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

Ph.D. Thesis booklet

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Összefoglalás

Az optikai spektroszkópiai módszerek közé tartozó Raman-spektroszkópia értékes információt képes biztosítani a molekulák szerkezetéről és konformációjáról, ami miatt egyre nagyobb figyelmet kap a biológiai objektumok diagnosztikájában és monitorozásában is. Az orvosdiagnosztikai és biológiai vizsgálatokban alkalmazott optikai eljárások, így a Raman-spektroszkópia esetében is problémát jelent a szövetekben jelentkező erős fényszórás és korlátozott mélységi fénybehatolás. Mindezek jelentős akadályai az optikai technikák klinikai gyakorlatban történő alkalmazásának. A múlt század 90-es éveiben vezették be és fejlesztették ki a detektálási mélység növelésére a szövetek optikai átlátszóságot javító folyadékokkal történő átitatásán alapuló optikai átlátszóságnövelési technikát. Jelen munkában az átlátszóságnövelési eljárás hatását vizsgáltam a szövetekben található kollagén Raman-sávjaira. Ehhez különböző biológiai objektumok, sertés agyhártya és bőr átlátszóságát javítottam, majd konfokális Raman-mikrospektroszkópia segítségével *ex vivo* vizsgáltam a kollagén szerkezetét. A konfokális Raman-mikrospektroszkópia alkalmazásával különböző mélységekben (0 és 250 μm között) és kezelési időknél meghatároztam a tiszta glicerin, mint optikai átlátszóságnövelő adalék hatását a kollagén Raman-csúcsainak intenzitására, valamint a kollagén dehidratációjára és disszociációjára [T1]. Monitoroztam az 50%-os glicerinoldat diffúzióját és az agyhártya kollagénjének víztartalom-változását különböző mélységek és kezelési idők esetén [T2]. Sertésbőrön végzett *ex vivo* Raman-méréseket végeztem az optikai átlátszóságnövelés hatékonyságának összehasonlító elemzésére 633 és 785 nm-es gerjesztéseknél a szövet különböző mélységeiben, továbbá vizsgáltam a dimetil-szulfoxid hatását a glicerinoldat optikai átlátszóságnövelő tulajdonságaira [T3].

1. Introduction

The field of biomedical optics and biophotonics has emerged as one of the fastest-growing disciplines in basic life sciences and biomedical applications, including diagnosis, imaging, therapy, monitoring, and surgery. It permits functional and structural examination of tissues, organs and cells with imaging resolution and contrast 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 *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 diagnostics and monitoring in cosmetic and medical research. 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, medicine and materials science. Additionally, Raman spectroscopy has emerged as a promising alternative or complementary method to the gold standard method of tissue biopsy, which allows to assess the changes related to tissue conditions with minimum sample preparation and handling requirements.

Raman spectroscopy provides valuable insights into the collagen structure and conformation related to different progressive diseases arising from collagen alterations, offering objective, quantifiable molecular information for diagnosis and monitoring. For instance, Raman spectroscopy can effectively discriminate between *dura mater* and meningioma based on Raman peaks correlated with the abundance of collagen in *dura mater* and on the increased lipid content in tumour. Moreover, Raman spectroscopy can monitor changes in collagen conformation within the skin-dermis related to disease or aging processes. Additionally, Raman spectroscopy has been utilized to evaluate collagen alterations in various human body sites such as heart valves, eye, lungs, epithelium, stomach, colon, and breast.

Biomedical optics and biophotonics including Raman spectroscopy are suffering from strong light scattering or limited light penetration depth, posing a significant challenge for implementing optical techniques in clinical practice for diagnosis and monitoring of biological tissues and organs. In the late 1990s, the optical clearing technique based on immersion of the tissue into an optical clearing agent was introduced and developed in order to increase the detection depth of optical techniques, as well as to improve the spatial resolution and contrast

of the resulting images, by suppressing the light scattering within the biological objects. optical clearing is recognized to be a promising technique to combine with the optical methods in the visible to near-infrared spectral range for clinical practice.

Currently, a diverse range of biomedical imaging techniques, such as Raman spectroscopy, confocal Raman micro-spectroscopy, 3D confocal microscopy, polarized microscopy, and optical coherence tomography, have the potential for common utilization in combination with various optical clearing agents such as glucose, dimethyl sulfoxide, glycerol, *uDISCO*, *ScaleS*, and *Scale* to increase sensitivity and reduce light scattering in order to enhance in-depth optical imaging resolution and contrast within biological objects.

