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Abstract. Dehydration induced by optical clearing agents (OCAs) can improve tissue optical transmittance; however, current studies merely gave some qualitative descriptions. We develop a model to quantitatively evaluate water content with partial least-squares method based on the measurements of near-infrared reflectance spectroscopy and weight of porcine skin. Furthermore, a commercial spectrometer with an integrating sphere is used to measure the transmittance and reflectance of skin after treatment with different OCAs, and then the water content and optical properties of sample are calculated, respectively. The results show that both the reduced scattering coefficient and dehydration of skin decrease with prolongation of action of OCAs, but the relative change in former is larger than that in latter after a 60-min treatment. The absorption coefficient at 1450 nm decreases completely coincident with dehydration of skin. Further analysis illustrates that the correlation coefficient between the relative changes in the reduced scattering coefficient and dehydration is ~1 during the 60-min treatment of agents, but there is an extremely significant difference between the two parameters for some OCAs with more hydroxyl groups, especially, glycerol or D-sorbitol, which means that the dehydration is a main mechanism of skin optical clearing, but not the only mechanism. [DOI: 10.1117/1.3621515]

Keywords: optical clearing; quantitative analysis; dehydration; partial least-squares; near-infrared reflectance spectroscopy; scattering; skin.

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1 Introduction

Optical techniques are widely used in the fields of medical imaging, diagnosis, and therapy,[1] but the limited penetration depth due to the high turbidity of tissue limits their clinical application. Recently, a tissue optical clearing technique has been developed to reduce the scattering properties and improve the light penetration depth by application of optical clearing agents (OCAs) with high refractive indices, hyperosmolarity, and biocompatibility.[2,3] This technique allows for significant improvement of spectroscopic and imaging techniques in application to tissue investigation and treatment control.

Among the investigations on the optical clearing of various tissues, skin optical clearing attracts much attention because skin is a highly scattering tissue for which OCA is easy to apply. The previous studies mainly concentrated on screening OCAs,[4] improving the delivery of agents into skin to enhance the optical clearing efficacy,[5] yet the mechanisms of skin optical clearing remain not entirely understood. In general, at OCA application, two counterdirectional fluxes of OCA into and interstitial or intracellular water out of tissue should be created.[3] Both processes lead to refractive index matching of tissue scatterers and surrounding media, and therefore, to less light scattering and optical clearing. The refractive index matching was regarded as the major mechanism of tissue optical clearing.[2] However, this opinion was not supported as the only mechanism of optical clearing, because optical clearing effects in skin did not correlate with OCAs' refractive indices and osmolality.[4,6–8] Tissue initial structure, in particular, the ability of ordering of its scattering components at dehydration may have a considerable inclusion in the optical clearing effect.[3,9] After having investigated six kinds of OCAs (i.e., DMSO, glycerol, 1,4-butanediol, 1,2-propanediol, PEG200, and PEG400), Wen et al.[7] also observed that the OCA with a higher refractive index could induce better optical clearing efficacy except for the last two. Because PEG200 and PEG400 can induce the solution to be more transparent by making the aggregation of intralipid particles and change the structure of sample, though they have lower refractive index compared to DMSO or glycerol. Actually, Yeh et al.[6] and Hirshburg et al.[10] also found the dissociation of collagen after performing glycerol on *in vitro* skin, but Wen et al. just observed the decrease in the fibril size without disassembly by *in vivo* study.[11] In contrast, the dehydration of skin has been proved to be an important mechanism in the optical clearing process and a widely acceptable opinion.[3,9,12–15]

In order to study the correlation between the dehydration and optical clearing efficacy, the dehydration of skin induced by OCAs used to be evaluated by the mass loss of skin.[5,16]

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Oliveira et al.[17] also recently obtained the percentage for water loss of the rat muscle by measuring the mass and the refractive index. However, the change of the sample's mass should not only include the efflux of interstitial fluid of skin but also the influx of OCAs into tissue.[18] Therefore, it is necessary to develop a quantitative analysis method to assess the water content of skin, which will be helpful to clarify and quantify the mechanism of optical clearing of skin. Two typical techniques, such as the electrical capacity measurement and the near-infrared reflectance spectroscopy, are proposed to evaluate tissue hydration. Especially, some noninvasive instruments based on electrical measurements have been developed and widely applied to detect the moisture of stratum corneum.[19] However, the dehydration of skin caused by OCAs is not only from the epidermis, but also the dermis. In contrast to the capacitance method, the near-infrared reflectance spectroscopy measures the water content directly and brings the information from depth of tissue, and provides information of the water content in skin.[12,20–24] Arimoto and Egawa showed a series of absorbance spectra transferred from reflectance spectra and thought the changes were relative to the water loss of porcine skin.[23] And Xu et al. investigated the water content of porcine tissue by use of measurements of differences in absorbance at two wavelengths (1100 and 1936 nm).[12,25]

Woo et al.[26] developed a good calibration model to predict the water content in forearm skin by means of partial least-squares (PLS) regression based on the NIR spectra in the 1150–1650 nm region and the relative water content values. And they obtained the standard water content by putting the skin sample in the desiccator with silica gels first and then by drying at 105°C to get constant weight. While Vornheder and Brabbs[27] indicated that a 105°C oven or toluene distillation would induce the decomposition of carbohydrates while a vacuum oven and near-infrared procedure could overcome this problem. Moreover, PLS is a currently used valid multivariate calibration method for the development of a calibration model for the water content of skin in the NIR spectroscopy.

