Journal of Biomedical Optics

SPIEDigitalLibrary.org/jbo

Optical coherence tomography monitoring of enhanced skin optical clearing in rats *in vivo*

Elina A. Genina
Alexey N. Bashkatov
Ekaterina A. Kolesnikova
Marina V. Basko
Georgy S. Terentyuk
Valery V. Tuchin

SPIE

Optical coherence tomography monitoring of enhanced skin optical clearing in rats *in vivo*

Elina A. Genina,[a] Alexey N. Bashkatov,[a] Ekaterina A. Kolesnikova,[a] Marina V. Basko,[b] Georgy S. Terentyuk,[a,b,c] and Valery V. Tuchin[a,d,e]
[a]Saratov State University, Department of Optics and Biophotonics, 83, Astrakhanskaya Street, Saratov 410012, Russia
[b]Saratov State Medical University, 112, Bolshaya Kazachia Street, Saratov 410012, Russia
[c]Ulianovsk State University, 42, L. Tolstoy Street, Ulianovsk 432000, Russia
[d]Institute of Precise Mechanics and Control of RAS, 24, Rabochaya Street, Saratov 410028, Russia
[e]University of Oulu, P.O. Box 4500, Oulu 90014, Finland

Abstract. A comparative study of physical, chemical, and combined enhancement of transdermal transport of optical clearing agents (OCAs) is presented. As a physical enhancer of diffusivity, ultrasound (US) with a frequency 1 MHz and a power 1.1 W in the continuous mode was used, and dimethyl sulfoxide (DMSO) was used as a chemical enhancer. OCA (glycerol and polyethylene glycol-400 in equal proportion) was topically applied to the rat skin *in vivo* as alone or as together with the enhancers. Monitoring of skin optical clearing was implemented using an optical coherence tomography. The results have shown that the attenuation coefficient of intact skin dermis after the application of US-DMSO-OCA, US-OCA (both for 4 min), and DMSO-OCA (for 20 min) combinations decreased approximately by 31%, 19%, and 5%, respectively, while OCA alone did not induce a noticeable clearing effect for 20 min. Control skin sites with removed epidermis were used for modeling the upper limit of dermis optical clearing, i.e., maximal degree of optical clearing, by using the studied enhancers. They demonstrated that the attenuation coefficient decreases by 32%, 30%, 17%, and 16% at the action of US-DMSO-OCA, US-OCA, DMSO-OCA, and OCA, respectively. It can be concluded that US-DMSO-OCA combination only allowed reaching the upper limit of skin optical clearing. [DOI: 10.1117/1.JBO.19.2.021109]

Keywords: tissue optical immersion clearing; skin; dimethyl sulfoxide; low-frequency ultrasound; optical coherence tomography.

Paper 130445SSR received Jun. 27, 2013; revised manuscript received Sep. 4, 2013; accepted for publication Sep. 10, 2013; published online Oct. 8, 2013.

1 Introduction

For the last 20 years, optical clearing of tissues has been of great interest owing to its great potential in enhancing the capabilities of noninvasive light-based diagnostics and laser therapy.[1] The possibility of temporal clearing of the superficial skin layers is very useful in developing functional imaging and laser therapeutic techniques that rely on sufficient light penetration to a target embedded in tissue.[1,2] Optical clearing provides improvement in image quality and precision of spectroscopic and microscopic information from tissue depth.[1–5] In particular, optical clearing agents (OCAs) have been successfully employed in optical coherence tomography (OCT) to improve image quality at greater depths in skin tissue.[1,4,6] In laser surgery, the reduction of light scattering by a tissue results in a decrease of laser fluencies required for a therapeutic effect.[1,2,7]

However, a living epidermis of the thickness 75 to 150 μm and its upper-layer stratum corneum (SC) with the thickness in the range of 10 to 20 μm represent a major barrier, which makes the delivery of the OCAs deep into the skin a rather difficult problem.[8] These agents typically possess hydrophilic properties, and hence, cannot penetrate through the lipid-rich SC by the topical application. To reduce the barrier function of the skin for hydrophilic OCAs, a number of different chemical and physical methods have been proposed.[9–12]

Dimethyl sulfoxide (DMSO) is well known and widely used agent for the enhancement of transdermal drug delivery. It is a polar aprotic solvent of SC lipids.[13] It was reported that DMSO induced a high permeability of SC for both hydrophilic and lipophilic drugs.[14,15] It has a high-refractive index and has been shown to reduce the light scattering in skin due to the immersion of skin scatterers.[16] In addition, it interferes with the highly organized structure of thick collagen fibers, changing the interfibril spacing on a submicrometer scale[17] that plays an important role in optical clearing of skin. However, results of the studies of DMSO's optical clearing potential and safety are rather inconsistent.[16,18–20] It was found that optical clearing potential of DMSO is less than that of glycerol, propylene glycol, ethylene glycol, and some others OCAs.[18] A highly concentrated DMSO provokes a wheal accompanied by swelling of the SC and epidermal spongiosis.[19] On the other hand, it was noted that DMSO by itself is an effective topical OCA.[16] Any side effects of pure DMSO at 20-min topical application on the rat skin *in vivo* were not mentioned.[16] In spite of some inconsistency in results of its testing, it is widely used for skin optical clearing and also in mixtures with different OCAs.[13,13–18,21–23] Therefore, we believe that the study of DMSO as a chemical enhancer for transdermal OCA diffusivity is important for the development of skin optical clearing technique. At that, the concentration of DMSO in

Address all correspondence to: Elina A. Genina, Department of Optics and Biophotonics, Saratov State University, 83, Astrakhanskaya Street, Saratov 410012, Russia. Tel: 7 8452 210716; Fax: 7 8452 279710; E-mail: eagenina@yandex.ru

0091-3286/2014/$25.00 © 2014 SPIE

solution used for the optical clearing has to be low enough to prevent skin damage.

Low-frequency ultrasound (US) treatment (sonophoresis), as a noninvasive physical method, also exhibited enhancing skin clearing effect when topically applied with glycerol, polyethylene glycol, propylene glycol, and other agents.[24,25] The significant US-induced enhancement in OCT imaging depth and contrast of *in vitro* porcine skin and *in vivo* human skin was found.[26]

Cavitation is generally believed to be the dominant mechanism of sonophoresis and explains the permeability enhancement.[27] A theoretical analysis of surface interaction of cavitation bubbles with SC lipid bilayers has considered three modes of interaction: shock-wave emission, microjet penetration into the SC, and microjet impact on the SC.[28] During the growth process, neighboring bubbles may merge to create larger bubbles that continue to grow, creating channels. These channels, originating inside the SC, grow in time toward its boundaries, which leads eventually to internal transport paths.[27] These processes induce disorders in the lipid bilayers of the SC that leads to weakening of skin-barrier function and enhancement of OCA penetration into skin.[10]

Multimodal approaches, including the combination of sonophoresis with physical or chemical treatment, enhance the degree of optical clearing, for example: microporation and sonophoresis[29] or US and a chemical enhancer.[30,31] It was shown that the combined use of low-frequency US and enhancers, such as sodium lauryl sulfate and thiazone, promoted increasing of efficacy of transdermal transport of both hydrophilic and lipophylic agents.[30,31]

A comparative study of physical, chemical, and multimodal (physicochemical) approaches to transdermal transport enhancement of OCA is helpful for the development of new and the optimization of existing optical clearing methods. However, in spite of numerous publications related to the problem of the search of the most effective enhancers, it remains not clearly understood.

