Appendix 1

Form No. 24

Raman microspectroscopy in biomedical study

CODE NAME OF PROJECT OR COLLABORATION: **Biophotonics**

LIST OF AUTHORS IN THE ALPHABETICAL ORDER WITH INDICATION
OF INSTITUTES, COUNTRIES AND TOWNS OF LOCATION
(FOR JINR, INCLUDING LABORATORIES OF THE INSTITUTE)

Dubna, JINR, FLNP:
Arzumanyan G.M., Kučerka N., Mamatkulov K.Z., Vorobyeva M.Yu.

Armenia, Yerevan, Foundation ANSL, Yerevan Physics Institute
Harutyunyan V.V.

Belarus, Minsk, BSUIR
Bandarenko A.V.
Minsk, SOL Instruments
Kopachevski V.J.

Romania, Bucharest, NIMP
Baibarac M.

Russia, Moscow, GPI RAS
Smirnov V.V., Vereschagin K.A.
Moscow, MSU
Shaitan K.V.
Moscow, MONIKI
Volkov A.Yu.

Slovak Republic, Košice, PJSU
Hrubovčák P.

NAMES OF PROJECT LEADERS: **Grigory Arzumanyan and Norbert Kučerka**

NAME OF PROJECT DEPUTY LEADER: **Kahramon Mamatkulov**

DATE OF SUBMISSION OF PROPOSAL OF PROJECT TO SOD _____

DATE OF THE LABORATORY STC **14.05.2020** DOCUMENT NUMBER _____

STARTING DATE OF PROJECT 01.01.2021_____

Appendix 2

Form No. 25

PROJECT ENDORSEMENT LIST

Raman microspectroscopy in biomedical study

CODE NAME OF PROJECT OR COLLABORATION: **Biophotonics**

NAME OF PROJECT LEADERS: **Grigory Arzumanyan and Norbert Kučerka**

APPROVED BY JINR DIRECTOR _____ «____»____202

ENDORSED BY

JINR VICE-DIRECTOR _____ «___»____202

CHIEF SCIENTIFIC SECRETARY _____ «___»____202

CHIEF ENGINEER _____ «___»____202

HEAD OF SCIENCE ORGANIZATION DEPARTMENT _____ «___»____202

LABORATORY DIRECTOR _____ «___»____202

LABORATORY CHIEF ENGINEER _____ «___»____202

PROJECT LEADERS _____ «___»____202

_____ «___»____202

PROJECT DEPUTY LEADER _____ «___»____202

ENDORSED

RESPECTIVE PAC _____ «___»____202

Raman microspectroscopy in biomedical study

Content

- Abstract……..... 4
- Introduction……... 5
- State of the art of the science case proposed and literature ……..... 6
- Description of the proposed research…… 11
- Estimation of human resources and of the proposed budget…… 16
- Appendix 3, 4 …… 17
- Schedule proposal for the years 2021-2023…… 19
- Publications, conferences……... 20
- Review of the FLNPs' STC…… 23

Abstract

In the past several decades, Raman has emerged as a sensitive tool for biomedical study. Advances in instrumentation, methodology, and data analysis have enabled Raman microspectroscopy in a variety of applications from cellular analysis in vitro to in vivo clinical imaging. From advances in spontaneous Raman, to nanoparticle enhancements (SERS, TERS), and nonlinear Raman techniques (CARS, SRS), the use of Raman spectroscopy shows excellent prospects for a wide range of laboratory and clinical uses.

The main objective of the project is aimed at the application of modern Raman scattering methods in some biomedical tasks, which are inherently associated with biosensing and diagnostics. The project includes fundamental and applied segments. In part of basic research, the work will be aimed at identifying and understanding the mechanisms of the ratio of the intensities of antiStokes / Stokes components in surface-enhanced Raman scattering (SERS) spectrum. This will allow to formulate the conditions for obtaining reproducible SERS spectra during the development of biosensors. Applied tasks are related (i) with spectroscopic studies of netosis: in particular, with the search for Raman markers of this phenomenon, as well as with the identification of mechanisms for triggering the sterile activation of netosis under the influence of UV radiation, and (ii) lipid-protein interaction using a modern membrane mimetic – lipodisc.