The goal of this study is to establish an effective method for quantitative evaluation of water content during the skin optical clearing process by using NIR spectroscopy, which might be helpful to fully reveal the mechanisms of tissue optical clearing. Since alcohols have higher optical clearing potential,[8] several common agents (i.e., 1,2-propanediol; 1,4-butanediol; PEG200; PEG400; glycerol; D-sorbitol) were used instead of one or two chemical agents in the previous studies. On the basis of the measurements of transmittance and reflectance spectra after application of different OCAs, both the optical properties and water content of porcine skin were calculated. Combining the relative changes in water content with the optical properties of skin during the optical clearing process, their correlation coefficient and the significance test have been performed. Furthermore, the mechanism of dehydration was discussed.

2 Materials and Methods

2.1 *Materials*

2.1.1 *Preparation of skin samples*

The porcine skin samples were obtained from the local slaughterhouse within 2-h postmortem. All samples were stored at 4°C and sealed to prevent natural dehydration. Before the measurements, we cleaned the stratum corneum and the subcutaneous fat of all the skin specimens. The skin samples were cut into 50×50 mm^2 sections with surgical scissors and mounted with two slides. Each slide was 50×50 mm^2.

Table 1 Characteristics of the different alcohols.

OCA	Molecular weight	Refractive index
1,2-propanediol	76.1	1.433
1,4-butanediol	90.1	1.446
PEG200	190 – 210	1.461
PEG400	380 – 420	1.469
Glycerol	92.1	1.475
Glycerol (70% v/v)	92.1	1.437
D-sorbitol (70% w/w)	182.2	1.473

2.1.2 *Optical clearing agents*

Six kinds of common alcohols with high refractive indices as OCAs are used in this work [i.e., 1,2-propanediol, 1,4-butanediol, PEG200 (polyethylene glycol 200), PEG400 (polyethylene glycol 400), glycerol, and D-sorbitol (Jiangsu Qiangsheng Chemical Co., Ltd., Jiangsu, China) (see Table 1)]. Most solutions are pure (100%) except for D-sorbitol, whose saturated solubility is 70%. In order to compare the results of same concentration, 70% glycerol is also used. The refractive indices of the agents measured with an Abbe refractometer (WAY-2S, Shanghai YiCe Apparatus & Equipment Co., Ltd., Shanghai, China) at 589 nm, and the molecular weights are listed in Table 1.

2.1.3 *Instruments*

In order to weigh the skin samples, a precision balance (PL203 Mettler, Toledo, Ohio) was used. An electric vacuum drying oven (Yuhua Instrument Plant, Gongyi, China) was used to dry the skin sample to get the water content of skin. The thickness of the skin was determined by a Vernier caliper. A commercially available spectrophotometer (Lambda 950, PerkinElmer, Inc., Waltham, Massachusetts) with an integrating sphere of 150 mm diam was used to measure the transmittance and the reflectance of samples. The integrating sphere has an entranceport and an exitport of 1 in diam.

2.2 *Development of the Dehydration Model*

In order to develop a dehydration model for predicting the water content of porcine skin, a weight-loss experiment was implemented. The precision balance was used to weigh the mass of skin samples ($n=8$) in a certain interval of time, while the spectrophotometer with an integrating sphere was applied to measure the reflectance spectra over 1100–1700 nm with a 2-nm interval. During the period of 0–8.25 h, the skin samples were dried in natural conditions without any help (such as a hairdryer,[16] which

might induce some thermal effects). Then the samples were put into an electric vacuum drying oven at 30–40°C and 1.013×10^5 Pa to obtain the mass of dry skin samples. The total dehydrating time is 140 h, and the mass of dried skin was recorded as m_s to calculate the water content as follows:

$$C_{x\,\min} = \frac{m_{x\,\min} - m_s}{m_{x\,\min}} \times 100\%, \quad (1)$$

where $C_{x\min}$ is the water content of the skin samples at different time intervals, $m_{x\min}$ is the measured mass after x minutes of treatment in natural condition.

The PLS regression was applied to elucidate the relationship between water content and NIR spectrum by using A ($\log 1/R$) spectra where R is the diffuse reflectance spectra and A is the apparent absorbance. To compare the accuracies of PLS model and dual-wavelength method for the water-content prediction in porcine skin samples, the differentiation of the absorbance at $\lambda_1 = 1100$ and $\lambda_2 = 1450$ nm was done as follows:

$$C = K(A_2 - A_1) = K\ \log(R_1/R_2), \quad (2)$$

where C is the concentration of the analyte, K is a constant, and A_i and R_i are the absorbance and diffuse reflectivity for λ_i ($i = 1, 2$), respectively.

2.3 *Optical Clearing Agent Induced Optical Clearing of Skin*

2.3.1 *Experimental protocol*

To further study the relationship between the dehydration and optical clearing efficacy, all the skin samples ($n = 84$) were divided into seven groups for different agents in Table 1, with 12 samples in each group. Then, 1 ml of agent was topically applied onto the epithelium surface (50×50 mm^2) of skin sample for a period time ranging from 0 to 60 min. Prior to the measurements, the skin surfaces were wiped clean of clearing agent with Kimwipes™ (Shanghai ANPEL Scientific Instrument Company, Limited, Shanghai, China), and then mounted with two slides. The transmittance and reflectance spectra over 400–1700 nm were measured respectively by the commercially available spectrophotometer with an integrating sphere, and the thickness was measured by the vernier caliper at time intervals of 0, 10, 20, 30, 40, 50, and 60 min.