The goal of the work is to study using OCT technique the effect of low-frequency US, DMSO, and their combination on transdermal permeation of OCA through intact skin or skin with the partly removed epidermis.

2 Methods and Materials

2.1 *Immersion Solutions*

As an OCA, the mixture of dehydrated glycerol (Chemical Warehouse #1 Co., Staraya Kupavna, Moscow region, Russia) and polyethylene glycol with the MW 400 (PEG-400; Aldrich Co., St. Louis, Missouri) in equal proportion was prepared. The mixture was divided into two parts. DMSO (99%; Sigma, St. Louis, Missouri) was added to the first part to obtain 9%-DMSO-OCA solution. Refractive indices of the prepared mixtures were measured with an Abbe refractometer (Atago DR-M2/1550, Tokyo, Japan) at several wavelengths (450, 589, 680, 1100, and 1550 nm) at the temperature ~23°C and interpolated to the mean wavelength of the used OCT system (i.e., 930 nm). There were evaluated for DMSO-OCA and OCA mixtures as 1.423 and 1.421, respectively.

2.2 *Animals and Measuring Protocol*

In vivo experiments were carried out with white outbred laboratory rats. The age of the animals was nearly 12 months. Weight was 150 to 200 g. Before the experiment, they were subjected to general anesthesia by intramuscular injection of Zoletil 50 (Virbac, France). The dose was 0.18 ± 0.02 mL.

Eight rats were included in the experiments. On the back of each animal, two sites of approximately 1×1 cm^2 located symmetrically in relation to the dorsal spine were selected and marked. The hair on the skin was previously depilated using the depilatory cream Nair (Church & Dwight Co., Inc., Ewing, New Jersey). The rats were divided into four groups: I and II groups included the animals with intact skin, and III and IV groups included the animals with removed epidermis on the studied skin sites. A single-edged razor blade with a limiter was used for removing the upper skin layer with thickness 200 ± 20 μm, which includes epidermis and part of dermis. After the removal of epidermis, the sites were covered by a sterile tampon wetted with saline. Experiments were begun not later than 20 min after removing.

About 0.3 mL of OCA or DMSO-OCA was topically applied and uniformly distributed on the skin surface. The OCA with DMSO was applied on the rat skin from both the first and the third groups, and the OCA without DMSO was applied on the rat skin from both the second and the fourth groups (see Table 1).

The studied agent was applied on the both sites at the same time. One of the sites was exposed for 20 min. This period was chosen because it was shown that most of the DMSO dose permeates through SC within 20 min.[32] In this time, another site was treated with a sonicator "Dinatron 125" (Dinatronics, Danvers, Massachusetts) equipped with a 2.2-cm diameter

Table 1 Attenuation coefficient (μ_t, cm^{-1}) of the upper part of dermis at chemical, physical, and combined methods of OCA diffusion enhancement (averaged values with standard deviation).

	Experimental groups			
	Intact skin		Skin without epidermis	
	DMSO-OCA	OCA	DMSO-OCA	OCA
Type of the treatment	I	II	III	IV
Before treatment	93 ± 2	97 ± 9	96 ± 10	91 ± 6
20-min topical application	88 ± 9	96 ± 8	80 ± 2	76 ± 4
4-min US treatment	64 ± 4	79 ± 9	65 ± 2	64 ± 9

probe immediately after application of the solutions. The US frequency was 1 MHz, the power was 1.1 W in the continuous mode, and the time of sonication was 4 min. During sonication, the US probe was immersed into the applied solutions.

Monitoring of skin optical clearing was implemented using a commercially available spectral OCT system OCP930SR (Thorlabs, Newton, New Jersey) working at the central wavelength 930 ± 5 nm with 100 ± 5 nm full-width-at-half-maximum spectrum, a numerical aperture of 0.22, an optical power of 2 mW, a maximum image depth of 1.6 mm, and a length of scanned area 6 mm.[33] Axial and lateral resolutions were 6.2 and 9.6 μm in air, respectively. If we suppose that the average refractive index of dermis at 930 nm is about 1.383,[34] the axial and lateral resolutions can be evaluated as ~4.5 and ~7 μm, respectively.

The OCT system has the standard hand-held probe for skin imaging with a head piece 1×0.15 cm^2 and integrated camera, which allows targeting of the probe on the region of interest. At the measuring, the head piece contacted with the skin surface without compression. The size of the marked sites was made 1×1 cm^2 for increasing of precision of OCT targeting.

The studied sites were scanned before the application of OCA or DMSO-OCA and immediately after the 4-min US treatments or 20-min exposure. To exclude the influence of the displacements of the studied site from initial position on value of attenuation coefficient (μ_t), each OCT measurement included three B-scans, which were registered sequentially with a small shift inside each marked site. Values of μ_t were evaluated from data of each scan and averaged.

2.3 *Data Analysis*

The total attenuation coefficient of the tissue is the sum of absorption coefficient μ_a and scattering coefficient μ_s: $\mu_t = \mu_a + \mu_a$, and can be determined from the slope of the A-scan of the OCT signal measured for the region of interest.[35–38] In the superficial layers of many tissues, single-scattering model (SSM) can be adequately used.[37,38] In accordance with the SSM, the measured signal in OCT system is defined as[35–38]

$$\langle i^2(z)\rangle \approx \langle i^2\rangle_0 \exp(-2\mu_t z), \tag{1}$$

where $i(z)$ is the OCT signal, and z is the probing depth of tissue.[35,37,38]

In OCT, the intensity of optical reflection, $R(z) \propto (\langle i^2(z)\rangle)^{1/2}$, via the tissue probing depth z is measured. The OCT signal depends on the reflectivity of the tissue, $\alpha(z)$, at the depth z and the total attenuation coefficient of the tissue.[35] In the SSM, the reflected power can be proportional to $\exp(-\mu_t z)$,[35] i.e.,

$$R(z) = A\ \exp(-\mu_t z) + B, \tag{2}$$

where A is the coefficient of proportionality equal to $P_0\alpha(z)$, P_0 is the optical power launched into the tissue, and B is the background signal.

Figure 1 illustrates the selection of the region for data analysis. In each B-scan, 2000 consecutive in-depth A-scans were acquired. For calculation, we selected the region on the B-scan with plane surface. The region of interest included the layer of upper dermis with optical depth of about 140 μm (it was corresponded to physical depth ~100 μm) [red rectangle in Fig. 1(a)]. This is the depth at which the OCT intensity has decreased to $1/e$ of the skin surface OCT intensity. Fifty-one A-scans corresponding to ~150 μm in lateral direction [white rectangle in Fig. 1(a)] were averaged to get one intensity-depth profile [black curve in Fig. 1(b)]. The fitting of Eq. (2) to the experimental curve [red curve in Fig. 1(b)] in the region of interest allows evaluating the attenuation coefficient.