To realize the tasks of the project, we will employ the multimodal optical platform based on the unique "CARS" microscope, atomic-force microscopy (AFM), dynamic light scattering (DLS), electron microscopy (SEM, TEM), small-angle neutron scattering (SANS) and other instrumentations. Ultralow frequency Raman spectroscopy (~5 cm^{-1}) will become one of the key techniques of the project as well.

The project will involve a qualified and experienced team of the Raman Spectroscopy Sector of the FLNP JINR in close cooperation with interested partners from various countries and organizations. Financing of the project is requested at the level of ~130 k$ annually.

Introduction

The project is aimed at developing highly sensitive optical biosensorics, as well as spectroscopic characterization of the programmed cell death process - NETosis, by the method of spontaneous and enhanced Raman spectroscopy. For this purpose, it is planned the following:

- *study of the features of the Stokes and anti-Stokes components of the spectra of Surface—Enhanced Raman Spectroscopy (SERS) of analyte molecules on nanostructured surfaces of noble metals and the formulation of registration conditions for reproducible SERS spectra;*
- *stabilization of membrane proteins and studies of their structure using lipodisc by Raman scattering, electron microscopy and SANS;*
- *search for spectral / Raman markers of NETosis and study of the mechanisms of sterile formation of NETosis under the influence of UV radiation.*

Recently, there has been an intense growth of interest in the study of biosensorics by various optical methods in combination with nanomaterials, which are capable of detecting molecules in physiological fluids and living cells at extremely low concentrations (Fig. 1, inset).

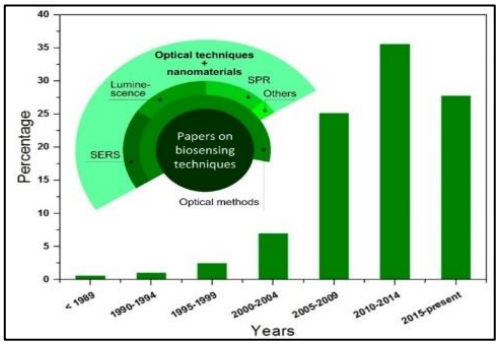


Fig.1. Number of works on biosensorics by years; the inset shows the distribution of biosensing based on optical methods (Google Academy, 30 November 2018)

The main objectives of these studies are non-invasive tests, medical diagnostics and treatment of various types of diseases, including oncology, virus-induced diseases, and other pathologies dangerous to human health. Accuracy and reliability are important biosensing requirements to minimize the risk of false results. In this regard, Raman spectroscopy, as one of the most informative, sensitive and non-invasive methods of light analysis of the molecular structures of condensed matter, is considered as a very effective tool for such purposes.

For a deeper understanding of the approaches in the development of biosensors, it is necessary, inter alia, to conduct studies in the anti-Stokes part of the Raman spectrum, where in some cases the ratios of intensities of the anti-Stokes and Stokes components of the SERS spectrum are different from the expected equilibrium (Boltzmann) values. The nature of these phenomena is not completely clear. Therefore, the goal of the upcoming experiments will be the identification of physical mechanisms leading to the observed anomalies in the SERS spectra. This is especially true to achieve the level of reproducible registration of SERS spectra when creating highly sensitive biosensors.

Of considerable interest is the question of a deep and comprehensive study of the peptide-membrane interaction, which, under certain conditions, can lead to dangerous neurodegenerative diseases. To study the spectroscopic features of this process, the project outlines the use of the innovative development of a membrane mimetic - lipodisc. The relevance of the task, of course, has high social significance.

Another application task of the project is to search for the spectral / Raman markers of NETosis - programmed cell death that has been little studied to date. Neutrophils are the first line of defense of the body against bacterial, fungal and, in some cases, viral infections. Early diagnosis of NETosis is extremely important in a number of diseases and pathologies.

