Confocal Raman micro-spectroscopy of collagen-containing tissues: the impact of optical clearing

Ph.D. Thesis booklet

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1. Introduction

The research field of biomedical optics and biophotonics has become one of the fastest-growing disciplines for basic life sciences and biomedical diagnosis, imaging, therapy, monitoring, and surgery. It permits functional and structural examination of tissues and cells with imaging resolution and contrasts unachievable by any other technique.

Light-based techniques, such as spectroscopy and optical imaging are widely accepted for the detection of physiological and pathological changes in cells and tissues by probing their optical properties during *in vitro* and *in vivo* studies or even *in situ*. Raman spectroscopy as one of the optical spectroscopic methods offers information about the molecular structure and conformation of biological objects and gains great attention for diagnosis and monitoring in the cosmetic and medical research fields. It could be utilized as a non-invasive, label-free, non-destructive, and even non-contact tool in various areas such as physics, chemistry, biology, and materials science. Moreover, it is deemed as an alternative and complementary method to the gold standards method of tissue biopsy, which allows to assess of the changes related to tissue conditions with minimum sample preparation and handling requirements.

Raman spectroscopy can assist in uncovering the collagen structures and conformation modifications related to progressive diseases caused by collagen molecule alterations and offers objective, quantifiable molecular information for diagnosis and monitoring. For example, Raman spectroscopy can discriminate between *dura mater* and meningioma based on Raman peaks correlated to rich collagen content in *dura mater* and on the increased lipid content in tumour and monitoring the changes of collagen conformation in skin-dermis related to disease or chronological aging process. However, the changes in collagen molecules have been analysed in various human body sites such as skin, heart valves, eye, lungs, epithelium, stomach, colon, and breast using Raman spectroscopy.

All biomedical optics or biophotonics including Raman spectroscopy are suffering from strong light scattering or limited in-depth light penetration, which is a significant hindrance for using the optical techniques in clinical practice for diagnosis and monitoring of biological tissues and organs. In the 90s of the last century, the optical clearing technique based on the immersion of the tissue into optical clearing agent was introduced and developed in order to increase the detection depth using optical techniques, as well as to improve the spatial resolution and contrast of the resulting images, by suppressing the light scattering in the biological objects. Thus, the optical clearing is recognized to be a promising technique to combine with the optical methods in the visible to NIR spectral range for clinical practice.

Currently, different biomedical imaging techniques like Raman spectroscopy, confocal Raman micro-spectroscopy, 3D confocal microscopy, polarized microscopy, and optical coherence tomography, are commonly utilized in combination with various optical clearing agents such as glucose, dimethyl sulfoxide, glycerol, *uDISCO*, *ScaleS*, and *Scale* to increase the sensitivity and reduce the light scattering in order to enhance the in-depth optical imaging resolution and contrast in biological objects.

However, the influence of optical clearing agents on different biological tissues and objects and their components (collagen in particular) remains an open question. Furthermore, the effect of optical clearing agent on water content, which is the major component of biological tissues (up to 80% of the volume), has not been sufficiently studied yet. Nonetheless, the main challenges are correlated with the need to improve imaging resolution and contrast even more to the subcellular level for organs or larger tissue blocks. The main motivation behind this thesis was to contribute to the knowledge and solutions to the above problems and questions. In addition, the development of the optical techniques in combination with optical clearing agents could pave the way for real-time molecular imaging within living cells.

2. Aims

The thesis aims to study the impact of the optical clearing agent on collagen in different biological tissues, such as *ex vivo* porcine *dura mater* and skin-dermis using in-depth confocal Raman micro-spectroscopy. Therefore, glycerol was chosen as one of the most commonly used optical clearing agent due to its efficiency, high refractive index, availability, biocompatibility, pharmacokinetics, biosafety, and low cost. The impact this optical clearing agent in different concentrations on collagen-related Raman peaks, dehydration, dissociation, as well as its concentration and diffusion coefficient in-depth are investigated using confocal Raman micro-spectroscopy.

To achieve this goal, the following tasks were defined:

1- Study the changes in collagen-related Raman peaks after the application of the optical clearing agent (glycerol) for *dura mater* and skin.
2- Investigate the in-depth changes in collagen hydration and dissociation in the *dura mater* after treatment with optical clearing agent.
3- Calculate the diffusion coefficient and concentration of the optical clearing agent in the *dura mater*.

4- Study the in-depth variation of the water content with respect to the strength of water hydrogen bond in the *dura mater* after optical clearing agent application.
5- Investigate the effect of optical clearing on *ex vivo* porcine skin through in-depth monitoring of collagen-related Raman peaks with different excitation wavelengths of 633 and 785 nm.