The attenuation coefficient has been obtained by the minimization of the target function

$$f(R_0, \mu_t, R_{max}) = \sum_{i=1}^{N_t} \{R_i^{exp} - [R_0\ \exp(-\mu_t z_i) + R_{max}]\}^2, \tag{3}$$

where R_{exp} is OCT signal measured on the depth z, and N_t is the number of measured points in the depth of the tissue (on the z-axis). To minimize the target function, the Levenberg–Marquardt nonlinear least-squares-fitting algorithm described in detail by Press et al.[39] has been used. For the minimization procedure, the following guest values have been used: $R_0 = R^{exp}\ (z=0) - R_{max}$; $R_{max} = R^{exp}\ (z = \text{maximal probing depth})$; and $\mu_t = \sum_{i=1}^{N_t}[z_i\ \ln(\frac{R_i^{exp}}{R_0})] / \sum_{i=1}^{N_t}(z_i)^2$.

The effectiveness of dermis optical clearing with chemical and physical enhancers was evaluated from the difference between the measured control and treatment values of μ_t according to[25]

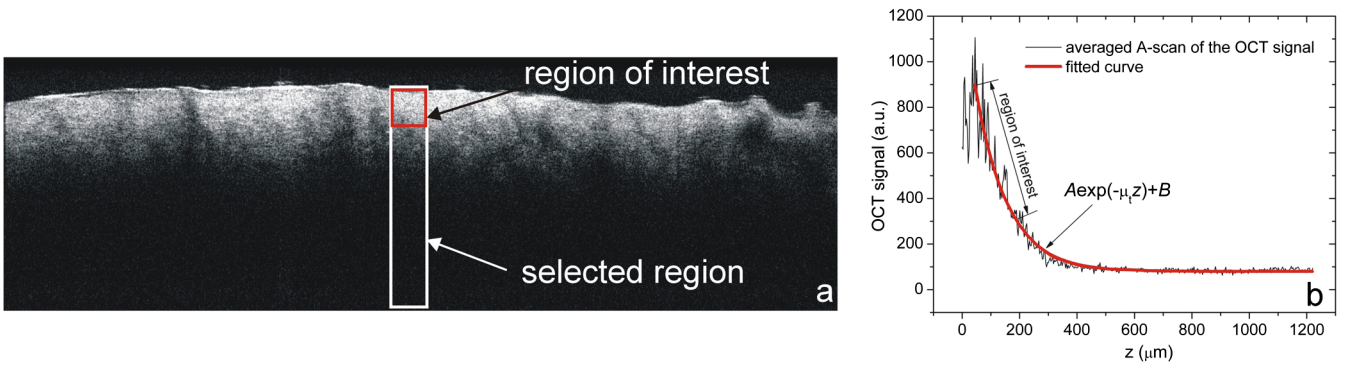

Fig. 1 Typical B-scan of skin without epidermal layer with marked selection regions (51 A-scans for averaging) and region of interest (a) and plot of the averaged A-scan and the fitted curve $R(z) = A\exp(-\mu_t z) + B$ using the single-scattering model (SSM) (b). For this case $A = 1230.4$, $B = 79.7$, and $\mu_t = 90.3$ cm^{-1}.

$$\Delta^{\mu_t} = \frac{\mu_{t(\text{treated})} - \mu_{t(\text{control})}}{\mu_{t(\text{control})}} \times 100\%. \quad (4)$$

The subscript 'control' refers to the skin with removed epidermis after the US-DMSO-OCA treatment, as much as we suppose that in this case, the best optical clearing can be achieved. It is an upper limit of optical clearing in our study. The subscript 'treated' refers to the skin with intact epidermis after the application of the OCA alone, DMSO-OCA, US-OCA, or US-DMSO-OCA.

3 Results

Typical A-scans of the OCT signals from the rat skin *in vivo* in the studied groups averaged in the region of 150 μm and smoothed with a median filter by five points are shown in Fig. 2. Figure 2(a) presents the OCT in-depth signal from intact rat skin together with the fitted curve [Eq. (2)] using the SSM. The epidermis and the upper part of dermis can be easily identified. The first-signal intensity peak results from the reflectivity of skin on the surface, and the second intensity peak can be assigned to the boundary between the epidermis and the dermis. Between the first and the second peaks, the epidermal layer is located. Figure 2(b) shows the OCT in-depth signal from the rat skin without epidermis together with the fitted curve using the SSM. In this case, the first peak corresponds to the reflectivity of dermis. A region of interest in both cases was the upper part of dermis; attenuation coefficient was calculated for this region. Epidermal layer was excluded from the study.

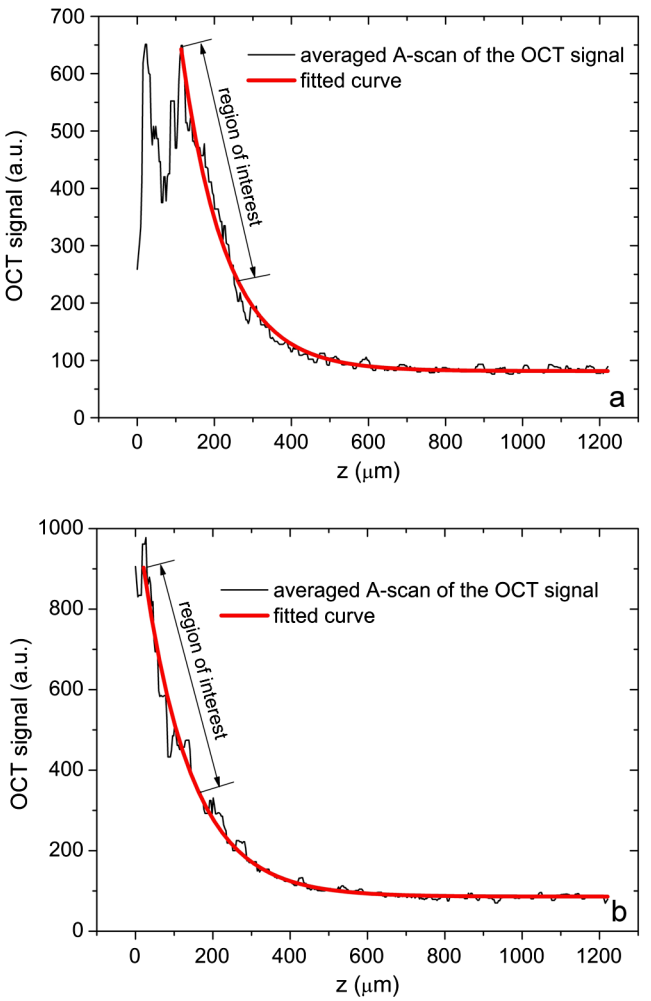

Fig. 2 The averaged optical coherence tomography (OCT) in-depth signal of the rat upper dermis *in vivo* and the fitted curve $R(z) = A\exp(-\mu_t z) + B$ using the SSM: (a) intact skin and (b) skin without epidermis.

