Journal of Innovative Optical Health Sciences
Vol. 3, No. 3 (2010) 189–194

DOI: 10.1142/S1793545810000964

ULTRASOUND ENHANCED SKIN OPTICAL CLEARING: MICROSTRUCTURAL CHANGES

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Our previous studies demonstrated the ultrasound-induced skin optical clearing enhancement with topical application of optical clearing agents on *in vitro* porcine skin and *in vivo* human skin. The objective of this study was to investigate the possible mechanisms of the enhanced skin optical clearing by ultrasound medications. Optical clearing effects of *ex vivo* guinea pig abdomen skin topically applied with 60% glycerol or the combination of 60% glycerol and ultrasound were studied by optical coherence tomography (OCT). Microstructure of skin surface was examined by scanning electron microscopy (SEM). Ultrasound with a frequency of 1 MHz and a power of 0.75 W over a 3-cm probe was simultaneously applied with glycerol solution for 15 min. The combination of 60% glycerol and ultrasound results in a 19% increase in OCT $1/e$ light penetration depth after 30 min, which is much better than 60% glycerol alone. SEM images demonstrated that changes in skin microstructure due to the tight order of the lipid bilayers in the stratum corneum disrupted and the separation of keratinocytes by the application of ultrasound contribute to the ultrasound-enhanced intact skin optical clearing effects.

Keywords: Enhancement of epidermal permeability; microstructure; optical coherence tomography; skin optical clearing; ultrasound.

1. Introduction

Optical clearing of tissues is a new approach that currently attracts much attention in the area of biomedical optics. Recently numerous works were conducted in the field, since it has a great potential in enhancing the capabilities of non-invasive light-based diagnostic and imaging techniques.[1–3] The possibility of selective translucence of the superficial skin layers is very useful in developing functional imaging techniques, including optical coherence tomography (OCT) and reflectance spectroscopy.[4] Skin optical clearing would aid the optical methods in the non-invasive visualization of cutaneous blood microvessels and small pathologic structures in tissues with high resolution. Another potential benefit of the optical clearing technique is the improvement of laser therapeutic techniques that rely on sufficient light penetration to a target embedded in tissue. Combining optical clearing with laser radiation could reduce laser fluences required for a therapeutic effect. Current challenges to skin optical clearing include epidermal penetration by optical clearing agents (OCAs). The outermost layer of the skin, the stratum corneum (SC), presents a significant barrier to the most topically applied OCAs and is hence responsible for the poor optical clearing effect. Slow diffusion of the index-matching agent through the skin barrier makes practical implementation of the approach difficult. To reduce barrier function of the skin during optical clearing, a number of different chemical and physical methods were proposed. Chemical

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penetration enhancers such as polyurethane prepolymers, oleic acid, azone (epsilon-Laurocapram) and propylene glycol into the transdermal formulation have shown to accelerate the skin permeability of OCA.[5–8] For physical methods, photo-thermal, sandpaper and microneedle arrays were applied to enhance transdermal skin clearing agent delivery.[9–13] Our recent studies have shown that sonophoretic delivery, as a non-invasive physical method, demonstrated enhancing skin clearing effects when applied topically with OCAs.[14] The significant ultrasound-induced enhancement in OCT imaging depth and contrast of *in vitro* porcine skin and *in vivo* human skin was found.[15] We further demonstrated the synergetic enhancing skin clearing effects when the combination of ultrasound and chemical enhancers was applied topically with OCAs onto skin.[16] Despite the demonstrated ultrasound enhanced effects, the exact mechanisms of the ultrasound-induced skin optical clearing are still unclear. In other words, how does ultrasound facilitate the penetration of OCAs into the intact skin through the stratum corneum barrier? In this study, we investigate the ultrasound-induced *ex vivo* guinea pig skin optical clearing by optical coherence tomography (OCT) and the skin microstructural changes during optical clearing by scanning electron microscopy (SEM).