All of the above mentioned is in mainstream of the world trends in the development of Raman spectroscopy and microscopy, and, thereby, determines the modern innovative nature of the proposed project.

State of the art of the science case proposed

1. Physical nature of the anomalous aSt / St ratio in the SERS spectra

One of the important and finally unresolved issues of SERS spectroscopy is the issue of the physical nature of the often anomalous ratio of the intensities of the anti-Stokes and Stokes components (hereinafter referred to as aSt / St), which is different from the expected equilibrium (Boltzmann) values. A number of studies have been devoted to solving this problem, but the bulk of the experiments were carried out using cw lasers. The use of picosecond lasers is very promising for solving this problem, since this duration corresponds to the scale of the times of flows and energy redistribution in nanostructured materials with analyte molecules adsorbed on them.

The attention of the SERS community to this problem was noticeably attracted in 1996 after the publication [1], where its authors for the first time reported an evidence of vibrational (Raman) pumping under SERS conditions (due to unexpectedly large SERS cross section). In [1] while studying Raman pumping, the laser power dependence of anti-Stokes to Stokes (aS/S) ratio (ρ) of Raman lines of Rhodamine 6G (Rh6G) and Crystal Violet (CV) using 830 nm laser excitation at room temperature was observed. Moreover, it was suggested that this method (of power dependent vibrational pumping) could become a possible tool for estimation of SERS cross sections.

The interpretation of these experimental results has been the subject of considerable debate in relevant scientific literature, with many authors denying the existence of Raman pumping and attributing the experimental observations to either laser-heating, various resonance effects, or combinations thereof [2-7] with a conclusion that no evidence for a SERS-induced nonthermal population distribution among the vibration states (vibration Raman pumping) of the adsorbed molecules was found. Nevertheless, ten years later the existence of vibration pumping under SERS conditions was confirmed by performing measurements of the aS / S ratio as a function of temperature down to 10 K at low intensity of laser excitation [8]. In the review [9], the underlying principles of vibrational pumping in surface enhanced Raman scattering (SERS) are summarized and explained within the framework of their historical development.

Despite these studies, the debate about ‘heating vs. pumping’ is still ongoing. Therefore, the goal of the upcoming experiments on this project will be to identify the physical mechanisms leading to the observed anomalies in the SERS spectra. To this end, a comparative study of the behavior of the intensities of the SERS lines of the analyte in the Stokes and anti-Stokes spectral regions will be performed depending on the pump radiation power in both pulsed and continuous modes. The identification of nonlinear behavior of line intensities in the SERS spectra, including those similar to coherent anti-Stokes Raman scattering [10-11], will also be in the focus of attention of the studies proposed in the project.

Nanostructures with controlled and well-defined computer-aided modeling structures are powerful tools for understanding the plasmonic properties of metallic nanostructures. However, compared to such structures, aggregated nanoparticle assemblies can exhibit unusual and unpredictable behavior. Therefore, an applied aspect of the solution of the

described problem is the formulation of the conditions for recording reproducible SERS spectra from analyte molecules deposited on the surface of nanostructured substrates, which must be observed when creating highly sensitive SERS biosensors.

According to our information, based on scientific literature, experimental work in this direction is not conducted in Russia.

The main world scientific competitors

1. Massachusetts Institute of Technology, Cambridge, USA (group of Prof. Kneipp)
2. University of California, Irvine, USA. (group of Prof. Abkarian)
3. Imperial College London, London, UK (group of Prof. Maher)

2. Membrane proteins and lipodiscs

The study of membrane proteins (MPs) is one of the major challenges in current research in molecular life sciences. It is known that membrane proteins make up 20-30% of the human proteome [12]. Knowledge of their structure helps the development of medicine and pharmacology - among proteins targeted by drugs, the proportion of membrane proteins is up to 50% [13-14]. At the same time, membrane proteins practically do not lend themselves to crystallization, and therefore, to study their structure, the traditional method of x-ray structural analysis is inapplicable. This project is devoted to the development of a method for stabilizing membrane proteins and studying their chemical structure by Raman spectroscopy using lipodisc - fragments of a lipid membrane (membrane mimetic) limited to amphiphilic polymers. On lipodiscs, in contrast to lipid-protein nanodiscs stabilized in solution with apolipoprotein or a special amphiphilic protein MSP (membrane Scaffold Proteins), there are relatively few studies, although these objects are of considerable interest from the point of view of structural biology and experiments with single molecules or their complexes.