However, the potential impact of optical clearing agents on various biological tissues and their components, particularly collagen, raises concerns regarding their safety, and this aspect remains a subject of ongoing research. Furthermore, the effect of optical clearing agents on the water content, the major component of biological tissues (up to 80% of their volume), has not been sufficiently investigated yet. Nonetheless, the main challenges are associated 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 related to the above mentioned unresolved issues and questions. In addition, the development of 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 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. Glycerol was chosen as optical clearing agent being one of the most commonly used agents due to its efficiency, high refractive index, availability, biocompatibility, pharmacokinetics, biosafety, and low cost. The impact of this optical clearing agent in different concentrations on collagen-related Raman peaks, dehydration, dissociation, as well as its concentration and diffusion coefficient in-depth was thoroughly investigated using confocal Raman micro-spectroscopy.

To achieve the above mentioned goals, the following tasks were defined:

1. Investigate the changes in collagen-related Raman peaks after the application of the optical clearing agent (glycerol) to both *dura mater* and skin-dermis.
2. Examine the in-depth changes in collagen hydration and dissociation in the *dura mater* after treatment with an optical clearing agent.
3. Calculate the diffusion coefficient and concentration of the optical clearing agent in the *dura mater*.
4. Analyze the in-depth variation of water content relative to water hydrogen bond strength in the *dura mater* after the application of the optical clearing agent.
5. Investigate the effect of optical clearing on *ex vivo* porcine skin through in-depth monitoring of collagen-related Raman peaks using different excitation wavelengths of 633 and 785 nm.

3. Materials and methods

3.1 Optical clearing agent

The optical cleaning efficiency in biological tissues depends upon various factors, including the refractive index, concentration, osmolarity, and physicochemical properties of the optical cleaning agent. 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 tissues. All these optical cleaning agents are generally considered to be non-toxic. However, it is important to note that prolonged exposure to optical cleaning agents can lead to some negative effects. These may include local hemostasis (impairment of blood flow), tissue shrinkage, and in extreme cases, necrosis (cell death) in living tissue.

Glycerol is a water-soluble triatomic alcohol comprising three carbon atoms covalently bound to the hydroxyl-group. Previous studies have demonstrated that the use of glycerol enhances the signal-to-noise ratio, improves the Raman signal quality, and reduces the systematic error that can arise from inaccurate determination of the surface and sub-surface spectra of the probing sample. Additionally, the optical cleaning efficacy is directly depending

on the number of hydroxyl groups (more hydroxyl groups result in better optical cleaning efficacy). This is described by their potential to disrupt and screen the hydrogen bonds in collagen triple helices, initiating the dissociation of the collagen structure.

3.2. Sample preparation

In this work, fresh *ex vivo* porcine tissues were used to study the impact of OC. 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 *dura mater*, taking into consideration features such as housing, gross anatomical structure, feasibility, and ethical concerns. Due to the similar structure of collagen in *dura mater*, its optical properties are comparable to the dermis of the skin and the sclera of the eye. The presence of a blood vessels network in *dura mater* is the only distinguishing feature from the sclera. All freshly harvested *ex vivo* porcine *dura mater* samples were obtained on the day of slaughter from a local accredited abattoir (Albertirsa, Hungary) and maintained cold on ice in phosphate buffered saline (Sigma Aldrich) during transportation to the laboratory. For the experiments the *dura mater* samples were gently wiped and dried with a paper towel prior the measurement, 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 treatment.

The porcine ear skin is morphologically, immunohistochemically, and histologically, comparable to the human skin. 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 slaughter from a local accredited slaughterhouse (Budapest, Hungary). The skin sample size was 1×1 cm^2 and the thickness was approx. 1.2 mm. It was manually cut with a scalpel and kept in a refrigerator for no more than two days at 5 °C. Before the measurements, the samples were allowed to acclimatize to the laboratory for 30 min at a temperature of 20 ± 1°C. Before the measurement, porcine the skin was thoroughly cleaned with water and then wiped dry with a paper towel. Furthermore, to increase the penetration of optical cleaning agents into the deeper layers of the *ex vivo* porcine skin, the hair was shaved off, and the skin was subjected to 20 tape stripping to remove the outermost layer on the surface. 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}) regions in the backscattered geometry by Renishaw inVia^{TM} confocal Raman microscope equipped with 633 and 785 nm laser sources, a 50× objective (NA = 0.9), and a 1200 l/mm grating, which provides spatial resolutions of 0.77 µm and 0.95 µm, respectively for the two excitations. All samples (*dura mater* and skin) were mounted on the motorized *xyz*-axis stage of the Raman microscope permitting automatic vertical displacement with micrometer resolution to acquire z-scan profiles in-depth.