3. Materials and methods

3.1 Optical clearing agent

The optical cleaning efficiency in biological tissues and objects depends upon various factors, including the refractive index, concentration, osmolarity, and physicochemical properties of the optical cleaning agent, etc. Additionally, the parameters related to the biological tissue such as its initial turbidity and permeability for the molecules of a selected optical cleaning agent, play a crucial role in determining the effectiveness of the optical cleaning process. In recent years, extensive research has been conducted on various substances and their combinations for use as optical cleaning agents in biological tissue optical cleaning. All these are generally considered as non-toxic agents. However, it's important to note that prolonged exposure time to optical cleaning agents can lead to some negative effects such as local hemostasis (impairment of blood flow), shrinkage of tissue, and, in extreme cases, tissue necrosis (cell death), irritation, and edema in living tissue.

In this thesis, glycerol was used as optical cleaning agent due to its efficiency, high refractive index, availability, biocompatibility, pharmacokinetics, biosafety, low cost —making it one of the most commonly used optical cleaning agents. Glycerol is a water-soluble triatomic alcohol composed of three carbon atoms covalently bound to the hydroxyl-group. Previous studies have been shown that glycerol administration on biological objects can efficiently enhance the Raman to fluorescence ratio and enhance signal quality. Additionally, the optical cleaning efficacy of optical cleaning agents is directly dependent on the number of hydroxyl groups (more hydroxyl groups result in better optical cleaning efficacy). This efficacy is described by their potential to disrupt and screen the hydrogen bonds in collagen triple helices, initiating the dissociation of the collagen structure. Furthermore, it has been observed that alcohols with hydroxyl groups positioned at longer distances along the carbon chain exhibit better optical cleaning efficacy than alcohols with hydroxyl groups located adjacent to each other.

3.2. Sample preparation

Fresh *ex vivo* porcine *dura mater* was used to study the impact of optical cleaning on *dura mater* collagen. Porcine *dura mater* serves as a suitable model for conducting *in vivo* investigations of human *dure mater*, taking into consideration features such as housing, gross anatomical structure, feasibility, and ethical concerns. The collagen structure in *dura mater* is largely similar to the dermis and sclera of the eyes, resulting in similar optical properties. The presence of a blood vessels network in *dura mater* is the only distinguishing feature from the sclera of the eyes. All the fresh *ex vivo* porcine *dura mater* samples were obtained on the day of sacrifice from a local accredited abattoir (Albertirsa, Hungary) and then kept cold on ice in Phosphate buffered saline (Sigma Aldrich) during transportation to the laboratory. For the experiments the *dura mater* sample was cleaned using a paper towel, then a leather punch was utilized for the excision of sample sections of 13 mm^2 in size and thickness of 0.4 ± 0.08 mm, which was measured with a digital micrometer before and after the treatment.

The porcine skin is morphologically, immunohistochemically, and histologically, comparable to the human skin. Nevertheless, despite numerous common properties, the *stratum corneum* of *ex vivo* porcine skin has a lower barrier function compared to *in vivo* human skin, which was recently found by confocal Raman micro-spectroscopy. To study the effect of optical cleaning, the measurements were made on *ex vivo* fresh porcine ear skin. The *ex vivo* fresh porcine ears skin was delivered to the laboratory on the day of sacrifice from a local accredited slaughterhouse (Budapest, Hungary) and kept in a refrigerator for no more than two days at 5 °C. Before the measurement, the porcine ears skin was cleaned out and washed with water, and then wiped with a paper towel. Furthermore, to increase the penetration of optical cleaning agents into the *ex vivo* porcine skin deeper layers, it was shaved to remove the hair, and 20 times of tapes were pressed onto the skin and removed. Subsequently, the skin samples were defatted by placing them in a bath of pure ethanol for 5 seconds.

3.3. Experimental setup

Raman spectra were acquired for the fingerprint (400-1800 cm^{-1}) and high wavenumber (2700-3800 cm^{-1}) (FP/HWN) regions in the backscattered geometry by Renishaw inVia™ confocal Raman microscope equipped with 633 and 785 nm laser sources, a 50× objective (NA = 0.9), and a 1200 g/mm grating, which provides spatial resolutions of 0.77 μm and 0.95 μm, spot sizes of 1.5 μm and 1.9 μm, respectively for the two excitations. All samples (*dura*

mater and skin) were placed on the motorized *xyz*-axis stage of the Raman microscope permitting automatic vertical displacement with micrometer resolution to acquire the in-depth z-scan profiles.