Recently, lipodiscs formed using a styrene-maleic acid copolymer (SMA, XIRAN) have gained large interest and popularity [15-20].

Advantages of lipodiscs:

Lipodiscs have several advantages over the traditional method of isolation and solubilization of membrane proteins using a detergent. Firstly, it is believed that lipodiscs allow you to keep a small amount of lipids around the protein that surrounded it in the membrane, and these lipids help keep the conformation of the membrane protein unchanged. Secondly, unlike nanodiscs that are similar in structure, formed using the MSP protein, lipodiscs formed using amphiphilic polymers can be formed with a minimum number of experimental steps - by adding an amphiphilic polymer to the membrane fraction of cells or directly to cells. Thirdly, two-component lipodiscs consisting of a lipid and a copolymer can be prepared, and for some analytical methods they act as a convenient reference sample.

To analyze the structure of the protein embedded in lipodiscs, transmission electron microscopy, TEM, is used. It allows to build a model of the distribution of electron density in a protein based on many images of individual molecules. The goal of this project is to improve existing procedures for isolating and studying the structure of membrane proteins using Raman spectroscopy, to obtain new information on the structure of lipodiscs with membrane proteins and empty lipodiscs.

The project provides for the solution of the following tasks in this area:

1. To develop a technique for obtaining Raman spectra from samples of lipodiscs with membrane proteins and “empty” lipodiscs.
2. Highlight characteristic lines in the spectra that are associated with the presence of each of the three components — lipid, protein, and copolymer.
3. Confirm the incorporation of membrane protein into lipodisc and determine the features of its structure.
4. To study the composition of proteins and lipids contained in lipodiscs.

It is expected that the proposed innovative lipodisc structure and sensitive Raman spectroscopy will make it possible to advance in such a complex process as lipid-protein interactions.

4. NETOSIS: spectral markers and UV-activation of neutrophils

Neutrophilic white blood cells are the first line of defense against bacterial and certain viral infections.

When the quantity or quality of these cells deviates from the norm, people are exposed to serious, often life-threatening infections. In addition, such disorders can be a marker of many systemic, autoimmune diseases. In this regard, the conduct of laboratory biomedical research studies, including of spectroscopic nature, are undoubtedly relevant and socially significant.

Unlike the well-known and investigated two other types of cell death - apoptosis and necrosis, netosis is currently poorly understood. This is especially true of spectroscopic, including Raman studies of this phenomenon. Pioneering work on netosis is considered the publication of Brinkman in 2004 [21]. He coined the term NETs - Neutrophil Extracellular Traps.

Neutrophils (also known as neutrocytes) are the most common type of granulocytes and the most common (60 to 70%) type of white blood cells in most mammals and form an important part of the innate immune system [22]. Neutrophils are cells such as phagocytes and are usually found in the bloodstream. They are very flexible and mobile, and are able to penetrate into those parts of the tissue where other cells are not capable. The main protective functions of neutrophils include: phagocytosis, chromatin degranulation and the formation of extracellular traps - NETs. These extracellular neutrophil traps include tissue made up of chromatin fibers that trap and kill extracellular pathogens.

