Fig. 5, skin integrity is compromised at micro-ablation sites, where parts of skin tissue ranging between 100 and 300 μm in diameter and up to 150 μm in depth are removed, which raises safety concerns. As this was an ex vivo study, no further examination into safety aspects of the laser-assisted transdermal drug delivery was conducted. However, in vivo studies have shown that after the use of fractional lasers skin tissue displays good epidermal barrier function. Namely, due to the very small area of ablation, the remaining untreated skin serves as a reservoir for healing, which leads to complete re-epithelialization of the stratum corneum within one day after laser treatment (Lee et al., 2010). Also in clinical setting, the use of fractional Er:YAG laser demonstrated significantly lower rate of (mild) adverse effects than when skin was treated with conventional (non-fractional) Er:YAG or CO_2 lasers (Graber et al., 2008). Nevertheless, even with this good safety profile of fractional Er:YAG lasers, concerns regarding transient increased skin permeability (the desired outcome of the treatment) remain. To avoid unwanted introduction of pathogens, caution should be advised for about a day after laser treatment until skin barrier function is fully restored.

4. Conclusions

Er:YAG laser with fractional output beam profile was used as enhancement method for transdermal drug delivery of three model molecules of different sizes: fluorescein isothiocyanate labeled dextrans (FITC-dextrans or FD) with average molecular weights of 4 (FD4), 10 (FD10) and 20 kDa (FD20). The Er:YAG laser is used for controlled removal of the thin dead outer layer of the skin, the stratum corneum, taking advantage of the ablative effects of laser light on tissue while causing minimal thermal damage, thus sparing viable underlying layers (epidermis, dermis). Further, laser beam of fractional lasers is split into microbeams, so even larger portion of viable skin tissue is spared. Pulses of different pulse energies, fluences and durations have been tried experimentally so far, however, to our knowledge, this is the first study directly comparing different pulse durations at constant or varying pulse energies, assessing the effect of different temporal pulse profiles on skin and consequently on molecular delivery. We experimented with Fotona LightWalker laser system with a fractional handpiece PS-01 able to modulate pulse duration at constant delivered energy, thereby changing the instantaneous power.

In the first part of the study, we used protocols among which pulse duration was varied while keeping pulse energy constant: shorter pulses of equal energy means higher peak power. Following that, we kept the duration of the pulses constant while varying their energy. Our results suggest that two mechanisms seem to play important part in the laser-enhanced transdermal delivery story. One is creation of differently sized microchannels, for which the energy of the delivered pulses is the most important parameter. The second mechanism is partitioning of the permeant into tissue, which differs with the extent of thermally-altered tissue due to laser pulses that can – at constant energy – be controlled predominantly by differing pulse duration/power. Namely, tissue ablation threshold is lowered at shorter pulse durations with higher power, which is preferred as it lowers thermal effects on viable skin layers. This is also shown by histological analysis of laser-treated skin and observation of laser-created microchannels after treatment with different laser pulse protocols. Especially for smaller molecules, transdermal delivery is

increased by increasing laser-created microchannel size, but also by making partitioning into tissue easier when less thermal damage is caused on tissue. Increasing molecular size, it becomes more difficult to separate the effects of these two mechanisms. For large molecules, increasingly difficult transport through the remainder of skin tissue (regardless of the properties of the laser-created microchannels through the outermost skin layers) lessens the control over molecular delivery by changing pulse parameters. However, had the parameter ranges been different than the ones we experimented with, we may also be able to see changes in transdermal delivery of the largest molecule, FD20, going from one experimental protocol to the next. Namely, an optimal pulse protocol is probably different for each molecule. Also, optimal protocols may lie outside of parameter ranges we experimented with, even for the smallest molecule, FD4.

In conclusion, finding optimal laser pulse parameters is not as straightforward as increasing pulse energy or maximizing power density, as it appears to be a multivariable process, which makes it difficult to isolate separate effects each parameter change exerts on tissue. Further, the story does not end with the properties of the laser-created microchannels such as their depth, surface and zones of thermally changed tissue. Adding properties of the permeant, such as – in our study – its molecular size, or even partition coefficient and ionization to the mix only increases the multivariable nature of laser-enhanced transdermal drug delivery. It is therefore important to be able to modulate as many laser pulse parameters as possible. Further research is needed in this field in order to find optimal pulse protocols for different permeants.

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