Study on the refractive index matching effect of ultrasound on optical clearing of bio-tissues based on the derivative total reflection method

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Abstract: In recent years, the tissue optical clearing (OC) technique in the biomedicine field has drawn lots of attention. Various physical and chemical methods have been introduced to improve the efficacy of OC. In this study, the effect of the combination of glycerol and ultrasound treatment on OC of in vitro porcine muscle tissues has been investigated. The refractive index (RI) matching mechanism of OC was directly observed based on the derivative total reflection method. A theoretical model was used to simulate the proportion of tissue fluid in the illuminated area. Moreover, the total transmittance spectra have been obtained by a spectrometer over the range from 450 nm to 700 nm. The administration of glycerol and ultrasound has led to an increase of the RI of background medium and a more RI matching environment was achieved. The experimental results support the validity of the ultrasound treatment for OC. The RI matching mechanism has been firstly quantitatively analyzed based on the derivative total reflection method.

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1. Introduction

With the development of biomedical photonics, a lot of interests have been attracted in the research of optical diagnosis, biomedical imaging, and laser therapy, etc. However, the optically turbidity, high scattering and absorption of bio-tissues, which limit the penetration of light, restrict the investigation depth of non-invasive optical imaging technique. Optical clearing (OC) technique proposed by Tuchin et al. can reduce the scattering of tissues by immersed in osmotic agents with high refractive index (RI) [1–4]. Different kinds of optical clearing agents (OCAs) have been used on biological tissues, i.e. glucose, glycerol, propylene glycol, dimethyl sulfoxide and mannitol [4–7]. To enhance the permeation of OCAs into tissues, plenty of physical and chemical methods have been introduced [5–7,9–15]. Many of them and their combinations have achieved remarkable OC effect on tissues with variety structures, such as iontophoresis, intensive pulsed light, microneedle rolling, application of ultrasound and their combination with chemical enhancers [5,9–14]. The application of OC technique has achieved a significant enhancement of resolution in photoacoustic technique in recently studies [16–18].

It’s commonly accepted that RI matching is the major mechanism for OC, while the scattering properties of tissues are mostly due to the RI mismatch between scattering...
components and the background medium [4,9,15]. The main constituents that contribute to the RIs of tissues, for scattering components, are cellular membrane, cell nucleus, organelles, collagen fibers and melanin, while for background medium, are interstitial fluid and cytoplasm [19]. The OCAs with high RI permeating into tissues partly replace background medium. It leads to a more RI matching environment which will diminish the scattering of light in tissues. Furthermore, dehydration and morphology change of collagen fibers are also proposed as important mechanisms for OC [4,6,7,9,15,20]. As common methods, optical coherent tomography (OCT) and Vis/NIR spectrograph have been applied in the investigation of OC to evaluate the efficiency [4–7,14,15].

The phenomenon of enhanced transdermal transport caused by the ultrasound application is reported as sonophoresis [5,11–14,21–25], which achieves prominent enhancement in OC technique and transdermal drug delivery. The investigation on the effect of ultrasound parameters indicates that the low-frequency sonophoresis can induce a more remarkable enhancement [11,14,21–24]. It’s widely agreed that the cavitation effect is the main mechanism for the low-frequency ultrasound [22,23]. The ultrasound treatment disorders the biological barrier to promote the penetration of OCAs into tissues. Sonophoresis is a promising noninvasive physical method for the OC technique. Although some insights have been gained through these studies, an in-depth evaluation of the correlation between the OC mechanisms and ultrasound treatment remains lacking.

In this study, the effect of imposing the combination of glycerol and ultrasound on porcine muscle tissues has been investigated by the derivative total reflection method (DTRM). With this method, we can observe the RI change of scattering components and background medium directly, which clearly explains the RI matching mechanism of OC. In addition, the proportion change of tissue fluid and muscle tissue at prism-sample interface can also reveal the process of OC. Therefore, the RI matching process in OC could be monitored by DTRM. It may provide valuable information for further study on OC.

2. Materials and methods
2.1 Materials
The experiments were performed in vitro with fresh muscle tissues taken from tenderloin. The tissue samples were booked from food market. During the 2 hours transportation from local slaughter house to our laboratory, the porcine tissue was preserved at −3~0 °C to retain freshness. The samples have been sealed and refrigerated before experiment. Then samples were cut along the muscle fiber direction into slices of approximately 2.5 × 3.5 cm² with the thickness of about 2 mm. The thickness was measured by a micrometer and obtained as averaging data from three different positions. After thawed under room temperature, the samples were placed on the surface of the prism with a minor pressure to avoid the existence of air gap on the interface [26]. Experiments were performed in four situations and the samples were divided into four groups as control group, experimental group 1, 2 and 3. For the control group, the sample was immersed in air while measured. Experimental group 1 was imposed by glycerol (>99%, 1.4709, Benchmark) but not subjected to ultrasound, while experimental group 2 and 3 were imposed by glycerol and then subjected to ultrasound for 5 and 10 min, respectively. For each group, the measurement was performed four times.