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 considered 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 fingerprint and high wavenumber 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, I investigated the impact of optical cleaning on collagen-related Raman peaks to control the optical properties of biological objects (*ex vivo* porcine *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 determined the impact of pure glycerol as an optical clearing agent on *ex vivo dura mater* tissue with confocal Raman micro-spectroscopy at different depths from 0 to 250 µm and treatment times [T1].

   i. The intensities of collagen-related Raman peaks were significantly enhanced across all depths 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, which suggests collagen shrinkage.

   ii. Deconvolution of the Raman spectra using Gaussian-Lorentzian functions revealed evidence of collagen dissociation. The observed upshift of the 1666 cm^{-1} Raman peak indicated structural alterations in the molecular geometry of the amide I group of collagen, suggesting dissociation of triple helix chains into simpler

structures such as single or double strings. This dissociation was attributed to the high concentration of glycerol.

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

   i. A new approach was developed to determine 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 ranged from 9.6×10^{-6} to 3.0×10^{-5} cm^2/s, and its concentration from 0.6 to 20% v/v at different depths confirming the effectiveness of the optical clearing method with a moderate concertation. This method can be applied for different optical cleaning agents and drugs.

   ii. The application of a 50% glycerol solution caused significant changes in the total water content of the *dura mater*, reflecting tissue dehydration during the optical cleaning process. Even at low concentrations of glycerol inside the sample. The most prevalent water states in the *dura mater* with highest concentration were the weakly and strongly-bound water types. These water forms play a crucial role in the optical cleaning process, influencing the glycerol-induced water migrations.

3- I determined the impact of various optical cleaning agents containing glycerol and dimethyl sulfoxide on the Raman spectra of *ex vivo* porcine skin-dermis using 633 and 785 nm excitations at different depths from 0 to 240 μm during the optical clearing for both 30 and 60 min, together with the influence of dimethyl sulfoxide as an enhancer for the optical cleaning process [T3].

   i. Optical cleaning significantly enhanced the excitation depth and the quality of Raman spectra obtained using 633 nm excitation confocal Raman micro-spectroscopy, exceeding the capabilities of 785 nm excitation without optical clearing. In addition, the results show enhancement in Raman peak intensities of *ex vivo* skin for both excitation wavelengths across all scanning depths after optical clearing treatment.

   ii. The addition of 5% dimethyl sulfoxide, as a penetration enhancer into the optical cleaning agent further increases the efficiency of the optical cleaning compared to the DMSO-free optical cleaning agent. The most substantial improvement in

optical cleaning efficiency values were obtained for Raman peaks at 1246 and 1271 cm^{-1} (2.6 and 2.7, respectively) at a depth of 200 μm after one hour treatment using 633 nm excitation.

Based on the result of this PhD thesis, future studies in this direction could involve optimizing the biocompatibility of optical clearing agent and integrating this method into clinical practices for the diagnosis and treatment of brain and skin diseases. This integration aims to enhance the diagnostic and therapeutic capabilities of optical analysis methods. These results could provide valuable insights into the structural alterations induced by glycerol treatment, shedding light on the impact of optical clearing processes on the molecular organization of biological tissues.

5. Publications related to the Ph.D. thesis

[T1] Ali Jaafar, Roman Holomb, Anton Y. Sdobnov, Zsombor Ocskay, Zoltán Jakus, Valery V. Tuchin and Miklós Veres; *Ex vivo* confocal Raman microspectroscopy of porcine *dura mater* supported by optical clearing; https://doi.org/10.1002/jbio.202100332; Q2; IF: 2.8, MTMT ID: 32587417.

[T2] 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: 3.2, MTMT ID: 33204848.

[T3] Ali Jaafar, Malik H. Mahmood, Roman Holom, László Himics, Tamás Váczi, 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: 2.3, MTMT ID: 32078778.

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, doi.org/10.1016/j.jallcom.2021.162467; Q1; IF: 5.3, MTMT ID: 32550898.

[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, MTMT ID: 33041627.

[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, MTMT ID: 34396683.

[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, MTMT ID: 34425261.

International conferences

[IC1] Ali Jaafar, Malik H. Mahmood, R. Holomb, L. Himics, V. V. Tuchin, Miklós 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 Miklós 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.