Influence of alcohols on the optical clearing effect of skin *in vitro*

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Abstract. The optical clearing technique has shown great potential in improving light penetration into biotissues. Among various optical clearing agents (OCAs) under study, the hydroxyl-terminated agents induce the highest optical clearing effect of skin, but the exact mechanism of optical clearing is still unclear. In consideration of several probable factors, such as the number of hydroxyl groups, the refractive index, and the molecular weight, we investigate the optical clearing effect of porcine skin after applying six alcohols to the epidermis and saline to the dermis. The dynamical transmission intensity of porcine skin is monitored by an integrating sphere system, and the thickness of skin samples is measured before and after experiments. The results show that the transmittance of skin increases significantly, but there is no significant change in thickness after the treatment of OCAs. The optical clearing effect of skin induced by alcohols is related to the number of hydroxyl groups. The refractive index or molecular weight of optical clearing agents does not correlate with the degree of optical clearing effect for a 60-min time interval of measurement. However, the behavior of skin transmittance after 60 min needs to be further investigated.
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Keywords: optical clearing agents; alcohols; skin; refractive index; molecular weight; number of hydroxyl groups.

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1 Introduction

The optical clearing of tissues by the use of biocompatible chemical agents is a new approach in the area of biomedical optics, which can effectively enhance the penetration of light in tissue.[1–19] This method combined with other forms of optical imaging, such as multiphoton microscopy imaging[20–22] and optical intrinsic signal imaging,[23,24] would improve the capabilities of noninvasive light-based diagnosis, which would result in a significant accomplishment in life sciences.

Among the investigations of the optical clearing of various tissues, skin optical clearing attracts extensive attention.[9,10,12–19] Different optical clearing agents (OCAs), such as glycerol, polyethylene glycol (PEG), butanediol, trimethylol propane, the combined PPG- and PEG-based prepolymer mixture, sorbitol, xylitol, glucose, dextrose, fructose, sucrose, oleic acid, dimethyl sulphoxide (DMSO), and so on, were applied to study the optical clearing effect of skin.[9,10,12–19] These chemical agents can be classified by their chemical structure as alcohols,[9,10,12,13,17–19] sugars,[10,15–19] organic acid,[9,14] and other organic solvents.[9,10,14,18]

To investigate the optical clearing mechanism of skin after application of agents directly to the dermis, Choi et al. inquired into the dependence of optical clearing potential (OCP) on refractive index and the osmolality of three different groups of OCAs,[9] i.e., hydroxy-terminated agents, organic solvents, and organic acids. They found that the mean agent OCP did not correlate with refractive index or osmolality. Among the three groups of OCAs, the hydroxy-terminated organic compounds, named alcohols, demonstrated the highest OCP. However, different alcohols also showed different OCP, which was not given attention.

In clinical applications, it would probably be more common that the OCAs are applied to the surface of epidermis and allowed to diffuse into the dermis. Hence the optical clearing effect of skin might depend on the quantity of an agent reaching the derma. Substances with low molecular weight (<500 Da) are able to penetrate skin tissue more easily.[25] Thus, the molecular weight of an agent may be a potential factor that influences the optical clearing effect. It has been reported that the optical clearing effect of blood is relative to the molecular weights of dextrans.[26] However, it is unclear whether the molecular weight of other OCAs, such as alcohols, influence the optical clearing of skin.

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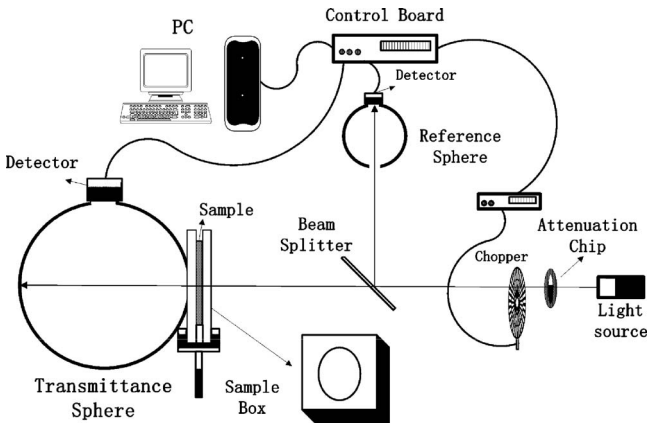

Fig. 1 Experimental setup (sample box shows the diffusion chamber).