2.2 Glycerol and ultrasound treatment
For the ultrasound treatment, the sonicator (B-629, Radium Beauty & Hairdressing Equipment Manufacturer, Guangzhou, China, 0.68 W/cm²) operated at a frequency of 1–1.1 MHz in pulsed mode. A cell which consists of the probe of the sonicator, a plastic vessel and a plastic plate was utilized as shown in Fig. 1. Since the experiments begun, the cell was filled with glycerol. There is a hole with diameter of 1.2 cm on the plastic plate to ensure the penetration of glycerol into tissues from only one side. The data at 0 min was obtained before
imposing glycerol. Once the glycerol was applied, the ultrasound treatment was performed for a period of 5 min and 10 min for experimental group 2 and 3, respectively. The tissue samples were exposed with glycerol for a total 60 min during experiments. After the application of glycerol, data were obtained at 15min, 30min, 45min and 60min. The operating steps presented above were utilized in both reflectance measurements based on DTRM and transmittance spectra measurements.

2.3 Measurements by DTRM

The experimental setup is schematically shown in Fig. 1. The measurement is based on the DTRM reported previously [26,27]. Light from a He-Ne laser (632.8 nm) is divided into two portions through the splitter M. One portion is received by detector D1 of a dual-channel power meter (PM320E, Thorlabs, New Jersey, USA) as monitoring, while the other reach the prism (ZF4, \( n_p = 1.723 \)) after propagates through the half-wave plate S, the polarizer P and aperture diaphragm A. After reflected from the prism-sample interface, the light is received by detector D2. The prism is fixed on the rotation stage (M-038, Physik Instrumente, Karlsruhe, Germany) to change the incident angle of light. By collecting the emergent light, the reflectance can be set as a function of the incident angle.

![Fig. 1. Schematic diagram of the refractive index measurement system: M-splitter; D1,D2-detector; S-half-wave plate; P-polarizer; A-aperture diaphragm](image)

2.4 Theoretical simulations

The angular positions of the maximum derivatives of reflectance curve are defined as critical incident angles [28,29]. According to the Snell’s law, the RI of the sample can be achieved as

\[
\sin \theta = \frac{n_s \sin \theta_c}{n_p},
\]

where \( n_s \) is the RI of prism, \( \theta_c \) is the critical incident angle at prism-sample interface.

According to Fresnel Formula, the reflectance for transverse magnetic wave (TM wave) at prism-sample interface can be expressed as

\[
R_{TM} = \frac{\left| n_s \cos \theta - n_p \cos \left[ \arcsin \left( \frac{n_p \sin \theta}{n_s} \right) \right] \right|^2}{\left| n_s \cos \theta + n_p \cos \left[ \arcsin \left( \frac{n_p \sin \theta}{n_s} \right) \right] \right|^2}
\]

(1)

Here, \( \theta \) is the incident angle at prism-sample interface.
At the prism-sample interface, the contacting area consists of muscle tissue and tissue fluid. By a model based on the DTRM we reported before [30], the proportion of tissue fluid at the illuminated area can be simulated. Besides the loss at prism-sample interface, the final reflectance measured by detector should include the losses at the air-prism and prism-air interface. Taking the proportions of tissue fluid \( m_1 \) and muscle tissue \( m_2 \) at prism-sample interface, in the illuminated area, into account, the final reflectance is modified as

\[
R_f = m_1 R_{f1} + m_2 R_{f2}
\]

(2)

\( R_{f1} \) and \( R_{f2} \) are the reflectance from background medium and scattering components, respectively. And the proportions should satisfy \( m_1 + m_2 = 1 \). Based on the Nelder-Mead simplex algorithm, a fitting program has been used to obtain \( m_1 \) and \( m_2 \) by minimizing the sum

\[
S(N) = \sum_{i=1}^{N} \left( R_{e,i} - R_{f,i} \right)^2
\]

(3)

\( R_{e,i} \) is the \( i \)th measured reflectance while \( R_{f,i} \) is the \( i \)th calculated reflectance. To evaluate the reliability of fitting, \( E^2 \) is introduced as

\[
E^2 = 1 - \frac{\sum_{i=1}^{N} \left( R_{e,i} - R_{f,i} \right)^2}{\sum_{i=1}^{N} \left( R_{e,i} - \bar{R} \right)^2}
\]

(4)

Here, \( \bar{R} \) is the mean value of measured reflectance over \( N \) values of incident angle. While \( E^2 \) is closer to 1, reliable fitting is obtained.