It should be mentioned that there are many publications in the literature on netosis devoted to the methods and mechanisms of neutrophil activation, as well as to the positive and negative consequences of this phenomenon [23-27]. Survey papers can be found in [28,29]. However, it should be noted that currently there are only few studies on spectral markers of this phenomenon and sterile UV-activation of netosis [30–33]. The latter is a very urgent topic, since humanity is exposed to the sun every day in one dose or another, including and UV spectrum exposure, which could be of negative consequences. These two tasks, namely the search for Raman markers of netosis and sterile UV-activation, are the key stages of the research of the proposed project in terms of netosis.

References

1. Kneipp K., Wang Y., Kneipp H., Itzkan I., Dasari R., Feld M. Population Pumping of Excited Vibrational States by Spontaneous Surface-Enhanced Raman Scattering. Phys. Rev. Lett. 1996, **76**, 2444−2447.
2. Haslett T. L., Tay L., Moskovits M. Can surface-enhanced Raman scattering serve as a channel for strong optical pumping? J. Chem. Phys. 2000, **113**, 1641−1646.
3. Brolo A. G., Sanderson A. C., Smith A. P. Ratio of the surface-enhanced anti-Stokes scattering to the surface-enhanced Stokes-Raman scattering for molecules adsorbed on a silver electrode. Phys. Rev. B: Condens. Matter Mater. Phys. 2004, **69**, 045424.
4. Maher R. C., Hou, J., Cohen L. F., Le Ru E. C., Hadfield J.M., Harvey J. E., Etchegoin P. G., Liu F. M., Green, M., Brown, R.J., Milton, M. J. Resonance contributions to anti-Stokes/Stokes ratios under surface enhanced Raman scattering conditions. J. Chem. Phys. 2005, **123**, 084702.
5. Le Ru E. C. and Etchegoin P. G. Vibrational pumping and heating under SERS conditions: fact or myth? Faraday Discuss., 2006, **132**, 63–75.
6. Maher R. C., Cohen L. F., Le Ru E. C., and Etchegoin P. G. A study of local heating of molecules under Surface Enhanced Raman Scattering (SERS) conditions using the anti-Stokes/Stokes ratio. Faraday Discuss., 2006, **132**, 77–83.
7. Kevin T. Crampton, Alexander Fast, Eric O. Potma, and V. Ara Apkarian Junction Plasmon Driven Population Inversion of Molecular Vibrations: A Picosecond Surface-Enhanced Raman Spectroscopy Study. Nano Lett., 2018, 9, 5791-5796
8. Maher R. C., Etchegoin P. G., Le Ru E. C., and Cohen L. F. A Conclusive Demonstration of Vibrational Pumping under Surface Enhanced Raman Scattering Conditions. J. Phys. Chem. B, 2006, **110**, 11757-11760.
9. Maher R. C., Galloway C. M, Le Ru E. C., Cohen L. F. and Etchegoin P. G. Vibrational pumping in surface enhanced Raman scattering (SERS). Chem. Soc. Rev., 2008, 37, 965–979.
10. S. Lefrant, I. Baltog, M. Baibarac Surface-enhanced Raman scattering studies on chemically transformed carbon nanotube thin films. J. Raman Spectrosc. 36, 676-698, 2005
11. I. Baltog, M. Baibarac, I. Smaranda, S. Lefrant Abnormal anti-Stokes Raman emission as a CARS process in disordered media. J. Phys. B At. Mol. Opt. Phys. 44, 095401, 2011.
12. Wallin E, von Heijne G (1998) Genome-wide analysis of integralmembrane proteins from eubacterial, archaean, and eukaryotic organisms. Protein Sci 7:1029–1038
13. von Heijne G (2007) The membrane protein universe: what's out thereand why bother? J Intern Med 261:543–557.
14. Overington JP, Al-Lazikani B, Hopkins AL (2006) How many drugtargets are there? Nat Rev Drug Discov 5:993–996.
15. Jonas M. Durr, Stefan Scheidelaar, Martijn C. Koorengeve, et al. The styrene–maleic acid copolymer: a versatile tool in membrane research. Eur Biophys J (2016) 45:3–21
16. N. Voskoboynikova, W. Mosslehy, A. Colbasevici, T. T. Ismagulova, D. V. Bagrov, K. V. Shaitan, et al. Characterization of an archaeal photoreceptor/transducer complex from Natronomonas *pharaonis* assembled within styrene–maleic acid lipid particles. RSC Adv., 2017, 7, 51324-51334
17. Long A.R., O'Brien C.C., Malhotra K., et al. A detergent-free strategy for the reconstitution of active enzyme complexes from native biological membranes into nanoscale discs. BMC Biotechnology 2013, 13, 41.
18. J. M. Dorr, S. Scheidelaar, M. C. Koorengevel, J. J. Dominguez, et al. The styrene-maleic acid copolymer: a versatile tool in membrane research. Eur. Biophys. J., 2016, 45, 3–21.