2.3.2 *Data analysis*

Because both the transmittance and reflectance depend on the thickness of sample, it is more significant to demonstrate the optical clearing efficacy by using the reduced scattering coefficient. On the basis of the measurements of diffuse transmittance and reflectance, the inversed adding-doubling (IAD) algorithm was adopted to derive the reduced scattering coefficient by assuming a constant value of anisotropy factor (g) as 0.9, which is a typical value for skin in the visible and NIR spectral ranges.[28] With the algorithm, as described in our previous study, the optical properties are obtained by iterating an adding-doubling solution of the radiative transport equation until the calculated values of the transmittance and reflectance match the measured ones. At the same time, we obtained the water content of porcine skin by the established predicting model. In addition, the correlation coefficient was calculated to evaluate the validation of dehydration model we developed and also to evaluate whether there is similar change tendency between the water content and the reduced scattering coefficient of skin after application of a kind of OCA, and significance analysis was performed to assess whether there is any significant difference between both values for each group.

3 Results and Discussions

In order to build a model to assess the dehydration of skin, the PLS regression was applied to elucidate the relationship between water content and NIR spectrum of skin. To illustrate the validation of the dehydration model, we compared the predicting effect of PLS to the dual-wavelength method. On the basis of the changes in optical clearing efficacy and dehydration caused by application of the agents, the correlation between both values was analyzed. Finally, possible mechanisms of dehydration induced by OCAs were discussed.

3.1 *Validation of the Dehydration Model*

In this study, the PLS model was developed using $\log 1/R$ spectra within the spectral range from 1100 to 1700 nm. Figure 1(a) shows typical reflectance spectra in the range from 1100 to 1700 nm during the drying process of skin. It can be seen that the reflectance decreases sharply with wavelength in the range of 1100–1400 nm and then keeps a low level for the longer wavelength of 1400–1700 nm. There are two troughs of wave at 1190 and 1450 nm, respectively. The reflectance goes down with losing water in the shorter wavelength, while increases in the longer wavelength. The above results may be explained as follows: For the shorter wavelength range, where absorption of skin is not high, but scattering is still high, the reflectance is strong. Dehydration of skin induces the decrease of reflectance, which may be due to reduction of scattering caused by water loss. With the increase of wavelength, the scattering of skin goes down, which reduces the reflectance. In addition, the water content in skin is usually very high, and water has a weak absorption peak at 1190 nm and a strong absorption peak at 1450 nm, which is the reason that we observe two troughs of reflectance, and the former is much less than the latter. For the range of 1400–1700 nm, where absorption is considerable with the respect to scattering, response to dehydration is opposite to that where scattering is higher than absorption.

The PLS regression was applied to elucidate the relationship between water content and NIR spectrum by using apparent absorbance ($\log 1/R$) spectra. The corresponding changes of apparent absorbance are shown in Fig. 1(b). The mass of skin reached the constant value after 140 h of drying process. Figure 1(c) shows the water content changes of porcine skin against time for the first 76 h, where the major changes of mass were taking place.

To demonstrate the validity of the PLS model, we compared its predicted result to that of the dual-wavelength method. Figure 1(d) is the scatter plot, showing the correlation between the measured water content of porcine skin and the predicted values by PLS (solid circles) and dual-wavelength methods (open circles) values.