2.5 Measurements by spectrometer

To validate the OC effect investigated by DTRM, the transmittance spectra measurements have been performed. Total transmittance spectra of samples of each group were obtained by a High-Resolution spectrometer (Ocean Optics HR4000) [5–7,12,13]. The measurements were performed over the range from 450 nm to 700 nm. As shown in Fig. 2, light from a xenon lamp transports through an aperture diaphragm A and a converging lens L to achieve parallel light. Then the light is divided into two portions through the splitter M. One portion is received by detector D as monitor, while the other illuminates the sample which is fixed on a
home-made plastic holder with a circle window. Transmitting through tissue sample, light is received by the integrating sphere. Then the light is collected by the optic fiber of the spectrometer. The glycerol was spread as a film on only one side of the tissue sample. The ultrasound treatment was performed by immersing the probe in the glycerol film on the tissue sample. Since the ultrasound treatment was completed, the probe was removed. Before measurement, the glycerol was removed, and spread on tissue sample right after measurement [5,6,12]. The spectra were obtained according to the experimental approach recommended above.

3. Results

3.1 Measured reflectance and the RIs of tissues

Based on the DTRM, the measured reflectance curves and the derivative of the reflectance curves of porcine muscle tissues for control group, experimental group 1, 2 and 3 are shown in Fig. 3, 4, 5, and 6. The error of measured reflectance which was mainly caused by errors of the vertex angle, the refractive index of prism, laser power fluctuation was less than 1%. Here, the fitting curves obtained by a model based on the DTRM matches the experimental data points very well. For each curve, the calculated $R^2$ is larger than 0.995.

For each of the derivative of the reflectance curves, two peaks appear, corresponding to the total internal reflection occur between prism-tissue fluid and prism-muscle tissue, respectively. With increased time of the experiment, the first peak shifts toward right for each group. In the meantime, the amplitude of the first peak related to the proportion of tissue fluid in the illuminated area decreases gradually. As the glycerol permeating into tissues, interstitial fluid is replaced partly by glycerol. The mixture of tissue fluid and glycerol gives a rise to the RI of the background medium, which induces the shift of the first peak toward right. For the second peak, it also shifts toward right since glycerol entering into cells. Therefore, the RI contributed mostly by the cells containing glycerol increases gradually. At the same time, the amplitude of the second peak rises with time increasing.

The changes of the position and the amplitude of the peak indicate the RI matching mechanism and water loss in the course of OC. Compared to the control group, the first peak of sample imposed with only glycerol moves faster and further. In other words, the penetration of glycerol into tissues leads to an increase of the RI of background medium. Although the second peak shifts towards right as well, these two peaks relatively move closer to each other. Therefore, it achieves a more RI matching environment with the administration of glycerol. As shown in Fig. 5 and Fig. 6, the incident angles, corresponding to the first peak position, are larger than that of experimental group 1 at 60 min. It reveals that the application of ultrasound has enhanced the permeation of glycerol.
Fig. 3. Measured reflectance and fitting curves, and the corresponding derivative of the reflectance curves of fresh porcine muscle tissues for control group obtained at the time intervals of 0 min, 15 min, 30 min, 45 min, 60 min.

Fig. 4. Measured reflectance and fitting curves, and the corresponding derivative of the reflectance curves of fresh porcine muscle tissues for experimental group 1 obtained at the time intervals of 0 min, 15 min, 30 min, 45 min, 60 min.
Fig. 5. Measured reflectance and fitting curves, and the corresponding derivative of the reflectance curves of fresh porcine muscle tissues for experimental group 2 obtained at the time intervals of 0 min, 15 min, 30 min, 45 min, 60 min.

Fig. 6. Measured reflectance and fitting curves, and the corresponding derivative of the reflectance curves of fresh porcine muscle tissues for experimental group 3 obtained at the time intervals of 0 min, 15 min, 30 min, 45 min, 60 min.
Figure 7 and Fig. 8 illustrate the time-dependent RIs of background medium and scattering components for each treatment. The difference of initial RI measured at 0min for each group was caused by the different initial state of samples. But the RIs of tissues were barely influenced by the minor mechanical pressure imposed on samples when the pressure was less than 0.35 MPa [26]. For the control group, the OC is induced mainly by dehydration. The RI of background medium increases slightly while there is nearly no change in the RI of scattering components. With the administration of glycerol, there is a remarkable increase of the RI of background medium from 1.356 to 1.381. Meanwhile, because of glycerol entering the cells, the RI of scattering components gets an increase from 1.388 to 1.403. Within 30 min since the application of glycerol, the RI has a significant increase. After 30min, the increase of RI is slower. Applying the glycerol and ultrasound simultaneously, the enhancements of the RI increases are greater comparing to that of experimental group 1. As shown in Fig. 7, there is a greater increase of the RI of tissue imposed by glycerol and 10 min ultrasound treatment from 1.359 to 1.396, while the RI increases from 1.357 to 1.388 for sample treated by glycerol and 5 min ultrasound treatment. Thus, it will achieve a greater enhancement of the permeation of glycerol into tissues with longer ultrasound treatment time.
3.2 Simulated proportions of tissue fluid at illuminated area