2. Materials and Methods

Figure 1 shows the schematic of the OCT system used in this study, which is a delay line OCT system with a 1310 nm central wavelength and 40 nm bandwidth light source (18 mW). The light source yields an 18-μm axial resolution in free space, or approximately 13 μm within tissue if the mean refractive index of bulk tissue is assumed to be 1.38; this determines the axial imaging resolution of the system. The transverse resolution was measured at 15 μm. For the scanning speed of the reference arm at 100 A-scan/s, a typical OCT image of 256 × 200 pixel size requires about 2 s.[15]

Adult guinea pigs were sacrificed for the experiments. The imaging was performed within 0–1 h right after the animals were sacrificed. The imaging sites were chosen at the abdomen, where thick hairs were removed to expose the skin directly to the air. Immediately after the first OCT image, 60% glycerol solution (60%G) or the combination of 60% glycerol solution and ultrasound (60%G/SP) was applied onto the skin surface and the tissue was allowed to absorb the chemical for 30 min. Another OCT image was then taken at the same site, allowing us to scrutinize the OCT expressions of tissue before and after the agent application. All the images presented in this paper are expressed in

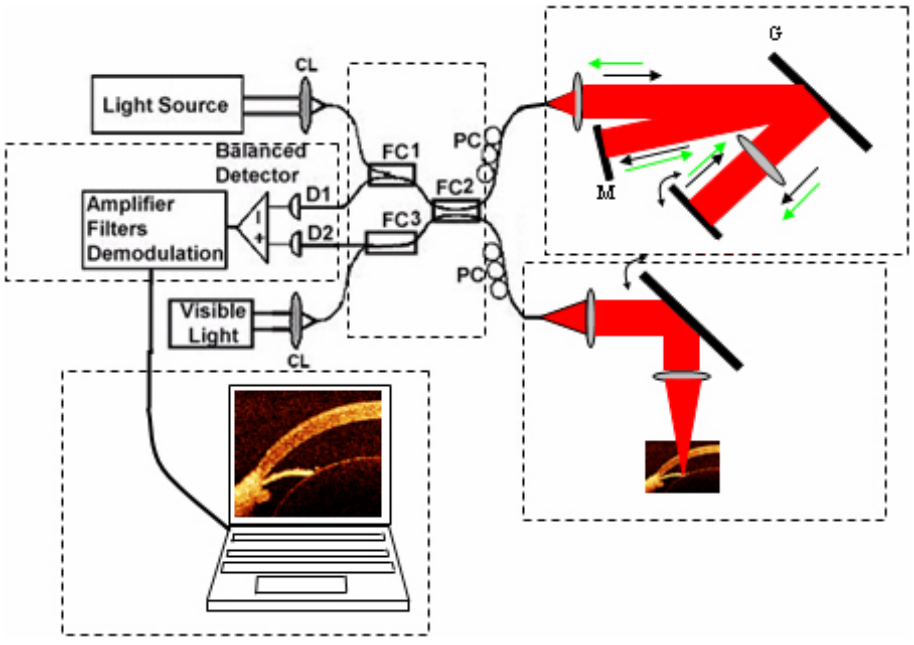


Fig. 1. Schematic of the OCT system, where CL is the collimating lens, FC the fiber coupler, PC the polarization controller, OL the objective lens, and D the detector.

units of millimeters, with the vertical axis representing the depth and the horizontal axis representing the spatial length.

B-328 sonicator (Beauty and Health Instrument Company, Ltd., Guangzhou, China) operating at a frequency of 1–1.1 MHz equipped with a 3-cm diameter probe was used for ultrasound application.

Once the glycerol solution was applied, ultrasound treatment was performed on the samples for a period of 15 min. During sonication, the ultrasound probe was immersed in the agent solution that was topically applied onto the skin. Ultrasound at 0.75 W over the 3-cm probe was applied in a pulsed mode at the frequency of 4 MHz to minimize thermal effects. After 15-min ultrasound-treatment, the sample was kept occluded with the solution for a total of 30 min. In other words, for the first 15 min of treatment, the skin sites were applied with the combination of 60% glycerol and ultrasound, while the control skin sites were only applied with 60% glycerol for the whole 30 min.

Digital reregistration of the uneven skin surface in the OCT data based on an edge-detection algorithm was conducted to obtain a flat surface. Quantitative data were obtained by averaging the linearized signal intensity across the lateral imaging range as a function of depth. A best fit exponential curve covering epidermis and dermis in depth was applied to the averaged and normalized signal intensity data from which the corresponding $1/e$ light penetration depth was derived.[15,16]

The native and treated skin samples with 60%G or 60%G/SP were cut for SEM observation after OCT imaging. After removal of subcutaneous tissue and fat as much as possible, the skin samples were fixed by immersion in 5% glutaraldehyde for 24 h at 4°C, post-fixed with 1% osmium tetroxide for 2 h at 4°C, and dehydrated in a gradient alcohol series. Then, the samples were covered with carbon and gold prior to examination in a SEM system of TM-1000_5748 (Hitachi Ltd., Tokyo, Japan).