Figure 3 presents the OCT images of the rat skin *in vivo* with intact epidermis and removed one before any treatments and after application of chemical, physical, or combined enhancers of OCA transport. Column numbers refer to the numbers of the experimental groups: I and II correspond to the groups with intact epidermis, and III and IV correspond to the groups without epidermis. The lettering of the rows corresponds to the different treatments of skin. In row a, there are the OCT images of skin before the OCA application; in row b, the images after 20 min exposure of the OCA are shown; and in row c, the images correspond to OCA application and sonophoresis. In the images of intact skin (Ia and IIa), epidermis with SC is well seen, and the boundary between epidermis and dermis is sharply differentiated. SC, epidermis "E", and dermis "D" are marked in the image Ia. In the images IIIa and IVa, the areas with disrupted epidermis and denuded dermis are well seen. They are also marked with the corresponding letters and arrows in the image IIIa. In the image IVa, the edge of damaged epidermis is marked.

In the images b and c in the columns I and II, we can see the result of the OCA delivery into the skin with intact epidermis. The hair follicles looked like dark inclined stripes on the light sites ("F" in the images IIb and IIc) and the OCA localization in the opening of the follicles ("OCA" in the images IIb and IIc) are well seen. It means that the OCA without DMSO has penetrated mainly in the follicles and decreased the scattering of neighboring tissue. In the case of using chemical enhancer, the DMSO-OCA mixture has penetrated through the intact epidermis and decreased the scattering not only in the areas of follicles, but also throughout dermis. After US treatment the significantly cleared tissue can be seen in the image Ic. In images b and c in the column III, we can see even optical clearing of dermis. Images b and c in the column IV show that the sites with damaged epidermis were more clear than other ones ("OC"—optical clearing).

The values of attenuation coefficient on the upper part of dermis obtained with the SSM at the different types of treatment are presented in Table 1. Before any treatment, the values of μ_t of the dermis in the groups with intact epidermis are approximately equal to that in the groups with the removed epidermis within precision of the measurements. The initial value of μ_t on the upper part of the dermis averaged over all groups (altogether 16 samples) was evaluated as 94 ± 3 cm^{-1}.

Relative changes of light attenuation coefficient in the studied groups can be evaluated from data in Fig. 4. It presents the normalized values of attenuation coefficient. Bars correspond to the standard deviation. In the figure, it is well seen that after topical application of OCA and 20-min exposure, the attenuation coefficient decreased in all studied groups; however, the degree of the optical clearing was differed. Reduction of the attenuation coefficient in the groups with intact epidermis was 5% and 1% at the action of DMSO-OCA and OCA, respectively. With the same treatments, the value of μ_t reduction of denuded dermis in III and IV groups was 17% and 16%, respectively. It can be concluded that using OCA had no noticeable effect on *in vivo* intact skin at the topical treatment for 20 min and that the difference in μ_t is insignificant between

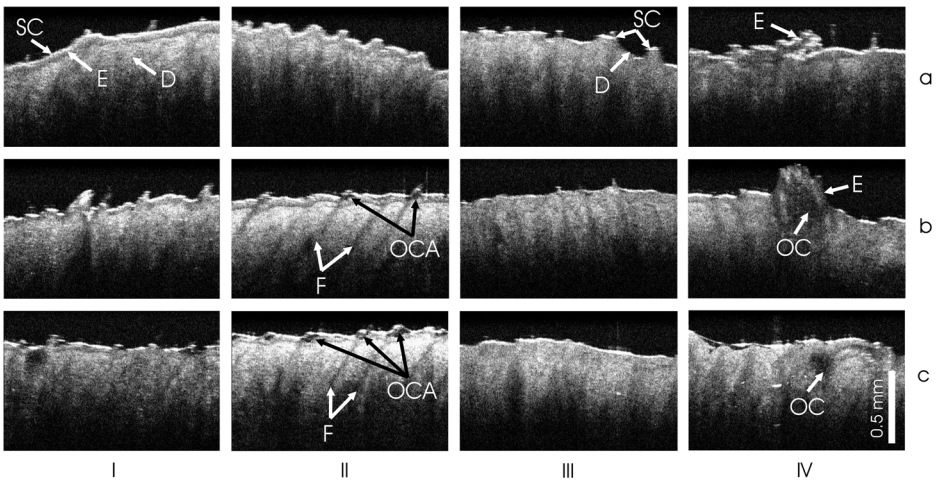

Fig. 3 OCT images of the rat skin *in vivo* with intact epidermis (columns I and II) and removed epidermis (columns III and IV) before any treatment (a), after OCA topical application and 20-min exposure (b), after OCA topical application and 4-min sonophoresis (c). The studied OCA included mixture of glycerol, PEG-400, and DMSO (columns I and III) or glycerol and PEG-400 (columns II and IV). SC is the stratum corneum, E is the epidermis, D is the dermis, F is the follicle, OC is the optical cleared areas, and OCA is the optical clearing agent.

the groups with removed epidermis at OCA (III) or OCA-DMSO (IV) topical application.

The application of US as a physical enhancer of diffusion led to a decrease of the attenuation coefficient of dermis by 19%; whereas, the combination of DMSO and US allowed enhancing the clearing effect by 31% in the groups with intact epidermis. In comparison with 20-min exposure of the OCA without DMSO, the change of the μ_t after US treatment was 18%; and with DMSO, it was 26%.

In the groups with removed epidermis, the following changes of μ_t relative to the initial values were observed: 30% after US treatment only and 32% after the combination of DMSO and sonophoresis. These differences can be considered as insignificant. It is well seen that in comparison with diffusion of OCA without any enhancers, the US treatment promoted the additional increasing of dermis clearing by 14%; whereas, DMSO adding to the OCA at the sonication had no extra influence on the diffusion.

Thus, Fig. 4 shows that DMSO adding to the OCA did not enhance optical clearing of denuded dermis as well as optical clearing degree of dermis at combined treatment US-DMSO-OCA are approximately equal in both groups with intact epidermis and without epidermis.