Figure 9 demonstrates the simulated proportions of tissue fluid $m_i$ for each treatment. For the control group, there is a small decrease of the proportion due to the dehydration. For the experimental groups, the decreases of the proportions are obvious faster than that of control group. As glycerol permeating into tissues, water from deeper layers gets out of the tissues, resulting in the decrease of the amount of tissue fluid at prism-sample interface. The initial proportion of tissue fluid for each group is determined by the pressure imposed on tissues [26]. The variation trend of the tissue fluid proportion matches well with the change of the RI discussed above.

![Proportion of tissue fluid for each treatment](image)

Fig. 9. Proportions of tissue fluid for each treatment.
3.3 Measured transmittance spectra

The transmitted spectra have been obtained and the total transmittances of samples for each treatment over the range from 450 nm to 700 nm are shown in Fig. 10. The enhancement of the total transmittance is illustrated for each group. Due to dehydration, there is a slightly enhancement for the control group during 60 min. Compared with the control group, the application of glycerol and ultrasound treatment has promoted the penetration of light into tissues. As illustrated in Fig. 10, the maximum enhancement is achieved during the first 15 min for experimental group 1, 2 and 3. For the sample imposed by the combination of glycerol and ultrasound, there is a greater enhancement of total transmittance compared to that imposed by glycerol only. With longer ultrasound exposure time, a greater enhancement will be observed. Hence, the ultrasound treatment has facilitated the process of the permeation of glycerol.

4. Discussion

As the glycerol permeating into tissues, interstitial fluid is partly replaced by the glycerol. Because of the high RI of glycerol, the mixture of interstitial fluid and glycerol gives a rise to the RI of background medium. Meanwhile, the entrance of glycerol into cells causes an increase of the RI of muscle tissues. Despite the increase of the RI for both background medium and scattering components, they match to each other better. The results of the RI changes indicate the RI matching process in OC directly. The results of the enhancement in OC induced by the combination of glycerol and ultrasound matches previous reported results very well [11,14].

Furthermore, the water loss is also an important mechanism for the OC process. As illustrated in Fig. 3 and Fig. 7, the dehydration of tissues causes a rise of the RI of background medium while the RI of scattering components barely changes. The phenomenon of the OC caused by dehydration agrees with previous study very well [15]. Furthermore, the
results in this study indicate that the OC induced by dehydration can be explained by the RI matching as well. For the experimental groups, the penetration of glycerol is also the cause of water loss. As the permeability rate of water through cytomembrane is much faster than that of glycerol, there is much more water getting out of cells while less glycerol entering cells. It causes the decrease of the proportion of tissue fluid in the illuminated area. Moreover, the water loss from tissues leading to a higher concentration of scattering components and denser background medium induces a more RI matching environment in tissues. The glycerol exposure has diminished the scattering of light in tissues.

The experimental results reveal that the administration of ultrasound has enhanced the RI matching and the intensity of transmitted light through tissues. The mechanical effect caused by ultrasound exposure exerts a variation pressure on tissues which induces the movement of the cells and the organelles in cells [23]. It may be a main mechanism for the facilitated permeation of glycerol into tissues and the entrance into cells. Besides, the thermal effect is an important mechanism that should be taken into account for the safety of clinical application. The absorption of ultrasound will cause a rise of temperature of tissues. It depends on the frequency, intensity and exposure time of ultrasound [22,23]. With longer treatment time of ultrasound, it will achieve a greater OC efficacy.

5. Conclusion

As a consequence, the application of ultrasound treatment for 10 min has led to a more effective enhancement in OC than that of ultrasound treatment for 5 min. The DTRM has provided a way to monitor the RI matching process in OC directly. While glycerol penetrating into tissues, the RIs of background medium and scattering components increased simultaneously. At the same time, the RIs matched better during OC. DTRM is a promising method to monitor the RI matching process in OC. The results in this study can provide valuable references to the application in fields of bio-sensing, optical imaging, diagnosis and clinical medicine.

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