Here we investigated the optical clearing effect of porcine skins after applying the epidermis with some alcohols based on the measurement of the dynamical transmission intensity. Considering several probable factors, such as hydroxyl group numbers, refractive index, and molecular weight, we discussed whether there was a correlation between the skin optical clearing effect and the three characteristics of OCAs.

2 Materials and Methods

2.1 *Preparation of Skin Samples*

Skin specimens were obtained from the porcine abdomen in a local slaughterhouse within 1-h postmortem. All samples were measured within 8 h and stored at 4 °C. The stratum corneum and the subcutaneous fat of the whole skin specimens were cleaned before the samples were dissected for measurements. The samples were cut into 50 × 50-mm sections, and clamped tightly between two apertures of horizontal diffusion chambers. The diffusion chambers were made of two colorimetric wares. Each chamber was 50 × 50 × 5 mm, with an aperture of 30 mm in diam on one side, as shown in Fig. 1. One chamber near the epidermis was filled with OCAs, and the other one was filled with isotonic saline. Both sides of the skin were able to react to the solution through the apertures. Because the osmotic pressure of isotonic saline was similar to *in vivo* physiological conditions, the diffusion chambers were able to simulate the physiological environment of skin *in vivo*.

In this work, all samples (n=50) were divided into seven groups, which included six experimental groups and a control group. The thickness of sample was measured by using a micrometer. The native thickness was about 1.61 ± 0.11 mm.

2.2 *Chemical Agents*

The hydroxy-terminated agents have high OCP,[9] small substances (<500 Da) easily penetrate the skin,[25] and the refractive indices of mammalian tissues are about 1.40,[27] so, in this study, we chose six alcohols with high refractive indices as OCAs, i.e., 1-butanol, 1, 4-butanediol, 1, 3-propanediol, PEG200, PEG400, and glycerol (Qiangsheng Chinese Chemicals, Limited, China). Their molecular weights range from 74 to 400 Da. At room temperature (~25 °C), the refractive index was measured with a refractometer (Digital Abbe Refractometer, WAY-2S, Shanghai, China). Table 1 shows refractive indices, molecular weights, and numbers of a hydroxyl group of the OCAs used in this study.

Table 1 Characteristics of the different alcohols and saline investigated in this study.

	Molecular weight	Refractive index	N (–OH)
1-butanol	74.12	1.40	1
1, 4-butanediol	90.12	1.45	2
1, 3-propanediol	76.10	1.43	2
PEG200	190 to 210	1.46	2
PEG400	380 to 420	1.47	2
Glycerol	92.09	1.47	3
Saline	58.0	1.33	0

2.3 *Experimental System*

An integrating sphere system was applied to monitor the transmission intensity of skin samples under the treatment of OCAs (see Fig. 1). A 1-mm-diam beam He–Ne laser (λ =632.8 nm, 5 mW, Melles Griot, Carlsbad, California) was chopped mechanically at 1-kHz (model SR 540), and then split into two beams by a beamsplitter. The reflected beam was a small fraction (20%) of the laser beam, which irradiated to a reference sphere of 70 mm in diam. The rest (80%) of the laser beam irradiated to the sample mounted in front of the port (ϕ=25 mm) of a big integrating sphere of 210 mm diam. The intensity of the transmitted light from the sample, including collimated and diffuse components of transmitted radiation, was collected by the integrating sphere. The signals from the Si PIN photodiodes (1223-01, Hamamatsu) in both integrating spheres were inputted into the control circuit, finally, sampled by PC. In this system, the reference measurements of laser power could reduce experimental error because of the fluctuance of laser power. The transmission intensity in the experiment was the ratio of the value detected by the big sphere to that by the reference sphere.[28]

2.4 *Measurement Method*

Experiments were conducted at room temperature (~25 °C). Each skin sample was clamped between two diffusion chambers, which were held together. 12 ml of OCAs was added to the donor cell in contact with the epidermal side of the skin, while 12 ml of isotonic saline was added to the receptor cell in contact with the dermal side. The chambers were set up with the receptor cell touching the port (ϕ=25 mm) of the integrating sphere. To determine the OCAs induced optical clearing of skin, the dynamical transmission intensity of samples was measured at 0, 1, 2, 3, 4, 5, 10, 15, 20, 25, 30, 40, 50, and 60 min after the application of OCAs. The relative transmittance can be deduced as the following formula:

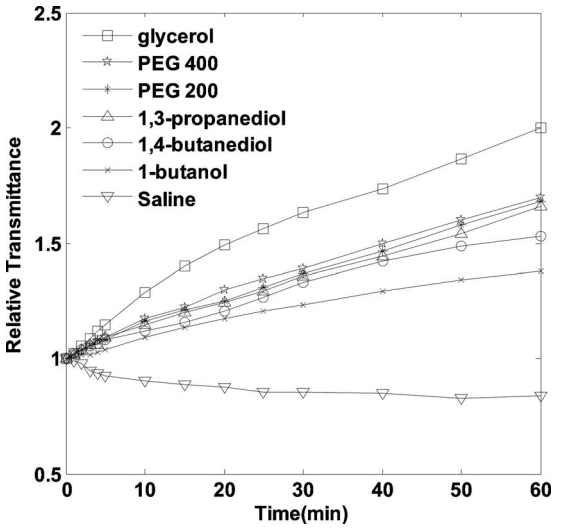

Fig. 2 Relative transmittance of porcine skins after treatment with six alcohols and saline solution.

$$T = \frac{I_t}{I_0}. \quad (1)$$

Here I_t and I_0 are the transmission intensity measured at time t and initially ($t=0$), respectively.

After the measurement, the skin samples were taken from the chambers and wiped dry. The surrounding exposed area was cut away, and then the thickness of skin was measured.

2.5 *Statistical Analysis*

SPSS 13.0 statistical software (SPSS Limited, Chicago, USA) was used to perform an F test among all the groups to compare the differences both in thickness changes and in relative transmittance after the experiment. Within each group, a paired-sample t-test was performed to compare skin thickness before and after an experiment. The Pearson correlation coefficient, relating relative transmittance to the refractive index and molecular weight of OCAs, was calculated.

3 Results

3.1 *Relative Transmittance of Porcine Skin*

Based on the measurements of transmission intensity, the relative transmittance of skin was deduced. Figure 2 shows the average changes of relative transmittance with time. The error bars in the plot are not shown because they are close in several groups.

The results indicate that the relative transmittance of skins increases gradually after treatment with the OCAs. In contrast, there is some decrease in the control group alone after treatment with the isotonic saline. Among the alcohols, glycerol shows the greatest changes in relative transmittance, and 1-butanol shows the poorest.

3.2 *Skin Thickness and the Corresponding Change after the Experiment*

Table 2 lists the thickness of samples before and after the experiment, and the corresponding change. It seems that there is slight shrinkage in all the experimental groups and a slight swell in controls. However, a paired-sample t-test shows that there is no significant difference within each group, and an F-test shows that there is no difference in thickness change among groups ($P > 0.05$).

Table 2 Skin thickness before and after experiments and the corresponding changes (d_0 is the thickness before experiments; d is the thickness after experiments; and Δd is the thickness changes after experiments).

	d_0 (cm)	d (cm)	Δd (cm)
1-butanol	1.56±0.11	1.54±0.13	−0.02±0.05
1, 4-butanediol	1.65±0.12	1.63±0.10	−0.02±0.02
1, 3-propanediol	1.68±0.08	1.57±0.03	−0.10±0.09
PEG200	1.63±0.12	1.58±0.09	−0.05±0.04
PEG400	1.54±0.08	1.50±0.20	−0.02±0.18
Glycerol	1.58±0.12	1.51±0.11	−0.07±0.11
Saline	1.66±0.10	1.74±0.05	0.07±0.01

3.3 *Correlation between the Optical Clearing Effect and the Number of Hydroxyl Groups in Chemical Structure*

According to the number of hydroxyl groups in the chemical structure, we can divide the OCAs used in this work into three categories: monohydric alcohols (1-butanol), diatomic alcohols (PEG400, PEG200, 1, 3-propanediol, and 1, 4-butanediol), and triatomic alcohols (glycerol). The maximal relative transmittance of the skin 60 min after treatment with three categories of alcohols is shown in Fig. 3. The maximum of the relative transmittance is almost 2.00 induced by glycerol, whereas the minimum is only 1.38 induced by 1-butanol. There is no statistical difference among the other four alcohols, diatomic alcohols, i.e., PEG400, PEG200, 1,3-propanediol, and 1,4-butanediol. Therefore, we can conclude the main features: 1. glycerol, triatomic alcohol, has the greatest effect; 2. 1-butanol, monohydric alcohol, has the poorest effect; and 3. the four diatomic alcohols are moderate. In other words, the more hydroxyl groups an OCA has in its chemical structure, the more effective it is in skin optical clearing.