3. Results and Discussions

Figure 2 shows the OCT images of *ex vivo* guinea pig skin exposed to air [Fig. 2(a)], topically applied with 60%G [Fig. 2(b)] and 60%G/SP [Fig. 2(c)] at 0 and 30 min. Native skin exposed to air for 30 min showed little changes in the OCT image depth [Fig. 2(a)]. From Fig. 2(c), it can be seen that light penetration depth for the skin treated with 60%G/SP was significantly enhanced after 30 min of treatment as the signal coming back from the stratum basale layer was increased. OCT imaging of the skin where only 60%G was applied has shown a little enhancement in imaging depth [Fig. 2(b)].

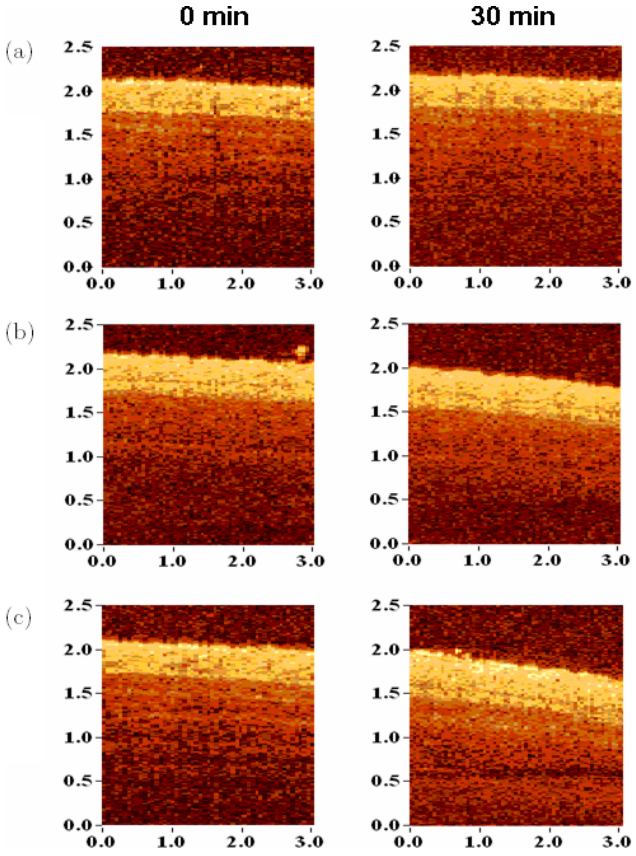
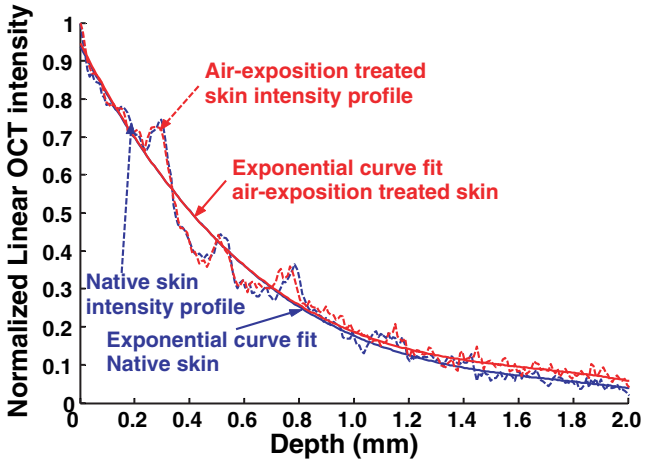
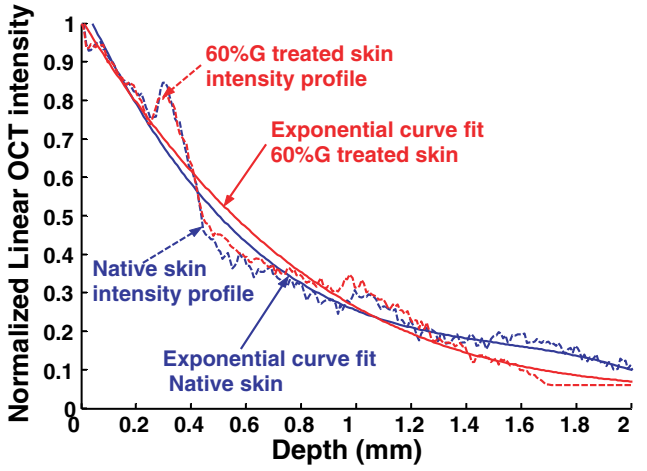


Fig. 2. OCT images of *ex vivo* guinea pig skin (a) exposed to air, (b) topically applied with 60% glycerol, and (c) 60% glycerol with 15-min ultrasound at 0 and 30 min.