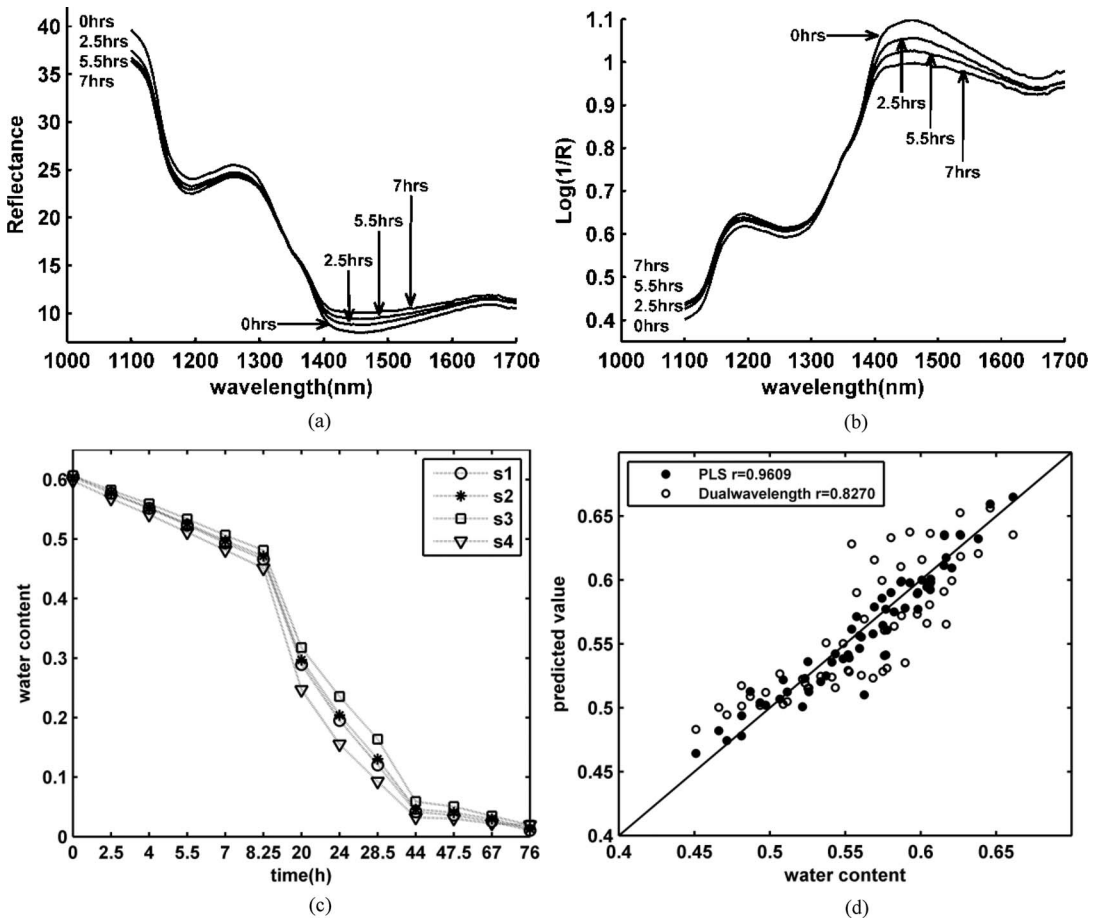

Fig. 1 The building process of water-content prediction model: (a) Reflectance spectrum at different time point in the dehydration process, (b) the corresponding transformed apparent absorbance spectrum, (c) water content changes of four porcine skin samples against time, and (d) scatter plot showing the correlation between the water content of porcine skin and the predicted by the PLS (solid circles) and dual-wavelength method (open circles) values.

Some previous studies adopted the dual-wavelength method to develop the model for acquiring the water content of porcine skin[16,25] (i.e., in the study by Xu et al.,[25] 1100 and 1936 nm were used). Here, the recordings at 1190 and 1450 nm (Ref. 24) were used to predict the value of water content. On one hand, 1450 nm is also one of the higher absorption peaks of water;[22] on the other hand, the SNR at 1936 nm is $\ll$ 1450 nm even though the commercial spectrophotometer (Lambda 950, PerkinElmer, Inc., Waltham, Massachusetts) with an integrating sphere was used to measure the reflectance of skin. It can be seen from Fig. 1(d) that the distribution of the predicted water content by PLS method had a smaller range than that by the dual-wavelength method. By computing the correlation coefficient of the predicted values of water content by these two methods regarding weighing measurements, the following data were received 0.9609 and 0.8270, respectively, for the PLS and dual-wavelength methods. Considering the uncertainty of the constant K, the error of the predicted results by the dual-wavelength method can be even more deviated in practical cases.

The dual-wavelength method just uses the recordings at two wavelengths, whereas the PLS method analyzes the whole spectral data, which includes more information about the water content. Furthermore, the uncertainty of K brings the analysis results a relatively large error. Therefore, PLS is better than dual-wavelength as the method for quantitative analysis of dehydration of the porcine skin.

3.2 *Optical Clearing Agent Induced Changes in Optical Properties of Skin*

To see the alterations of the reduced scattering coefficient induced by OCAs presented in Table 1, the transmittance and reflectance spectra over 400–1700 nm were measured and analyzed. Figures 2(a) and 2(b) illustrate typical spectral changes of transmittance and reflectance, respectively, as a function of time when the skin sample was treated with 1,2-propanediol as a typical example because the kinetic changes produced by the other agents have almost similar trends. The corresponding apparent absorbance spectra [Fig. 2(c)] in the wavelength range of 1100–1700 nm were used for the prediction of the water contents of skin samples during the optical clearing process. The reconstructed reduced scattering coefficient decreases with the time as shown in Fig. 2(d), which demonstrates the optical clearing process of the porcine skin. Figure 2(e) shows the changes of the reconstructed absorption coefficient corresponding to the measured transmittance and reflectance spectra.