Figure 5 demonstrates the result of the calculations of Δ^{μ_t} according to Eq. (4) on the basis of the data presented in Fig. 4. These results allow evaluating effectiveness of different enhancers of OCA diffusivity with regard to the control, which corresponds to the animal group with removed epidermis after US-DMSO-OCA treatment. For this group, $\Delta^{\mu_t} = 0$. All columns correspond to the animal groups with intact epidermis after the treatments with OCA ("OCA"), OCA with DMSO ("DMSO-OCA"), OCA with sonophoresis ("US-OCA"), and combined treatment with DMSO and sonophoresis ("US-DMSO-OCA"). It is well seen that the column OCA has a maximal height. It means that OCA application is the least effective treatment as compared with the others. Δ^{μ_t} has

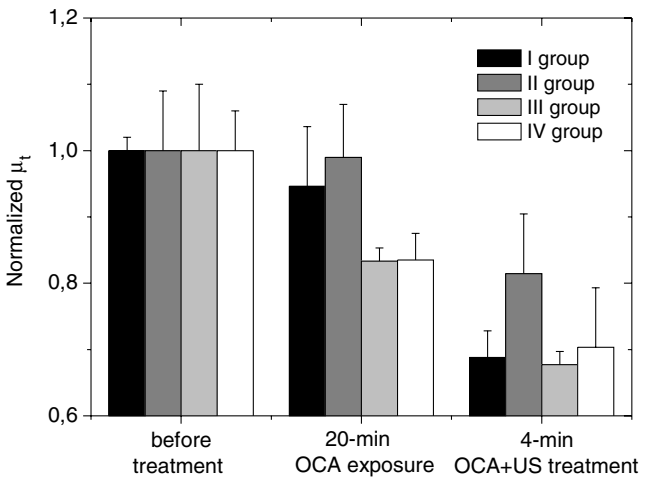

Fig. 4 Normalized light attenuation coefficient of the upper dermis in the studied groups of rats.

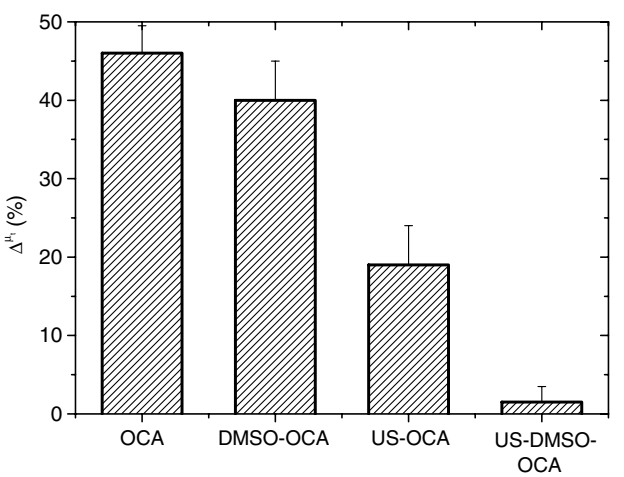

Fig. 5 Difference between normalized attenuation coefficients of dermis of the rat skin *in vivo* with removed epidermis after US-DMSO-OCA treatment and dermis of the rat skin with intact epidermis after glycerol and PEG-400 solution 20-min exposure (OCA), OCA with 9% DMSO 20-min exposure (DMSO-OCA), OCA with 4-min ultrasonic treatment (US-OCA), and OCA with combined DMSO and US treatment (US-DMSO-OCA).

a minimal value in the column US-DMSO-OCA. Thus, we can conclude that combined treatment with chemical (DMSO) and physical (sonophoresis) enhancers of OCA diffusivity leads to the most effective optical clearing of intact skin, as it causes the maximal decrease of the dermis attenuation coefficient that is closed to the μ_t at the upper limit of the optical clearing. In addition, we can see that 4-min sonophoresis provides more effective penetration of OCA into intact skin than 20-min application of OCA with DMSO.

4 Discussion

At present, two models have been frequently used for the description of OCT signal: the single-scattering model (SSM) and the multiple-scattering model (MSM).[36–38] The SSM is based on the assumption that only the light that has been scattered once remains coherent and contributes to the OCT signal. Therefore, the SSM is valid for low-scattering tissues and in the regime where the single-scattering events dominate, i.e., for optical depths $\theta = \int_0^s \mu_t ds < 4$.[35] The last requirement is also met in the superficial layers of high-scattering tissues such as dermis. In this case, the results of the SSM and MSM are nearly the same.[38]

To confirm the adequacy of the used model for μ_t evaluation, we have used data of spectrophotometric measurements of the rat skin optical properties *ex vivo* presented in the Ref. 40. From the data, the value of μ_t at 930 nm was evaluated as 87 cm^{-1}.[40] Result of μ_t extraction from OCT measurements of the rat skin *in vivo* obtained by us with the SSM is 94 ± 3 cm^{-1}. Comparing these values, it is well seen that μ_t obtained from the spectrophotometric and OCT measurements with SSM model are close to each other. Insignificant differences between the presented μ_t values deal with differences in the methods of measurements, skin state (*ex vivo* or *in vivo*), and natural variability of skin optical properties. Thus, we can conclude that the SSM can be used for the treatment of data of the OCT measurements for the estimation of optical properties of the upper part of dermis on the depth down to 100 μm.

It is well seen that the fitted curves in Fig. 2 are in a good agreement with the slope of the OCT signals from the upper dermis. In addition, the scattering of skin decreases during the optical clearing, and therefore, the use of the SSM is valid for μ_t evaluation for bigger depths.

In Table 1, we can see the decreasing of the attenuation coefficient after OCA action in comparison with the initial values in all animal groups. It is related to optical clearing. However, mechanisms of the optical clearing of intact skin and skin with removed epidermis evidently differ. These differences influence the degree of the optical clearing at similar treatments.

In our study, we used the mixture of glycerol and PEG-400. Both agents are widely used as OCAs for the reduction of skin scattering.[40–43] However, topical application of the agents did not result in any significant clearing because it is difficult for them to permeate through intact skin. One of the major protective functions of the skin is derived from low permeability of SC and living epidermis. The diffusion resistance of the SC makes the transdermal delivery of OCA and water loss through skin difficult.[44] We supposed that the reduction of attenuation coefficient after the OCA 20-min topical application in the II group was not observed, because the depth of the OCA penetration into intact skin was limited only by the thin superficial layer of epidermis. It is verified by the results of *ex vivo* skin clearing presented by Matsui et al.[45] Histological analysis of the cross-sections of the samples has shown that glycerol treatment resulted in the shrinkage of the SC due to the reduction of the air gaps within the SC, although, no notable consistent changes were noted in the dermis or other layers of the epidermis.[45]

Because of its properties of strong solvent, addition of DMSO to the mixture of glycerol and PEG-400 enhanced transport of the OCAs through SC. We obtained that after 20 min, μ_t reduction was about 5% in comparison with the initial moment. According to the results presented in Refs. 46–48, addition of DMSO to propylene glycol, glycerol, and others agents provided effective immersion of dermis. It can be supposed that in this case, immersion can be accompanied by the dehydration of dermis induced by high-hygroscopic properties of glycerol.[49] At the same time, viscosity of the glycerol-PEG-400 solution is also high. Consequently, we can assume that the rate of water flow from dermis can exceed the rate of the OCA spreading inside dermis.