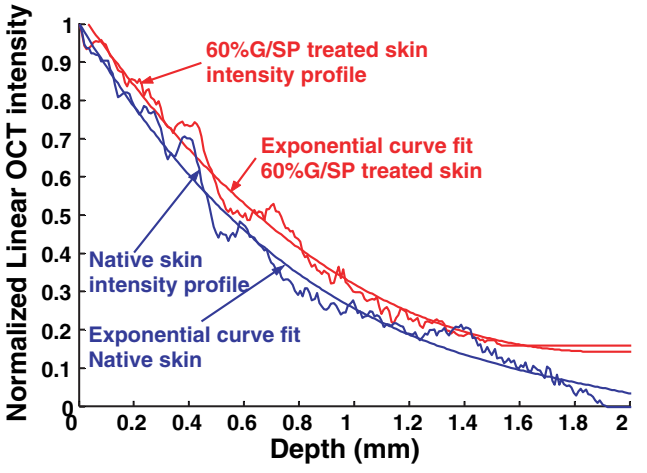
In order to define imaging depth more quantitatively, the OCT depth intensity profiles with corresponding exponential best fit curves of untreated, 60%G and 60%G/SP treated *ex vivo* skin have been shown in Fig. 3. Figure 3(a) shows that there was almost no changes in the $1/e$ light penetration depth of the native skin exposed to air for 30 min. The $1/e$ light penetration depth of epidermis and dermis increased by roughly 8.7% and 18.6% from the native to the optically cleared state with 60%G [Fig. 3(b)] and 60%G/SP [Fig. 3(c)] at 30 min, respectively. The results demonstrated that the combination of glycerol and ultrasound was much more effective on optical clearing of *ex vivo* guinea pig skin than glycerol alone. The results are in agreement with our previous findings of enhanced *in vivo* human skin optical clearing with ultrasound.[15] Glycerol alone had some effects on *ex vivo* skin in this study. However, it



(a)



(b)