3.4 *Correlation between the Optical Clearing Effect and the Refractive Indices of Optical Clearing Agents*

The refractive indices of the OCAs used in the work vary between 1.40 and 1.47. Figure 4 illustrates the relation between the maximal changes in the relative transmittance of skin and the refractive indices of OCAs. The results show that the correlation coefficient is only 0.12. To eliminate the effects caused by the chemical structure of OCAs, we further discussed diatomic alcohols' induced optical clearing effect of skin. The correlation coefficient between their refractive indices and the corresponding maximal relative transmittance is

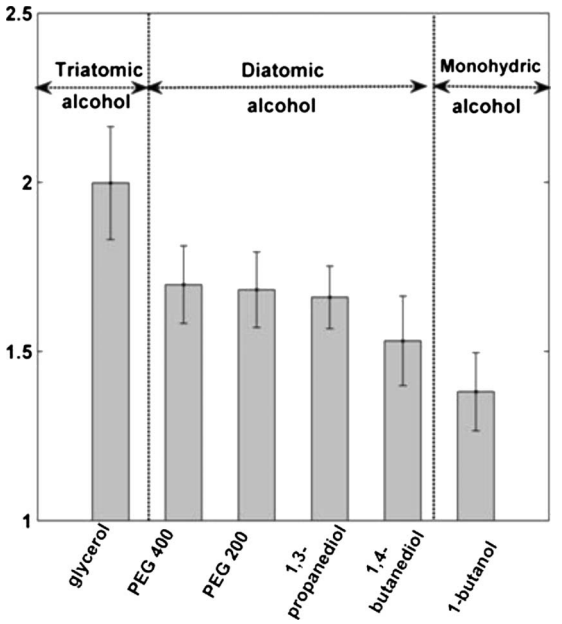

Fig. 3 The maximal relative transmittance of skin samples treated by three categories of alcohols: triatomic, diatomic, and monohydric alcohols.

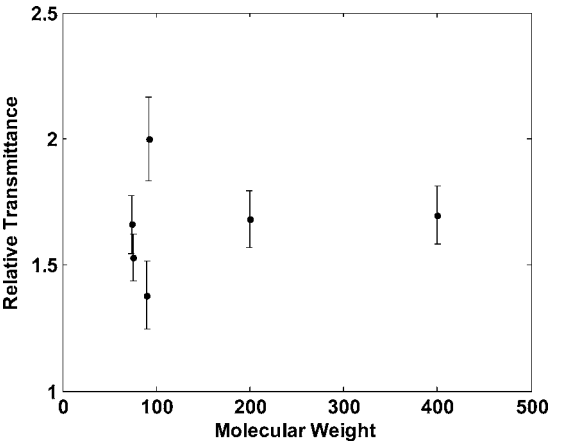

Fig. 5 Correlation between the maximal relative transmittance of skin and OCA molecular weights.

0.22. Therefore, the optical clearing effect of skin treated for 60 min has no correlation with the refractive index of OCAs.

3.5 *Correlation between Optical Clearing Effect and Molecular Weights of Optical Clearing Agents*

The OCAs' molecular weights and the corresponding relative transmittance of skin at 60 min are shown in Fig. 5. The correlation coefficient is only 0.08. Though small substances (<500 Da) easily penetrate the skin, the molecular weights of alcohols do not affect the optical clearing of skin directly when they range from 74 to 400 Da.