In comparison with intact epidermis, damaged epidermal layer cannot provide effective barrier function. Therefore, OCA can diffuse more or less freely into deeper skin layers and induce effective optical clearing due to the matching effect between dermal collagen fibers and interstitial fluid[1–7] that is demonstrated by groups III and IV. These groups are the control ones. They allow evaluating the effectiveness of enhancers for skin barrier overcoming. The use of DMSO as chemical enhancer of diffusivity has not allowed obtaining the same clearing degree of intact skin as that of damaged skin. The values of the attenuation coefficient of dermis in III and IV groups are less than that in I group by about 12%. In that, DMSO has not enhanced diffusivity of OCA in dermis. The values of attenuation coefficient were close in III and IV groups. It indicates that in dermis, DMSO served only as an immersion agent.

US increased the permeability of the dermis for OCA in both cases, at the intact epidermis and at the missing of the epidermis. However, significant differences in the degree of the optical clearing were observed in the series with intact epidermis between groups with DMSO and without DMSO. It is well seen that with the combined action of chemical and physical diffusion enhancers, the degree of optical clearing of the upper part of the dermis of the intact skin coincided with that of the denuded dermis.

Permeation of DMSO in skin was studied in detail. As a hydrophilic molecule, DMSO penetrates the skin via the protein phase.[50] It was shown that DMSO disrupts lipid organization as well as progressively modifies keratin.[51] It associates with polar portions of lipids and replaces water molecules as hydrogen bond acceptor, thereby, associating with the N—H and C═O protein moieties.[32]

Used in literature, concentration of DMSO varies in the wide range from 2% to 99.7%.[13–23] Most often, the authors use pure DMSO[16–18,19,21,22] or high-concentrated (with a concentration >10%) DMSO solutions.[13,14,16,20,23] Under the information from the Material Safety Data Sheet for DMSO,[52] it actually has low toxicity and is only a mild skin irritant. DMSO is FDA-approved for the treatment of interstitial cystitis[53] at a concentration of 50% w/w (Rimso-50, Bioniche Pharma USA LLC, Lake Forest, Illinois). In this treatment, 50 mL of the 50% DMSO solution is instilled directly into the bladder, which suggests that human biological tissue can safely tolerate both large quantities and relatively high concentrations of DMSO.

Furthermore, several groups report that high concentrations of DMSO are well tolerated by humans. Ludwig et al.[54] applied a bandage with 90% DMSO to eight subjects and observed mild skin irritation as the only complication. Bertelli et al.[55] studied 144 patients who were treated with a 7-day regimen involving 99% DMSO, designed to prevent further extravasation of chemotherapeutic agents. The only side effect involved is mild skin irritation and the characteristic garlic odor, which occurs with DMSO absorption by the body.

On the other hand, several groups provided reasons to caution against clinical use of DMSO. With intravenous administration of DMSO, blood serum changes were observed in one subject.[56] Jacoby and Weiss[57] provided preclinical data which suggested that DMSO can either promote or inhibit tumor formation, which depends on the manner in which DMSO was utilized. Serbye et al.[58] demonstrated that DMSO can irritate gastric tissue when applied directly, even at concentrations as low as 5%. As a drug-delivery enhancer, DMSO is used in pharmacology and cosmetology with lower concentrations (~2%).[15]

Based on the peer-reviewed literature,[13–23] we can see that high-concentrated DMSO can be used as effective OCA to improve of the quantitation of structural properties of biological tissues due to high refractive index of the chemical. We used the DMSO concentration 9% in the OCA, because it was effective enough for the enhancement of transdermal OCA delivery and, in that, it did not induce any noticeable side effects at the topical application. Besides, refractive index of the used mixture (1.423) was close to the refractive index of hydrated collagen fibers (~1.42)[59] that provided a high immersion of dermal tissue.

Effect of low-frequency US on the transdermal permeation of some alcohols: glycerol,[10,24–26,31,59] propylene glycol, butanediol, butanol, and polyethylene glycol (PEG-200 and PEG-400),[60] was investigated. For example, 60% glycerol and 60% PEG-400 with US exhibited 1.5- and 1.6-fold clearing capability of glycerol and PEG-400 alone, respectively.[60]

It was also shown that after US treatment with a frequency of 1 to 1.1 MHz and a power of 0.75 W over a 3-cm probe for 15 min, the following changes on the skin surface arose: widened fractures, loose cell arrangements in the SC, and the expanding hair follicles and sweat gland cells.[10] These derangements lead to a weakened skin barrier function, which contribute to the rapid penetration of OCA into the skin. In addition, rise of temperature promotes increasing diffusion rate of OCA components.

The effect of chemical enhancers, sonophoresis, and combined treatments on the skin optical clearing is widely studied by many scientific groups. For example, mixture of PEG-400 and thiazone was shown to decrease the reduced scattering coefficient at wavelength of 630 nm to 60% of the initial value after 40 min of topical treatment on skin *in vitro* and enhance imaging of dermal blood flow through the intact rat skin *in vivo*.[3] The enhanced effect of DMSO on skin optical clearing has been demonstrated by Jiang and Wang.[46] After 30- and 60-min treatment with 80% propylene glycol–50% DMSO mixture, transmittance at 1278 nm has increased by 38% and 53%, respectively.

The attenuation coefficients evaluated from the A-scans of OCT images of skin tissues after the application of 40%, 60%, and 80% glycerol solutions with sonophoresis decreased approximately to 11.8%, 18.5%, and 20.0% at 15 min compared with 40%, 60%, and 80% glycerol alone, respectively.[25] In these works, sonicators with a frequency of about 1 MHz were used because they are most commonly used in cosmetic treatments and transdermal drug delivery.[24–26,60]

It was also found that the enhancing effect was more significant when US and chemical enhancers were applied simultaneously on the skin. For example, 5% Azone in 60% glycerol caused a 1.4-fold increase in skin transmittance at 1276 nm for 60-min elapsed time in Ref. 61; this mixture with US led to a 2.3-fold increase of optical clearing degree, while 60% glycerol with US to a 1.6-fold increase only.[24]

The diffuse reflectance of the human skin *in vivo* after the treatment with 0.25% thiazone–PEG-400 solution in combination with US for 5 min decreased in about 2.2-fold in comparison with 1.2-fold decrease with the use of 0.25% thiazone–PEG-400 solution alone (at 540 nm after 60 min of the action). Simultaneously, 0.25% thiazone and PEG-400 solution with US resulted in 41% increase in OCT $1/e$ light penetration depth at 1310 nm after 60 min.[31]

Our results are in a good agreement with the data presented in the cited works, taking into account that we carried out the measurements after 20-min OCA application or just after 4-min sonophoresis in contrast to 30- to 60-min OCA exposure used in the works cited above. Besides, combinations of OCA and chemical enhancer were unique in the works. However, it results from all presented works that combined treatment of skin with chemical enhancer and US has more significant clearing effect than the treatment with separate enhancers.

Thus, we have shown that US-DMSO-OCA combination is much more effective than DMSO-OCA mixture and US-OCA. Due to the combination of US with DMSO, which enhance penetration effect and US cavitation with temperature effect, more quantity of the OCA can penetrate the skin, and the OCA can penetrate more quickly to achieve more degree of skin optical clearing. It is possible that longer application periods may yield further decreases in dermis attenuation coefficient. Such optimization experiments will be performed in future work.