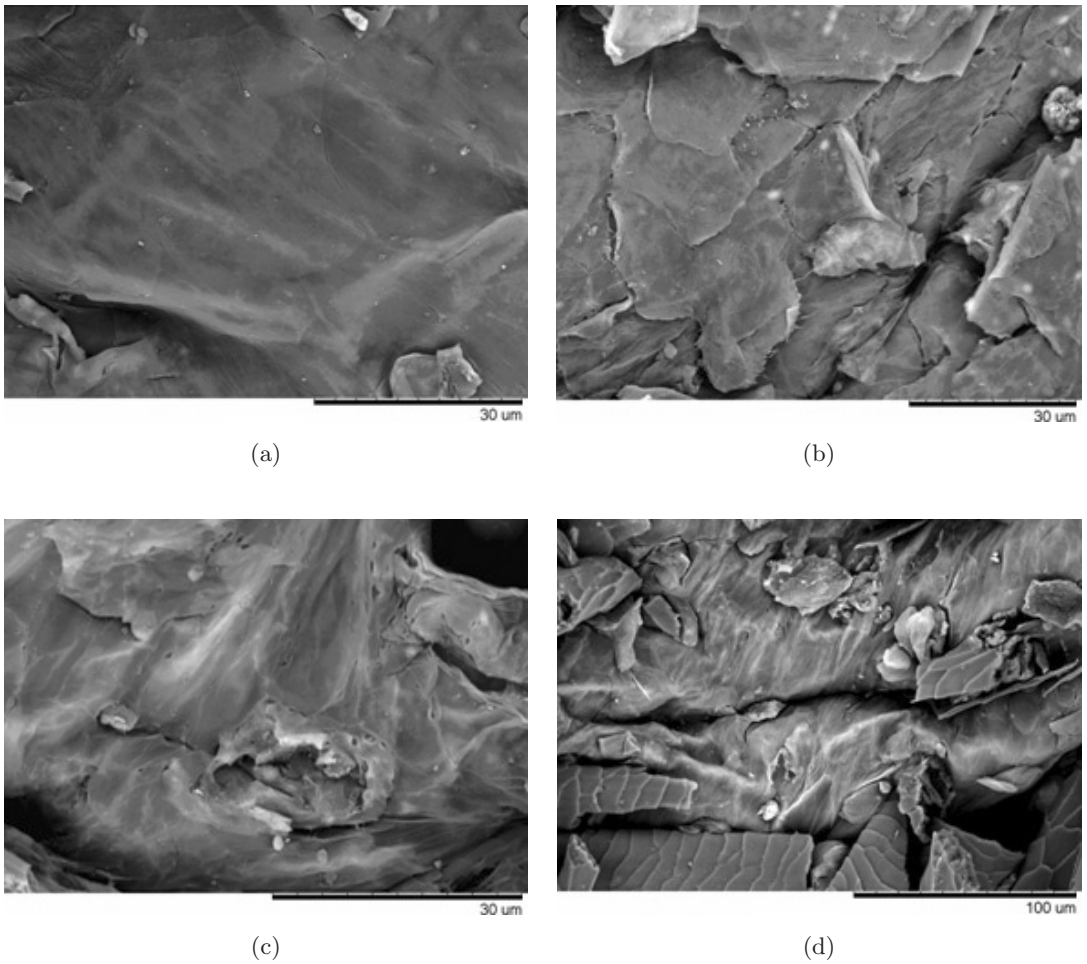


(c)

Fig. 3. OCT intensity profiles of (a) native guinea pig skin exposed to air for 30 min, (b) the skin with 60% glycerol, and (c) the skin with 60% glycerol and 15-min ultrasound at 1310 nm.

is extremely difficult for glycerol alone to permeate into *in vivo* human skin without ultrasound enhancement.[15] Glycerol alone could easily penetrate into *in vitro* porcine skin and is an effective clearing agent for *in vitro* skin.[14,16] This is probably because the microscopic structures within the *in vitro* skin stratum corneum have been naturally destroyed after slaughter.

As known, stratum corneum in the skin surface is mainly composed of the keratinocytes and lipid matrix composition. The keratinocytes in the stratum corneum lipid matrix are like the brick wall structure, i.e., the keratinocytes are piled with intervals in a continuous substrate of specific lipid composition. That stratum corneum lipid composition as membranous structures fill in keratinocytes interval space is a major factor in the formation of the skin barrier.[17] The main functions are to prevent the loss of water and nutrients, and to resist the invasion of external harmful substances. Therefore, the stratum corneum barrier function limits the penetration of transdermal drugs and optical clearing agents into intact skin. Figure 4 shows the SEM images of native skin (a), 60%G treated skin (b), and 60%G/SP treated skin (c) and (d). From Fig. 4(a), we can see that the stratum corneum of the guinea pig skin exposed to air for 30 min was intact without any damage and the cell boundaries were not obvious. Figure 4(b) shows the microstructure changes in the skin surface topically applied with 60% glycerol for 30 min. The cell boundaries were more evident and some tiny cracks were found. This allowed the penetration of glycerol into the skin, thereby contributing to the *ex vivo* skin optical clearing to some degree. The most significant changes in microstructure occurred when the skin was topically applied with the combination of 60%G and ultrasound, shown in Figs. 4(c) and 4(d). The cell boundaries within the stratum corneum and changes in layered arrangement structures of the lipid matrix were clearly seen. There was a notable separation of the keratinocytes or even broken cell, causing a discontinuous stratum corneum, and more fractures, larger hair follicles and sweat glands. The results indicate that widened fractures, loose cell arrangements in the stratum corneum, and the expanding hair follicles and sweat gland cells after ultrasonic medication lead to a weakened skin barrier function, which contribute to rapid penetration of glycerol into the skin. More glycerol penetrates the skin to achieve more refractive index



(a) (b)

(c) (d)

Fig. 4. SEM images of (a) native skin, (b) skin with 60% glycerol, and (c), (d) skin with 60% glycerol and 15-min ultrasound for 30 min.

matching in the skin,[18] and to cause decrease in the thickness of dermis and the corresponding more regular packing of tissue fibers,[19] therefore more effectively reducing light scattering in the skin.

4. Conclusions

We demonstrate in this paper that changes in *ex vivo* guinea pig skin microstructure due to the tight order of the lipid bilayers in the disrupted stratum corneum and separation of the keratinocytes by the application of ultrasound contribute to the ultrasound-enhanced intact *ex vivo* skin optical clearing effects.

Acknowledgment

This research was supported by research grants from the National Natural Science Foundation of China (Nos. 30470426, 30870675).

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