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Abstract. Confocal micro-Raman spectroscopy is employed to study the diffusion process of dimethyl sulfoxide (DMSO) in porcine skin optical clearing. The variation of DMSO concentration with time at different depths of the skin was obtained and then the DMSO diffusion coefficient with the passive diffusion model was calculated. Results show that it has a significant difference at different depths of the skin. Also, the DMSO concentration with the depth at different times was obtained and the same method was used to find the change law of the DMSO diffusion coefficient. Results indicate that it also changes with the treatment time. The experimental results are consistent with the theoretical model in a previous study. The current results demonstrate that Raman spectroscopy has the ability to quantitatively monitor the process of optical clearing. © 2013 Society of Photo-Optical Instrumentation Engineers (SPIE) [DOI: 10.1117/1.JBO.18.2.020507]

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Applying optical techniques in the medical field is becoming more and more important.¹ However, most biological tissues scatter light strongly, which limits the penetrating depth of light in the tissues. The difference of refractive index between the scattering particles and the ground substances of tissue is the major reason to give the biological tissues a strong optical scattering effect.² The optical property of biological tissues can be controlled by using appropriate chemical agents, called optical clearing agents (OCAs). Glycerol, glucose, propylene glycol, dimethyl sulphoxide (DMSO), and their combinations are frequently used as OCAs.³ OCAs have been successfully employed

in many optical techniques to improve image and spectroscopy quality at greater depths in tissue.^{4,5}

Although numerous studies have revealed that application of OCA to highly scattering biological tissue could cause optical clearing effect, the understanding of clearing mechanisms is still relatively poor,^{6,7} especially diffusion in multilayered biological tissue, such as skin. The percutaneous absorption dynamics had been studied by Fourier transform infrared spectroscopy (FTIR), optical coherence tomography, and second harmonic generation.⁸⁻¹⁰ Unfortunately, these methods were not able to resolve the problem on diffusion coefficient change. In this article, we try to use confocal micro-Raman spectroscopy to solve this problem.

Raman spectroscopy is a potential nondestructive measurement technique with many advantages, including high sensitivity, high spatial resolution, resistance to autofluorescence and photobleaching, relative insensitivity to water, simple to perform, and fully automated.^{11,12} Based on inelastic light scattering, Raman spectroscopy measures molecular vibrations and provides fingerprint signatures for various biomolecules in tissues, such as collagen, blood, proteins, lipids, and nucleic acids.^{13,14} Meanwhile, Raman spectra usually show narrow bands associated with the analyte, and, in theory, the intensity of an analyte band is linearly proportional to the analyte concentration.¹⁵ It makes Raman quantification promising.

The Raman system used in this study has been described previously.¹¹ In this study, the power of the laser beam focalized on the sample is measured and maintained at 50 mW. The spectral range is from 600 to 1800 cm^{-1} , and collect with 2 s exposure time. All the data are collected under the same conditions.

DMSO is purchased from the Tianjin Damao Chemical Reagent Factory (China) with a purity of 99.90%. Concentrations of DMSO solutions ranging from 0.31% to 40% (volume/volume) are obtained by dilution. Fresh porcine skin obtained from a local slaughterhouse is cleaned with doubly deionized water. The tissue samples are stored at 4°C for no longer than 12 h before measure. For micro-Raman spectroscopy analysis, each sample is cut into $2 \times 2 \text{ cm}^2$ pieces. The role of the filter paper is to prevent the solution from flowing down along the lateral edge of the porcine skin and make the liquid permeate uniformly. The filter paper and the skin sample are the same size.

In this study, we measure the concentrations of DMSO at the profile of the porcine skin. For this purpose, instrument is connected with a Leica microscope objective (NA = 0.4) of magnification 20 \times by the 90 deg adaptor. The measurement is set into two groups. First, record the progress of the changes of DMSO concentration with time at 50, 150, 250, and 350 μm under the surface. Set the focal position at $t = 0$ as (0, 0, 0). Raman spectra are taken after dropping DMSO on the filter paper. Second, longitudinal Raman scan (Z scan) of the porcine skin's profile with a step length of 100 μm between 50 and 850 μm , at 5, 10, 30, and 50 min, respectively. To reduce system error, DMSO (40%) is used as the external standard. And the Raman spectrum is baseline corrected by the software R 2.8.1 which is provided by Renishaw. Using Nonlinear Curve Fit of Origin 8.5 (OriginLab Corporation, Northampton, Massachusetts) to fit the data which is obtained in our experiment.