5 Conclusion

We have presented research and comparative analysis of combined and separated action of low-frequency US and 9% DMSO-OCA solution on the transport of the OCA (glycerol and PEG-400 in equal proportion) in the rat skin *in vivo* with both intact epidermis and damaged epidermis on the basis of OCT data. Through the analysis of attenuation coefficient of dermis, we have evaluated the efficacy of skin optical clearing and demonstrated optical clearing ability of a new combination of US and DMSO–glycerol-PEG 400 mixture for skin *in vivo*. The results have shown that the attenuation coefficient of intact skin dermis after the application of US-DMSO-OCA, US-OCA, and DMSO-OCA combinations decreased approximately by 31%, 19%, and 5%, respectively, while OCA alone did not induce a noticeable clearing effect. At this, optical clearing of denuded dermis at combined US-DMSO-OCA action increased by 32%. Thus, effectiveness of dermis optical clearing was maximal at the US-DMSO-OCA combination.

Acknowledgments

The work was carried out under the partial support from the Russian Foundation for Basic Research (grant 13-02-91176); RF Governmental contract 14.B37.21.0728; FiDiPro, TEKES Program (40111/11), Finland; and 224014 Photonics4life-FP7-ICT-2007-2.

References

1. E. A. Genina, A. N. Bashkatov, and V. V. Tuchin, "Tissue optical immersion clearing," *Exp. Rev. Med. Devices* **7**(6), 825–842 (2010).
2. M. H. Khan et al., "Optical clearing of in vivo human skin: implications for light-based diagnostic imaging and therapeutics," *Lasers Surg. Med.* **34**(2), 83–85 (2004).
3. D. Zhu et al., "Imaging dermal blood flow through the intact rat skin with an optical clearing method," *J. Biomed. Photonics* **15**(2), 026008 (2010).
4. K. V. Larin et al., "Optical clearing for OCT image enhancement and in-depth monitoring of molecular diffusion," *IEEE J. Sel. Top. Quantum Electron.* **18**(3), 1244–1259 (2012).
5. R. He et al., "Effects of optical clearing agents on noninvasive blood glucose monitoring with optical coherence tomography: a pilot study," *J. Biomed. Opt.* **17**(10), 101513 (2012).
6. M. Bonesi, S. G. Proskurin, and I. V. Meglinski, "Imaging of subcutaneous blood vessels and flow velocity profiles by optical coherence tomography," *Laser Phys.* **20**(4), 891–899 (2010).
7. G. Vargas, J. K. Barton, and A. J. Welch, "Use of hyperosmotic chemical agent to improve the laser treatment of cutaneous vascular lesions," *J. Biomed. Opt.* **13**(2), 021114 (2008).
8. H. Schaefer and T. E. Redelmeier, *Skin Barrier*, Karger, Basel (1996).
9. X. Wen et al., "Enhanced optical clearing of skin in vivo and optical coherence tomography in-depth imaging," *J. Biomed. Opt.* **17**(6), 066022 (2012).
10. X. Xu and C. Sun, "Ultrasound enhanced skin optical clearing: microstructural changes," *J. Innovative Opt. Health Sci.* **3**(3), 189–194 (2010).
11. N. Roxhed et al., "Painless drug delivery through microneedle-based transdermal patches featuring active infusion," *IEEE Trans. Biomed. Eng.* **55**(3), 1063–1071 (2008).
12. C. Liu et al., "1064 nm-Nd:YAG lasers with different output modes enhancing transdermal delivery: physical and physiological mechanisms," *J. Biomed. Opt.* **18**(6), 061228 (2013).
13. J. Jiang et al., "Penetration kinetics of dimethyl sulphoxide and glycerol in dynamic optical clearing of porcine skin tissue in vitro studied by Fourier transform infrared spectroscopic imaging," *J. Biomed. Opt.* **13**(2), 021105 (2008).
14. J.-M. Andanson, K. L. A. Chan, and S. G. Kazarian, "High-throughput spectroscopic imaging applied to permeation through the skin," *Appl. Spectrosc.* **63**(5), 512–517 (2009).
15. A. P. Funke et al., "Transdermal delivery of highly lipophilic drugs: in vitro fluxes of antiestrogens, permeation enhancers, and solvents from liquid formulations," *Pharm. Res.* **19**(5), 661–668 (2002).
16. A. K. Bui et al., "Revisiting optical clearing with dimethyl sulfoxide (DMSO)," *Lasers Surg. Med.*, **41**(2), 142–148 (2009).
17. M. Zimmerley et al., "Following dimethyl sulfoxide skin optical clearing dynamics with quantitative nonlinear multimodal microscopy," *Appl. Opt.* **48**(10), D79–D81 (2009).
18. B. Choi et al., "Determination of chemical agent optical clearing potential using in vitro human skin," *Lasers Surg. Med.* **36**(2), 72–75 (2005).
19. A. Pagnoni et al., "Optical coherence tomography in dermatology," *Skin Res. Technol.* **5**(2), 83–87 (1999).
20. R. Samatham, K. G. Phillips, and S. L. Jacques, "Assessment of optical clearing agents using reflectance-mode confocal scanning laser microscopy," *J. Innovative Opt. Health Sci.* **3**(3), 183–188 (2010).
21. G. Vargas et al., "Use of osmotically active agents to alter optical properties of tissue: effects on the detected fluorescence signal measured through skin," *Lasers Surg. Med.* **29**(3), 213–220 (2001).
22. S. Karma et al., "Enhanced fluorescence imaging with DMSO-mediated optical clearing," *J. Innovative Opt. Health Sci.* **3**(3), 153–158 (2010).
23. P. Liu et al., "Discrimination of dimethyl sulphoxide diffusion coefficient in the process of optical clearing by confocal micro-Raman spectroscopy," *J. Biomed. Opt.* **18**(2), 020507 (2013).
24. X. Xu and Q. Zhu, "Feasibility of sonophoretic delivery for effective skin optical clearing," *IEEE Trans. Biomed. Eng.* **55**(4), 1432–1437 (2008).
25. H. Zhong et al., "In vitro study of ultrasound and different-concentration glycerol-induced changes in human skin optical attenuation assessed with optical coherence tomography," *J. Biomed. Opt.* **15**(3), 036012, (2010).
26. X. Xu and Q. Zhu, "Sonophoretic delivery for contrast and depth improvement in skin optical coherence tomography," *IEEE J. Sel. Top. Quantum Electron.* **14**(1), 56–61 (2008).
27. I. Lavon et al., "Bubble growth within the skin by rectified diffusion might play a significant role in sonophoresis," *J. Controlled Release* **117**(2), 246–255 (2007).
28. A. Tezel and S. Mitragotri, "Interactions of inertial cavitation bubbles with stratum corneum lipid bilayers during low-frequency sonophoresis," *Biophys. J.* **85**(6), 3502–3512 (2003).
29. J. Yoon et al., "A physical method to enhance transdermal delivery of a tissue optical clearing agent: combination of microneedling and sonophoresis," *Lasers Surg. Med.* **42**(5), 412–417 (2010).
30. J. Kushner, IV et al., "Dual-channel two-photon microscopy study of transdermal transport in skin treated with low-frequency ultrasound and a chemical enhancer," *J. Invest. Dermatol.* **127**(6), 2832–2846 (2007).
31. H. Zhong et al., "Synergistic effect of ultrasound and thiazone—PEG 400 on human skin optical clearing in vivo," *Photochem. Photobiol.* **86**(3), 732–737 (2010).
32. P. J. Caspers et al., "Monitoring the penetration enhancer dimethyl sulfoxide in human stratum corneum in vivo by confocal Raman spectroscopy," *Pharm. Res.* **19**(10), 1577–1580 (2002).
33. Thorlabs, "Spectral Radar OCT Systems," Catalog, 2007, http://www.thorlabs.com/images/Catalog/V19_07_Micros_Img.pdf (1 June 2013).
34. H. Ding et al., "Refractive indices of human skin tissues at eight wavelengths and estimated dispersion relations between 300 and 1600 nm," *Phys. Med. Biol.* **51**(6), 1479–1489 (2006).
35. R. K. Wang and V. V. Tuchin, "Optical coherence tomography. light scattering and imaging enhancement," Chap. 16, in *Handbook of Coherent-Domain Optical Methods: Biomedical Diagnostics, Environmental Monitoring, and Material Science*, V. V. Tuchin, Ed., 2nd ed., Vol. 2, p. 665, Springer, New York, Heidelberg, Dordrecht, London (2013).
36. D. Levitz et al., "Determination of optical scattering properties of highly-scattering media in optical coherence tomography images," *Opt. Express* **12**(2), 249–259 (2004).
37. D. J. Faber et al., "Quantitative measurement of attenuation coefficients of weakly scattering media using optical coherence tomography," *Opt. Express* **12**(19), 4353–4365 (2004).
38. P. Lee, W. Gao, and X. Zhang, "Performance of single-scattering model versus multiple-scattering model in the determination of optical properties of biological tissue with optical coherence tomography," *Appl. Opt.* **49**(18), 3538–3544 (2010).
39. W.H. Press et al., *Numerical Recipes in C: The Art of Scientific Computing*, Cambridge University Press, Cambridge (1992).
40. E. A. Genina et al., "Optical clearing of skin under action of glycerol: ex vivo and in vivo investigations," *Opt. Spectrosc.* **109**(2), 225–231 (2010).
41. Z. Mao et al., "Influence of alcohols on the optical clearing effect of skin in vitro," *J. Biomed. Opt.*, **13**(2), 021104 (2008).
42. X. Wen et al., "In vivo skin optical clearing by glycerol solutions: mechanism," *J. Biophotonics* **3**(1–2), 44–52 (2010).
43. T. Yu et al., "Quantitative analysis of dehydration in porcine skin for assessing mechanism of optical clearing," *J. Biomed. Opt.* **16**(9), 095002 (2011).
44. E. A. Genina et al., "Optical clearing of human skin: comparative study of permeability and dehydration of intact and photothermally perforated skin," *J. Biomed. Opt.* **13**(2), 021102 (2008).
45. A. Matsui, S. J. Lomnes, and J. V. Frangioni, "Optical clearing of the skin for near-infrared fluorescence image-guided surgery," *J. Biomed. Opt.* **14**(2), 024019 (2009).
46. J. Jiang and R. K. Wang, "Comparing the synergistic effects of oleic acid and dimethyl sulfoxide as vehicles for optical clearing of skin tissue in vitro," *Phys. Med. Biol.* **49**(23), 5283–5294 (2004).
47. J. Jiang, L. Zhang, and R. K. Wang, "Synergistic effect of hyperosmotic agents under topical application on optical clearing of skin tissue in vitro," *Proc. SPIE* **5696**, 80–90 (2005).
48. K. Moulton et al., "Use of glycerol as an optical clearing agent for enhancing photonic transference and detection of Salmonella typhimurium through porcine skin," *J. Biomed. Opt.* **11**(5), 054027 (2006).