The laser power delivered to the surface of the sample was maintained at 10 mW and 50 mW for 633 and 785 nm, respectively, which is non-destructive for biological objects. Raman measurements were acquired for 5 and 1 seconds at different points on each sample during the optical clearing process for the FP and HWN regions, respectively. The system was calibrated using 520 cm^{-1} Raman band of a silicon wafer before acquiring the Raman spectra. All the obtained data were recorded under the same conditions at room temperature of 20 ± 1°C.

4. New scientific results

In this thesis work, I studied the impact of optical cleaning on the collagen-related Raman peaks to control the optical properties of biological objects (*dura mater* and skin-dermis) in-depth using confocal Raman micro-spectroscopy. The scientific results achieved in this research work are summarized in the following points:

1- I investigated the impact of pure glycerol as optical clearing agent on the optical clearing in ex vivo *dura mater* tissue with confocal Raman micro-spectroscopy in different depths from 0 to 250 μm [T1].

   i. The intensities of collagen-related Raman peaks were enhanced across all depths from 0 to 250 μm after glycerol application. This enhancement was attributed to collagen dehydration, as evidenced by the change in the I_{937}/I_{926} Raman peak intensity ratio induced by glycerol (indicating collagen shrinkage).

   ii. The deconvolution of the Raman spectra using Gaussian-Lorentzian functions indicated the dissociation of the collagen. The observed upshift of the 1666 cm^{-1} Raman peak showed modifications in the molecular geometry of the amide I group of the collagen, suggesting the dissociation of triple helix chains into simpler structures such as single or double strings, which was attributed to the high concentration of glycerol.

2- I studied the impact of the moderate concertation of 50% glycerol, as an optical clearing agent on the *dura mater* collagen at different depths, including the diffusivity of glycerol and the changes in the water content [T2].

i. The collagen-related Raman peak intensities were enhanced across all depths (50, 100, 150, and 200 μm) after the glycerol application, confirming the effectiveness of the optical clearing method with a moderate concertation for the *dura mater* tissue.

ii. A new approach was developed to calculate the diffusion coefficient and concentration, by measuring the actual concentration of glycerol using the Raman peak intensity of the immobilized proteins as a reference. The diffusion coefficient of 50% glycerol was ranging from 9.6×10^{-6} to 3.0×10^{-5} cm^2/s, and its concentration varied from 0.6 to 20% v/v in different depths. This method can be applied for different optical cleaning agents and drug.

iii. The application of 50% glycerol caused significant changes in the total water content of the *dura mater*, related to tissue dehydration during the optical cleaning process, even at low concentrations of glycerol. The most prevalent water states in the *dura matter* with highest concentration are the weakly and strongly-bound water types. These play a crucial role in the optical cleaning process, influencing the glycerol-induced water migrations.

3- I investigated the impact of various optical cleaning agents on the Raman spectra of *ex vivo* porcine skin-dermis using 633- and 785-nm excitations at different depths and treatment times, together with the influence of dimethyl sulfoxide as an enhancer for the optical cleaning process [T3].

i. The results revealed the significant enhancement in the main Raman peak intensities of *ex vivo* skin for both excitations across all scanning depths from 0 to 240 μm during the optical clearing for both 30 and 60 minutes.

ii. The addition of 5% dimethyl sulfoxide, as a penetration enhancer to the optical cleaning agent further increases the efficiency of the optical cleaning.

iii. The optical cleaning allows to increase the excitation depth and the quality of the Raman spectra of 633 nm excited confocal Raman micro-spectroscopy above the levels of 785 nm excitation without optical cleaning method.

Based on the findings of the PhD work, future studies in this direction could involve optimizing the biocompatible optical clearing agent and integrating this method into real clinical practices for brain and skin diseases, in order to enhance the diagnostic and therapeutic capabilities of optical analysis methods. These results could contribute to a deeper understanding of the structural changes induced by glycerol treatment, shedding light on the impact of OC processes on the molecular organization of biological tissues.

5. Publications related to the Ph.D. thesis

[T1] Ali Jaafar, Maxim E. Darvin, Valery V. Tuchin and Miklós Veres; Confocal Raman Micro-Spectroscopy for Discrimination of Glycerol Diffusivity in *Ex vivo* porcine *dura mater*; Life 2022, 12(10), 1534; https://doi.org/10.3390/life12101534; Q2; IF: 3.253.

[T2] Ali Jaafar, Roman Holomb, Anton Y. Sdobnov, Zsombor Ocskay, Zoltan Jakus, Valery V. Tuchin and Miklos Veres; *Ex vivo* confocal Raman microspectroscopy of porcine *dura mater* supported by optical clearing; J.Biophotonics.2022; 15:e202100332.https://doi.org/10.1002/jbio.202100332; Q1; IF:3.390.