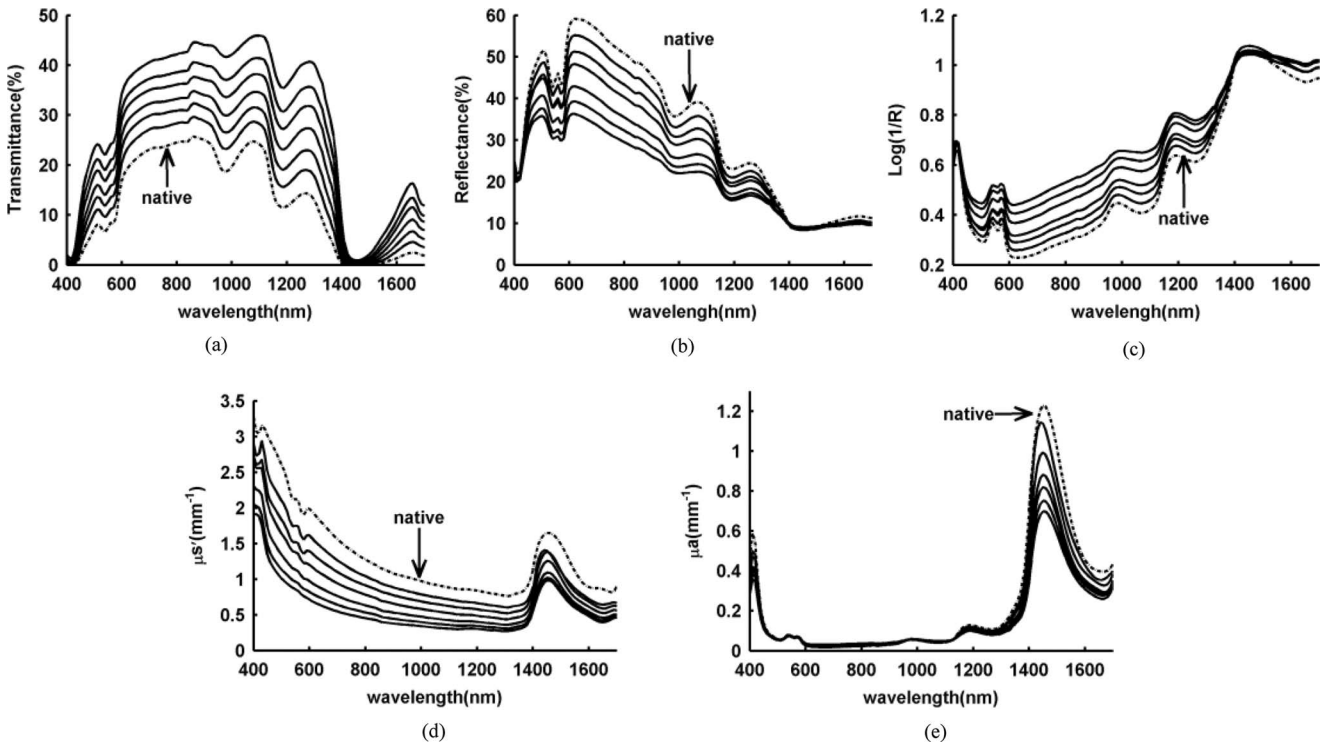

Fig. 2 Spectral changes for the skin sample before and after treatment by 1,2-propanediol, over the wavelength range of 400–1700 nm: (a) Transmittance after treatment by the agent at time intervals of 0, 10, 20, 30, 40, 50, and 60 min (from bottom to top); (b) corresponding reflectance spectra (from top to bottom); (c) corresponding apparent absorbance (log 1/*R*) (from bottom to top); (d) corresponding reconstructed reduced scattering coefficient spectra (from top to bottom); and (e) corresponding reconstructed absorption coefficient spectra (from top to bottom).

As shown in Fig. 2, the reflectance decreases with the passage of time in the range from 400 to 1370 nm, whereas the transmittance increases over the wavelength range under study (400–1700 nm). The transmittance and reflectance spectra of the porcine skin were obtained at time intervals of 0, 10, 20, 30, 40, 50, and 60 min. The reduced scattering coefficients at different time intervals were calculated with the IAD algorithm for each OCA action. The dashed lines in Fig. 2 represent the spectra of the native samples. The transmittance and reflectance spectra are transformed to be respectively lower and higher with the time course. The steps in the increase of transmittance and decrease of reflectance were almost uniform over a period of time ranging from 0 to 60 min, while the decrease of reduced scattering coefficient had the biggest step between a 0- and 10-min treatment by the agent.

It is noted that Fig. 2(b) shows a similar change trend of reflectance in the range of 1100–1400 nm to that in Fig. 1(a), but the numbers are different because clearing technologies are different–via direct dehydration or OCA application. However, for the longer wavelength range, the change in reflectance caused by OCA is not as obvious as that by pure drying. This may be the reason that pure drying only induces water loss and spatial correlation of scatterers with more effective packing or ordering, while OCA application would induce a more complex process with OCA-skin interactions.

It can be seen in Fig. 2(d) that the reduced scattering coefficients decrease with wavelength in the range of 400–1300 nm and have a relative big step that is beneficial to distinguish the optical clearing effect during the 60-min period, but there is a peak at a 1450-nm wavelength. It may be explained as a cross-talk between scattering and absorption values within the same band at 1450 nm [Fig. 2(e)], where absorption is strong, because of the calculation error of the IAD algorithm used. Previous investigations show the reduced scattering coefficient calculated by the IAD algorithm might be larger than the real value when the absorption is very strong and the scattering is weak, whereas the calculated value might be less than the real value when both the absorption and scattering are strong.[29,30] However, another more basic reason may have an effect. This is the anomalous dispersion of water within the absorption band, which may influence the refractive index of water (making it less for the shorter wavelengths within the band and higher for the longer wavelengths). Suppose that the main portion of water is in the interstitial space then we may expect that scattering coefficient will be increased first and further decreased as the wavelength changes through the absorption band. Anomalous dispersion effects at optical clearing evidently need more detailed theoretical and experimental study. At this time, it would be more valid to demonstrate the optical clearing efficacy of skin by analyzing the reduced scattering coefficient within the range of optical window (i.e., from 600 to 800 nm).

It is also interesting to note that optical clearing also affects spectral properties of absorption coefficient for both shorter (hemoglobin bands) and longer (water bands, i.e., 980, 1190, and 1450 nm) [Fig. 2(e)]. From the largest absorption peak at the 1450-nm wavelength, we find that the absorption coefficient

Table 2 Relative changes in skin thickness, reduced scattering coefficient at 760 nm, and water content after treatment of different OCAs, and the correlation coefficient between the latter two parameters.