49. J. W. Wiechers, J. C. Dederen, and A. V. Rawlings, "Moisturization mechanisms: internal occlusion by orthorhombic lipid phase stabilizers–a novel mechanism of action of skin moisturization," Chap. 9, in *Skin Moisturization*, A. V. Rawlings and J. J. Leyden, Eds., pp. 309–321, Taylor & Francis Inc. (2009).
50. Ch. W. Freudiger et al., "Label-free biomedical imaging with high sensitivity by stimulated Raman scattering microscopy," *Science* **322**(5909), 1857–1861 (2008).
51. A. N. C. Anigbogu et al., "Fourier transform Raman spectroscopy of interactions between the penetration enhancer dimethyl sulfoxide and human stratum corneum," *Int. J. Pharm.* **125**(2), 265–282 (1995).
52. "Material Safety Data Sheet for Dimethyl Sulfoxide," http://www.herpes-coldsores.com/dmso.pdf (21 May 2013)
53. J. Sea and J. M. H. Teichman, "Paediatric painful bladder syndrome/interstitial cystitis diagnosis and treatment," *Drugs* **69**(3), 279–296 (2009).
54. C. U. Ludwig et al., "Prevention of cytotoxic drug induced skin ulcers with dimethyl sulfoxide (DMSO) and alphatocopherole," *Euro. J. Cancer Clin. Oncol.* **23**(3), 327–329 (1987).
55. G. Bertelli et al., "Topical dimethylsulfoxide for the prevention of soft-tissue injury after extravasation of vesicant cytotoxic drugs—a prospective clinical-study," *J. Clin. Oncol.* **13**(11), 2851–2855 (1995).
56. P. Yellowlees, C. Greenfield, and N. McIntyre, "Dimethylsulphoxide-induced toxicity," *Lancet* **316**(8202), 1004–1006 (1980).
57. W. T. Jacoby and H. S. Weiss, "Inhibition and enhancement of skin tumors in mice by dimethyl sulfoxide depending on method of application," *J. Natl. Cancer Inst.* **77**(4), 983–987 (1986).
58. H. Serbye et al., "Gastric-mucosal injury and associated changes in mucosal blood-flow and gastric fluid secretion caused by dimethylsulfoxide (DMSO) in rats," *Dig. Dis. Sci.* **38**(7), 1243–1250 (1993).
59. D. W. Leonard and K. M. Meek, "Refractive indices of the collagen fibrils and extrafibrillar material of the corneal stroma," *Biophys. J.* **72**(3), 1382–1387 (1997).
60. X. Xu, Q. Zhu, and C. Sun, "Assessment of the effects of ultrasound-mediated alcohols on skin optical clearing," *J. Biomed. Opt.* **14**(3), 034042 (2009).
61. X. Xu and Q. Zhu, "Evaluation of skin optical clearing enhancement with azone as a penetration enhancer," *Opt. Commun.* **279**(1), 223–228 (2007).