[T3] Ali Jaafar, Malik H. Mahmood, Roman Holom, Laszló Himics, Tamas Vaczi, Anton Y. Sdobnov|, Valery V. Tuchin and Miklós Veres; *Ex-vivo* confocal Raman microspectroscopy of porcine skin with 633/785-NM laser excitation and optical clearing with glycerol/water/DMSO solution; Journal of Innovative Optical Health Sciences Vol. 14, No. 05, 2142003,2021, https://doi.org/10.1142/S1793545821420037; Q2; IF: 2.396.

International conferences

[IC1] Ali Jaafar, Malik H. Mahmood, R. Holomb, L. Himics, V. V. Tuchin, M Veres; Confocal Raman microspectroscopy of porcine skin ex vivo using laser excitation at 633 nm and optical clearing with glycerol/water/DMSO solution; for Internet Poster presentation, Conference on Internet Biophotonics XIII, annual conference Saratov Fall Meeting SFM'20, September 29-October 1, at Saratov, Russia, 2020.

[IC2] Ali Jaafar, Ágnes N. Szokol, Malik H. Mahmood, István Rigó, Anton Y. Sdobnov,Valery V. Tuchin and Miklos Veres; *Ex vivo* confocal Raman microspectroscopy of porcine dura mater using 532 nm excitation and optical clearing; for Internet Poster presentation, Conference on Internet Biophotonics, annual conference Saratov Fall Meeting SFM21, September 29- October 1, at Saratov, Russia , 2021.

[IC3] Malik H. Mahmood, **Ali Jaafar,** László Himics, László Péter,Ágnes Nagyné Szokol,István Rigó, Shereen Zangana, Attila Bonyár, Miklós Veres; Surface-enhanced Raman scattering substrates for DNA detection based on nanogold-capped poly(DEGDMA) microparticles; proceedings of the 25th Saratov Fall Meeting Conference, Laser Physics and Biophotonics, at Saratov, Russia, September 27- October 1, 2021.

[IC4] Ali Jaafar, Abbas Albarazanchi, Maxim E. Darvin, Valery V. Tuchin, Miklós Veres; Impact of e-cigarette liquid on porcine lung tissue – ex vivo confocal Raman micro-spectroscopy study; oral presentation, The 2nd Spring Biophotonics Conference 15-18, June, Espinho, Portugal, 2023.

Further scientific publications

[F1] Roman Holomb, Oleksandr Kondrat, Volodimir Mitsa, Alexander Mitsa, David Gevczy, Dmytro Olashyn, László Himics, István Rigó, **Ali Jaafar**, Malik H. Mahmood, Tamás Váczi, Aladár Czitrovszky, Attila Csík, Viktor Takáts, Miklós Veres; Gold nanoparticle assisted synthesis and characterization of As-S crystallites: scanning electron microscopy, X-ray diffraction, energy-dispersive X-ray and Raman spectroscopy combined with DFT calculations; Journal of Alloys and Compounds, 2021, doi.org/10.1016/j.jallcom.2021.162467; Q1; IF: 5.3.

[F2] Malik H Mahmood, **Ali Jaafar**, László Himics, László Péter, István Rigó, Shereen Zangana, Attila Bonyár and Miklós Veres; Nanogold-capped poly (DEGDMA) microparticles as surface-enhanced Raman scattering substrates for DNA detection; Journal of Physics D: Applied Physics,2022,Volume 55, Number 40; DOI 10.1088/1361-6463/ac7bba; Q2; IF: 3.4.

[F3] Ali Jaafar, Abbas Albarazanchi, Mohammed Jawad Kadhim, Maxim E Darvin, Tamás Váczi, Valery V Tuchin and Miklós Veres; Impact of e-cigarette liquid on porcine lung tissue–ex vivo confocal Raman micro-spectroscopy study; J. Biophotonics, 2023, e202300336. https://doi.org/10.1002/jbio.202300336; Q2; IF: 2.8.

[F4] Marowa Yass, Ahmed Al-Haddad, Mohammed Jassim Mohammed Ali, **Ali Jaafar** and Miklós Veres; Effectiveness of Green Synthesized Zinc Oxide Nanoparticles against Extensively Drug-resistant Klebsiella pneumoniae; Biomedical and Biotechnology Research Journal 7(3):p 497-503, Jul–Sep 2023. DOI: 10.4103/bbrj.bbrj_167_23; Q3; IF: 1.4.