OCA		10 min	20 min	30 min	40 min	50 min	60 min	*R*
1,2-PG	d/d_0	0.95 ± 0.02	0.93 ± 0.01	0.91 ± 0.03	0.89 ± 0.03	0.87 ± 0.03	0.86 ± 0.04	0.995
	μ_s'/μ_{s0}'	0.91 ± 0.04	0.82 ± 0.04	0.73 ± 0.03	0.66 ± 0.04	0.57 ± 0.04	0.48 ± 0.02	
	w/w_0	0.90 ± 0.03	0.81 ± 0.03	0.73 ± 0.03	0.68 ± 0.02	0.62 ± 0.02	0.56 ± 0.01	
1,4-BG	d/d_0	0.96 ± 0.01	0.95 ± 0.02	0.94 ± 0.02	0.92 ± 0.02	0.91 ± 0.02	0.90 ± 0.03	0.990
	μ_s'/μ_{s0}'	0.90 ± 0.02	0.81 ± 0.03	0.75 ± 0.03	0.70 ± 0.04	0.63 ± 0.03	0.57 ± 0.02	
	w/w_0	0.87 ± 0.01	0.80 ± 0.02	0.75 ± 0.03	0.71 ± 0.02	0.67 ± 0.02	0.64 ± 0.02	
PEG200	d/d_0	0.95 ± 0.03	0.93 ± 0.02	0.93 ± 0.03	0.92 ± 0.04	0.91 ± 0.04	0.89 ± 0.05	0.992
	μ_s'/μ_{s0}'	0.91 ± 0.02	0.83 ± 0.02	0.74 ± 0.02	0.68 ± 0.04	0.63 ± 0.03	0.58 ± 0.02	
	w/w_0	0.88 ± 0.02	0.80 ± 0.01	0.76 ± 0.04	0.70 ± 0.03	0.64 ± 0.02	0.61 ± 0.03	
PEG400	d/d_0	0.96 ± 0.01	0.94 ± 0.02	0.91 ± 0.01	0.91 ± 0.01	0.90 ± 0.02	0.88 ± 0.02	0.989
	μ_s'/μ_{s0}'	0.94 ± 0.02	0.86 ± 0.03	0.80 ± 0.03	0.71 ± 0.04	0.65 ± 0.02	0.60 ± 0.03	
	w/w_0	0.91 ± 0.02	0.86 ± 0.02	0.81 ± 0.03	0.77 ± 0.03	0.73 ± 0.03	0.68 ± 0.03	
Glycerol	d/d_0	0.98 ± 0.02	0.94 ± 0.03	0.93 ± 0.03	0.91 ± 0.04	0.89 ± 0.03	0.89 ± 0.04	0.998
	μ_s'/μ_{s0}'	0.88 ± 0.01	0.77 ± 0.01	0.66 ± 0.02	0.55 ± 0.03	0.46 ± 0.04	0.38 ± 0.02	
	w/w_0	0.89 ± 0.02	0.81 ± 0.02	0.73 ± 0.02	0.66 ± 0.02	0.60 ± 0.03	0.54 ± 0.02	
D-sorbitol	d/d_0	0.96 ± 0.01	0.94 ± 0.02	0.92 ± 0.03	0.91 ± 0.03	0.90 ± 0.03	0.89 ± 0.03	0.996
	μ_s'/μ_{s0}'	0.88 ± 0.02	0.78 ± 0.02	0.68 ± 0.01	0.60 ± 0.03	0.50 ± 0.02	0.42 ± 0.01	
	w/w_0	0.89 ± 0.02	0.83 ± 0.02	0.76 ± 0.02	0.71 ± 0.02	0.66 ± 0.01	0.59 ± 0.01	

of sample evidently decreases during the 60-min period. The absorption coefficient reduces to 57% after 60-min application of OCA, which is completely coincident with the predication of water content by using the developed dehydration model based on the reflectance spectra. Thus, we conclude that changes in the absorption coefficient at 1450 nm can reflect the dehydration of skin because there is an absorption peak of water at 1450 nm. Comparing the two methods to predict changes in the water content of tissue, the absorption coefficient at 1450 nm is more direct, while the PLS model based on NIR spectrum is valid for *in vivo*. The reason for that is not only algorithmic, but also physical: a decrease in scattering causes decreasing absorption due to shorter paths of photon tracing with the media with absorption.

3.3 *Correlation between Dehydration and Optical Clearing of Skin*

In order to quantitatively compare the skin optical clearing effect mediated by OCAs, we calculated the mean relative changes in reduced scattering coefficient at a particular wavelength of 760 nm and predicted the water content by the developed dehydration model at 0, 10, 20, 30, 40, 50, and 60 min after treatment by 1,2-propanediol, 1,4-butanediol, PEG200, PEG400, and glycerol in 100% and D-sorbitol in 70%, respectively. Data are from at least six pieces of samples. Table 2 summarizes the mean values and deviations of the skin thickness, reduced scattering coefficient, and water content after treatment of different OCAs and correlation coefficients between the latter two serials of parameters.

The skin thickness data were used in the IAD algorithm for calculation of the reduced scattering coefficient. It can be seen from Table 2, the skin thickness reduces slightly, whereas both the water content and reduced scattering coefficient decrease significantly with the treating time of OCAs. The relative changes in the reduced scattering coefficient and water content are very close for 1,2-propanediol, 1,4-butanediol, or PEG200; however, for PEG400, glycerol, or D-sorbitol, the reduced scattering coefficient decreased more quickly than the skin dehydration in the course of the 60-min treatment. Even though the relative changes in both parameters are not completely coincident, the correlation coefficient is almost close to 1, which means the direct relationship between the optical clearing efficacy and dehydration degree of skin.

A statistical analysis was applied to analyze the significant difference between the optical reduced scattering coefficient and