4 Discussion

The response of tissue to chemical agents is a reduction in light scattering and corresponding increase in optical clarity.[29]

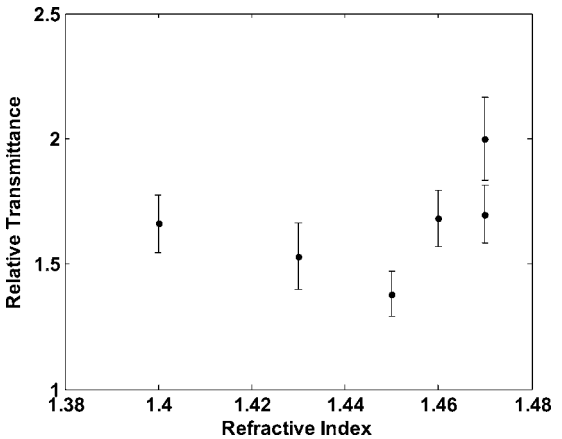

Fig. 4 Correlation between the maximal relative transmittance of skin and OCA refractive indices.

The tissue optical clearing technique has shown potential in optical diagnostics and therapy. However, the investigation about the mechanism of skin optical clearing is still localized on the *in vitro* situation.[6,9,14–16,29–31] The dehydration and shrinkage of skin was marked when the OCAs were directly placed on the dermis.[29,31] In clinical applications, the OCAs would probably be applied to the epidermis, and then diffuse into the dermis. The action of OCAs is very similar to transdermal drug delivery under physiological situations, so we apply diffusion chambers to simulate transdermal OCA delivery *in vivo*. In contrast with Choi et al., one chamber near the epidermis was filled with OCAs, and the other one near the dermal side was filled with isotonic saline in this work. The OCAs cannot be effective unless they penetrate from epidermis to dermis; on the other hand, part of the OCAs in the dermis was replaced by saline in the receptor cell for the substance exchange. Therefore, the effective OCAs in the dermis in this work may be less than that in previous studies,[9,14,29,31] and the corresponding dehydration and shrinkage of skins may also be milder. As a result, there is no significant change in skin thickness after treatment of OCAs.

Human skin consists of three layers: epidermis, dermis, and subcutaneous tissue. The epidermis is hydrophilic, so the hydroxy-terminated agents are able to penetrate the epidermis to the dermis easily.[9,25,30] The experimental results indicate that the number of hydroxyl groups in the chemical structure is relative to the optical clearing effect of skin. If an OCA has more hydroxyl groups, it can penetrate the skin more easily, and the corresponding transmittance of skin increases.

The high light scattering of tissue is mainly from refractive indice mismatches between cellular components and the extracellular fluid. However, the light scattering can be reduced due to refractive indices matching of the scatterers and the ground matter by immersing in hyperosmotic and refractive index matching agents.[2,3,32] The refractive indices of the OCAs used in this work range from 1.40 to 1.47. The experimental results show that the clearing effect has no correlation with the refractive index. It means the refractive index is not a direct factor that affects the optical clearing of skin. This result is consistent with previous studies.[9]

Smaller substances (<500 Da) are able to penetrate skin more easily,[25] and this means that the molecular weight may affect the penetrability of agents. However, the experimental results indicate no correlation between the optical clearing effect and the molecular weight when the molecular weight of the OCA is less than 400 Da. It demonstrates that the molecular weight of OCAs does not influence the degree of optical clearing for smaller alcohols directly.

As seen in Fig. 2, optical clearing does not complete in 60 min, and so, it is possible that the OCAs with large molecular weights (PEG200 and PEG400) do not penetrate into the area of detection. Thus, after a longer observation time, the degree of optical clearing by PEG200, PEG400, and glycerol may be equal, thus the behavior of skin transmittance after 60 min needs to be further investigated.

5 Conclusion

We investigate the skin optical clearing effect after application of six alcohols to the epidermal side, and application to the dermal side with saline in view of the number of hydroxyl groups, the refractive index, and the molecular weight. The results show that the optical clearing effect of skin caused by alcohols is related to the number of hydroxyl groups. The more hydroxyl groups an OCA has in its chemical structure, the more effective it is in skin optical clearing. The refractive index or the molecular weight of optical clearing agents does not correlate with the degree of optical clearing effect for a 60-min time interval of measurement. However, the skin optical clearing process is not complete and the behavior of the skin transmittance after 60 min is unclear. Therefore, a long-term observation needs to be further investigated.

Acknowledgments

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