Before we analyze the diffusion coefficient, we obtain the Raman spectra of porcine skin and DMSO [Fig. 1(a)].

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The bond of carbon and sulphur (c-s) stretch at 680 cm^{-1} is chosen to reliably extract the DMSO concentration from the observed Raman signal.¹⁶ Then the Raman spectra of different concentrations of DMSO are measured (total number of sample concentrations, 20). The calibration curves of DMSO solution are shown in Fig. 1(b). It has a correlation coefficient (R^2) of 0.9983. y and x denote the Raman intensity and the concentration of DMSO, respectively. These calibration curves can be used to calculate the concentration of DMSO.

In the following, we analyze the changes of the diffusion coefficient in the process of optical clearing.

For different depths below the porcine skin surface, 50, 150, 250, and 350 μm , respectively, we obtained the variation of DMSO concentration which is shown in Fig. 2. From Fig. 2, we find that the diffusion curves become more smoother, tending to a straight line as the depth increases, which is consistent with the theoretical model in previous study.¹⁷

In order to detect the changes of DMSO diffusion coefficient responding to the depths of the skin, we use the passive diffusion model in this study. As to the fibrous structures such as human skin dermis and muscle, it is quite reasonable to assume that the dynamics of fluid diffusion within this tissue is well described by free diffusion. To analyze concentration profiles under vitro condition, according to Fick's second law of diffusion, we have^{17,18}

$$\frac{\partial C_{\text{OCA}}(x, t)}{\partial t} = D \frac{\partial^2 C_{\text{OCA}}(x, t)}{\partial x^2}, \quad (1)$$

where D is the transport diffusion coefficient describing the mass transport of the OCA through the tissue; t is the time after topical application of the OCA; x is the depth in the tissue. A solution of Eq. (1) for a semi-infinite medium (tissue samples) is

$$C_{\text{OCA}}(x, t) = C_{\text{OCA, sat}} \text{erfc}(x/2\sqrt{Dt}), \quad (2)$$

where erfc is the complementary error function and $C_{\text{OCA, sat}}$ is the maximum OCA concentration that reached in tissue at saturation condition. In this letter, we use C_0 instead of the saturation concentration.

Using the model mentioned above, according to the custom curve fitting in software Origin 8.0 and the calibration curves of DMSO, we obtain the fitting parameters, which are shown in Table 1. From Table 1, we find that the correlation coefficients (R^2) of the fitted curves are all above 97%, except for the one at 50 μm with 82.40%, demonstrating that the formula has made

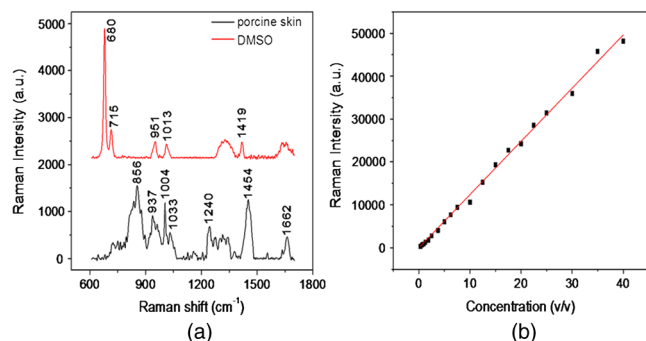


Fig. 1 (a) Raman spectra of 2.5% DMSO (top) and porcine skin (bottom). (b) Relative Raman intensity at 680 cm^{-1} for increasing volume fraction of DMSO in water.

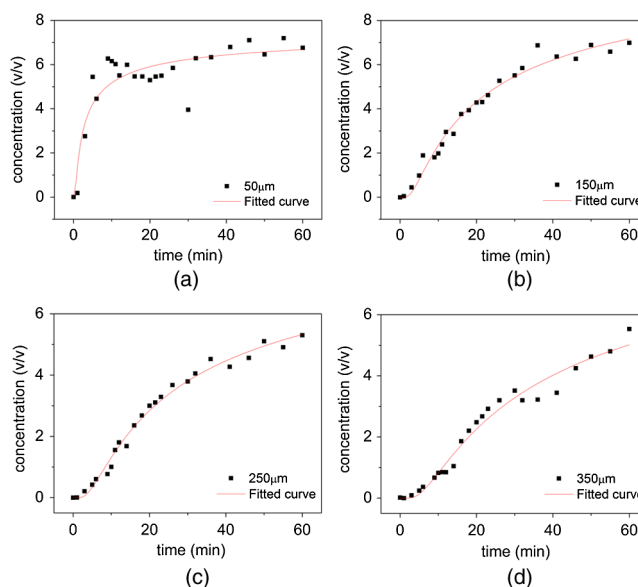


Fig. 2 The changes of DMSO concentration with time at different depths of (a) 50 μm , (b) 150 μm , (c) 250 μm , and (d) 350 μm .