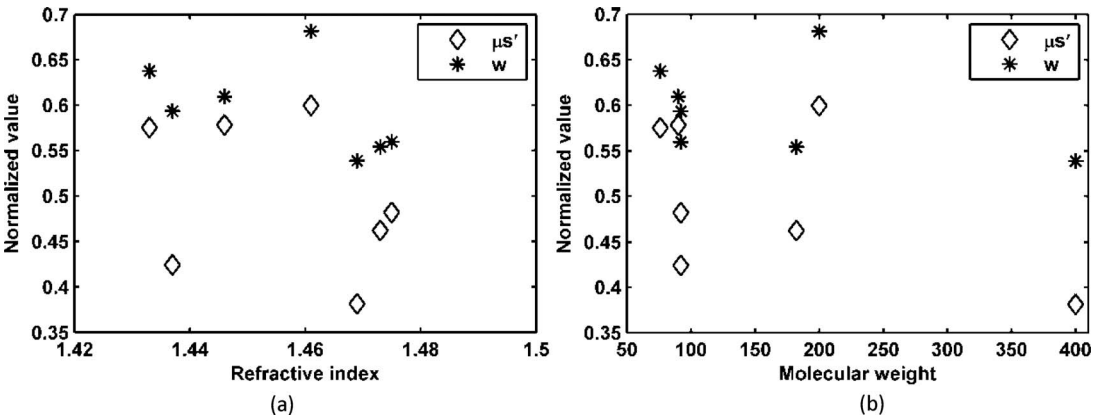

Fig. 3 Scatter plot showing the correlation between (a) the refractive index and water content (star) or reduced scattering coefficient (open diamond) of porcine skin and (b) the molecular weight and water content (star) or reduced scattering coefficient (diamond) at 60 min after application of different OCAs.

the water content induced by each agent. After topical application of glycerol, or D-sorbitol, there is an extremely significant difference ($P<0.01$) between the relative changes in reduced scattering coefficient and the water content, and there is significant difference ($P<0.05$) for PEG 400. Whereas, for 1,2-propanediol, 1,4-butanediol, or PEG200, there is no statistical difference between the two sets of data ($P>0.05$). Hence, we conclude that dehydration is the main mechanism of skin optical clearing for 1,2-propanediol, 1,4-butanediol, or PEG200 during the 60-min topical treatment, whereas for PEG400, glycerol, or D-sorbitol, some other mechanisms might exist that lead to further clearing besides the dehydration.

Actually, dehydration-induced transparency of skin has been demonstrated by both pure drying and OCAs application experiments,[3,9,12–15] and also proved in this work in the visible and NIR range, far enough from strong absorption bands. For pure drying of skin, the dehydration is only from the efflux of interstitial fluid; whereas for optical clearing of skin, the dehydration may be from the interaction between collagen and chemical agents besides the efflux of interstitial fluid. It should be pointed out that the dehydration of skin caused by OCAs is just a macroscopic phenomenon of mechanism of skin optical clearing. One of the mechanisms should be the matching of refractive indices of the scatterers and base material due to an increase of the index of refraction of the interstitial space by influx of OCAs with higher refractive index and efflux of interstitial fluid with lower refractive index, thus less overall scattering of tissue. The second one might be from tissue shrinkage (less thickness) and corresponding spatial correlation of scatterers (more effective packing or ordering) caused by dehydration, which also gives clearing effect due to constructive interference in the forward direction of all scattered waves by spatially correlated collagen fibers, as is characteristic to an absolutely transparent eye cornea.[28] The last mechanism should correlate with more regular packing or ordering of scatterers,[11] which increase the anisotropy factor g.[31] For the molecular mechanism of dehydration caused by OCAs, Hirshburg et al.[32,33] gave a reasonable explanation with the molecular dynamical simulation and demonstrated that the dehydration process is involved with the propensity of forming hydrogen bond bridges, which can be evaluated by collagen solubility of OCAs.[32] Besides the dehydration, the optical clearing agents entered into the dermis may induce collagen-fiber dissociation[33] or alteration of scatterer structure,[31] which also enhanced the optical clearing efficacy of skin. All these or some other possible mechanisms do not take effect independently but interact on each other and contribute to the ultimate clearing efficacy together.

3.4 *Other Possible Mechanisms of Optical Clearing or Dehydration*

In order to discuss other possible mechanisms of optical clearing of skin and factors of dehydration, we further consider the relation between optical clearing efficacy of skin or dehydration and other factors, such as the refractive index, molecular weight, or number of hydroxyl group of optical clearing agent.

Here, we draw scatter plots that illustrate the relation between the water content or reduced scattering coefficient after a 60-min application of OCAs and the refractive index [Fig. 3(a)] or the molecular weight [Fig. 3(b)] of OCAs. It can be seen that the optical clearing efficacy neither correlates with the refractive index nor molecular weight of the OCAs, which is consistent with Mao's conclusion.[4] Similarly, the dehydration of skin after a 60-min treatment of OCAs neither depends on the refractive index nor on the molecular weight of OCAs. Because all the OCAs used in this work are molecularly small, it is possible that the amount of OCAs entered in dermis mainly depends on the molecular permeability of OCAs.

Figures 4(a) and 4(b) show the relative changes of reduced scattering coefficient and water content at 10, 30, and 60 min after the application of chemical agents, such as 1,2-propanediol, 1,4-butanediol, PEG200, PEG400, glycerol, and 70% glycerol, D-sorbitol. As can be seen from the data, the water content reduces by 46, 41, 45, 44, 36, 39, and 32%, respectively; and the optical clearing effect after a 60-min treatment with the OCAs decreased gradually on the order of glycerol, D-sorbitol, 70% glycerol, 1,2-propanediol, 1,4-butanediol, PEG200, and PEG400 with the decrease of reduced scattering coefficients by 62, 58, 54, 52, 43, 42, and 40%, respectively. This does not mean that the more serious the skin dehydrates, the better the optical clearing efficacy is which also proves there must be some other