considerable fitting results for the data. We also realize that $C_0 = 7.76\%$ to 12.01% , which is much lower than 40%. The reasons may lie in two aspects, one is the dilution of the DMSO concentration due to the tissue dehydration, and the other is the barrier function of the corneum.⁷ The fluctuant variation of C_0 may be attributed to the dehydration of the tissue micro-environment, while the increasing trend of diffusion coefficient (D) may be relevant to the hierarchical structure of the skin.¹⁹

Figure 3 shows the changes of DMSO concentration with the depth at 5, 10, 15, 30, and 50 min after DMSO application onto the surface of porcine skin. Using the passive diffusion model and Origin 8.0, we obtained Table 2. From Fig. 3 and Table 2, we find that the longitudinal diffusion curves become smooth as the treatment time increased, which is agreed with the result of theoretical model.¹⁷ All the correlation coefficients are above 93.81%, which shows a good fitting between the fitting formula and the data. C_0 (6.43% to 9.04%) is also much less than 40%. The value of diffusion coefficient (D) shows a trend from ascent to descent with respect to time and reaches maximum at 30 min. It might be caused by the dehydration of tissue constituents and/or the structural modification of collagen.²⁰

In conclusion, confocal micro-Raman spectroscopy provides a highly sensitive way to characterize DMSO diffusion coefficient in porcine skin. Our results confirm the following: (1) The diffusion coefficients of DMSO in porcine skin are varying at

Table 1 Calculated results in different depths by passive diffusion model.

Depth (μm)	C_0 (v/v)	R^2	$D(10^{-7}\text{ cm}^2/\text{s})$
50	7.7635	0.8240	1.1222
150	12.0092	0.9793	1.1183
250	9.8692	0.9887	2.3065
350	10.6380	0.9725	3.2800

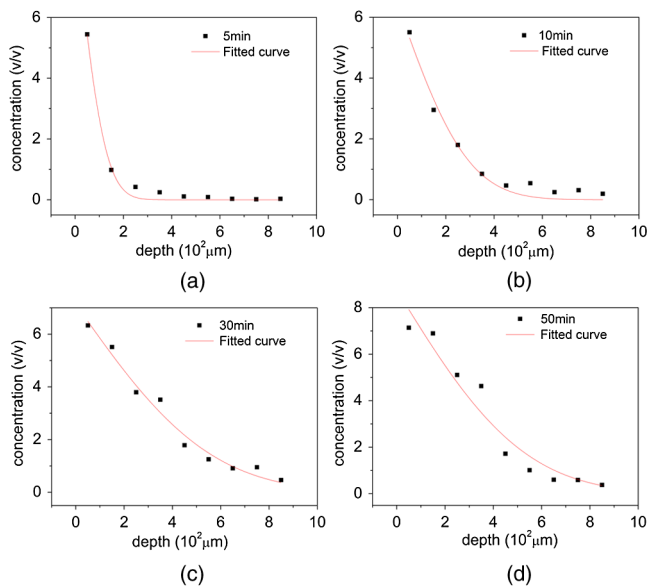


Fig. 3 The changes of DMSO concentration with the depth at different times of 5 min (a), 10 min (b), 30 min (c), and 50 min (d) after application of DMSO onto the surface of porcine skin.

Table 2 Calculated results in different time by passive diffusion model.

Time (min)	C_0 (v/v)	R^2	$D(10^{-7} \text{ cm}^2/\text{s})$
5	9.0416	0.9906	1.5267
10	6.4318	0.9750	3.6671
30	7.1349	0.9771	5.3198
50	8.7639	0.9381	2.8589

different depths of skin (different layer in porcine skin). (2) The diffusion coefficients of DMSO in porcine skin changed with the treatment time, and it shows a time-related trend from ascent to descent and reaches maximum at 30 min. (3) The maximum DMSO concentration (C_0) reached in tissue is about 10%, much lower than 40%, because of the tissue dehydration and the barrier function of the corneum. The current results show that Raman spectroscopy has the ability to quantitatively monitor the process of optical clearing. The experimental results also verify the theoretical model.¹⁷ It demonstrates that Raman spectroscopy techniques may have great potential in further studies of the optical clearing process.

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