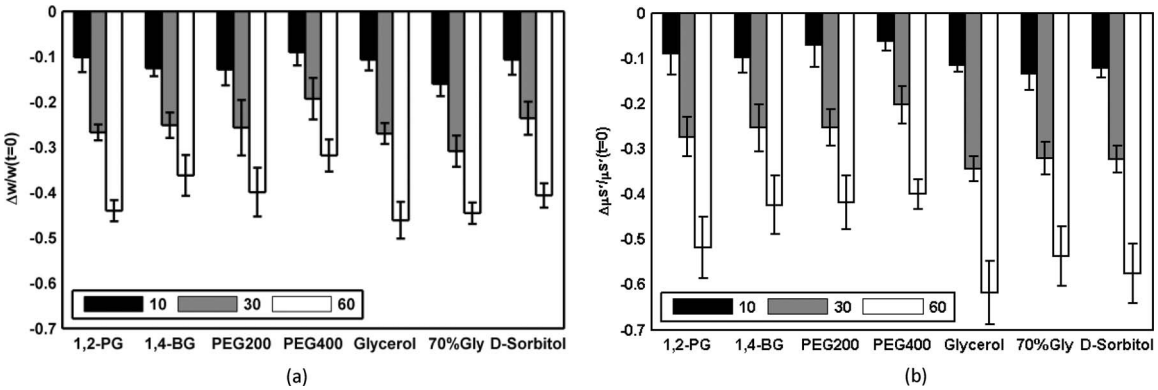

Fig. 4 Relative reduction of (a) water content and (b) reduced scattering coefficient after 10-, 30-, and 60-min treatment with the OCAs listed in Table 1. (70% Gly stands for 70% glycerol solution.)

factors enhancing the optical clearing efficacy of skin besides dehydration.

Our previous work finding indicates that the efficacy of skin optical clearing caused by OCA depends on the number of hydroxyl groups in its chemical structure for the studied monobasic alcohol, diatomic alcohols, and triatomic alcohols.[4] Here, we almost observe the same result with the six OCAs listed in Table 1. D-sorbitol and glycerol have more hydroxyl groups than the other four agents. However, as a hexahydric alcohol, the optical clearing effect of D-sorbitol seems to be worse than that of the pure glycerol, a triatomic alcohol, while better than that of the 70% glycerol solution as can be seen from the data. The reason for the worse optical clearing efficacy caused by D-sorbitol could be from its low-saturated solubility (70%). Hirshburg et al.[32] performed the molecular dynamics simulations of collagen peptides and found that the rate of tissue optical clearing correlated with the preferential formation of hydrogen bond bridges between agent and collagen. They indicated that glycerol prefers to form type II hydrogen bond bridges, whereas sorbitol prefers type IV bridges, which span more extensively across the collagen surface and lead to greater disruption of the collagen hydration layer than type II.[32] For glycerol, or D-sorbitol, there might be collagen solubility during the optical clearing process that could also induce the homogenization of skin refractive index besides dehydration.

It is noted that the change of the skin after 60 min is not observed and the study was just performed for *in vitro* experiment. In order to describe the mechanism of skin optical clearing more precisely, first, further investigation needs to be carried out after treatment by OCAs for a longer time. Second, the reduced scattering coefficient (μ_s') was used to evaluate the optical clearing efficacy in this work. As we know, the decrease of μ_s' should be caused by increase in anisotropy factor of scattering (g) or decrease in scattering coefficient (μ_s). Because of the limitation of the measurement instrument, we could not measure the collimated transmittance; thus, we just obtained the reduced scattering coefficient (μ_s'), instead of anisotropy factor of scattering (g) and scattering coefficient (μ_s), separately. In order to calculate μ_s' with the IAD algorithm, the anisotropy factor was fixed at 0.9, which is a typical value for skin.[28] As claimed in the work by Samatham et al.,[31] OCAs induced tissue transparency may be due to increasing of the anisotropy factor, so different anisotropy factors ($g = 0.7, 0.8, 0.99$) were tested in the IAD algorithm, and the results showed that the differences between the calculated values of μ_s' with $g = 0.9$ (used in this work) and $g = 0.99$ (or $g = 0.7, 0.8$) are $<3\%$. However, further work should adopt some other method[31] to obtain g and μ_s, which might be beneficial to understand the mechanism of optical clearing of skin caused by dehydration. Third, the histological approach by observing the microstructure of skin may determine the distribution of water in the skin at a microscopic level.[34] Fourth, the *in vivo* experiments should be performed because there is evident difference in skin status between *in vivo* and *in vitro* treatment by OCA.[11]

4 Conclusions

The role of dehydration on the optical clearing of skin tissue has been commonly admitted in many previous studies with some qualitative results. In this study, a dehydration model was developed first to quantitatively evaluate water content of skin with the partial least-squares method based on the measurements of near-infrared reflectance spectroscopy and weight of porcine skin. It has been concluded that the PLS method is better than dual-wavelength in the establishment of the method. A commercial spectrometer with an integrating sphere was used to measure the reflectance and transmittance of skin after treatment with different OCAs, and then, the water content was predicted with the established model and optical properties of sample were calculated with the IAD algorithm, respectively. The results show that both the reduced scattering coefficient and dehydration of skin decrease with prolongation of action of OCAs, but the relative change in the former is larger than that in the latter after a 60-min treatment. The absorption coefficient at 1450 nm decreases completely coincident with the predicted dehydration of skin during the optical clearing. Further analysis illustrates that the correlation coefficient between the relative changes in reduced scattering coefficient and dehydration is ~ 1 during the 60-min treatment of agents, but there is an extremely significant difference between both parameters for some OCAs with more hydroxyl groups, especially, glycerol, or D-sorbitol, which means that the dehydration is a main mechanism of skin optical clearing, but not the only mechanism. This work provides an analysis method to assess the water content of skin not

only *in vitro,* but also *in vivo* during the optical clearing process. The study on the relationship between dehydration and optical clearing efficacy will be meaningful to reveal the mechanism of skin optical clearing.

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