19. S. C. Lee and N. L. Pollock, Membrane proteins: is the future disc shaped? Biochem. Soc. Trans., 2016, 44, 1011-1018.
20. T. J. Knowles, R. Finka, C. Smith, Y. P. Lin, T. Dafforn and M. Overduin. Membrane proteins solubilized intact in lipid containing nanoparticles bounded by styrene maleic acid copolymer. J. Am. Chem. Soc., 2009, 131, 7484–7485.
21. Brinkmann V, Reichard U, Goosmann C, Fauler B, Uhlemann Y,Weiss DS, et al. Neutrophil extracellular traps kill bacteria. Science. 2004; 303:1532–1535.
22. Scapini P., Lapinet-Vera J. A., Gasperini S., Calzetti F., Bazzoni F., Cassatella M. A. The neutrophil as a cellular source of chemokines. Immunological Reviews. 2000; 177: 195–203.
23. Segal, AW. How neutrophils kill microbes. An. Rev Immunol. 2005, 23 (5), 197–223.
24. Fuchs, TA; Brill, A, Duerschmied, D, Schatzberg, D, Monestier, M, Myers DD Jr, Wrobleski, SK, Wakefield, TW, Hartwig, JH, Wagner, DD (Sep 7, 2010). "Extracellular DNA traps promote thrombosis". Proceedings of the National Academy of Sciences of the USA. 107 (36): 15880-5. Bibcode, 2010, PNAS,10715880F.
25. Khandpur R, Carmona-Rivera C, Vivekanandan-Giri A, Gizinski A, Yalavarthi S, Knight JS, et al. NETs are a source of citrullinated autoantigens and stimulate inflammatory responses in rheumatoid arthritis. Sci Transl Med. 2013, 5.
26. Konig MF, Andrade F. A critical reappraisal of neutrophil extracellular traps and NETosis mimics based on differentialrequirements for protein citrullination. Front Immunol. 2016, 7, 461.
27. Khan MA, Palaniyar N. Transcriptional firing helps to drive NETosis. Sci Rep. 2017, 7, 41749.
28. Galluzzi L, Vitale I, Aaronson SA, Abrams JM, Adam D, Agostinis P, et al. Molecular mechanisms of cell death: recommendations of the nomenclature committee on cell death 2018. Cell Death Differ. 2018, 25, 486–541.
29. Stojkov D, Amini P, Oberson K, Sokollik C, Duppenthaler A, Simon HU, et al. ROS and glutathionylation balance cytoskeletal dynamics in neutrophil extracellular trap formation. J Cell Biol. 2017, 216, 4073–4090.
30. Elsa Neubert, Katharina Maria Bach, Julia Busse et al. Blue and Long-Wave Ultraviolet Light Induce in vitro Neutrophil Extracellular Trap (NET) Formation. Frontiers in Immunology, 2019, 10:2428.
31. Dhia Azzouz, Meraj A. Khan, Neil Sweezey and Nades Palaniyar. Two-in-one: UV radiation simultaneously induces apoptosis and NETosis. Cell Death Discovery, 2018, 4:51
32. Marcin Zawrotniak, Dominika Bartnicka, Maria Rapala-Kozik. UVA and UVB radiation induce the formation of neutrophil extracellulartraps by human polymorphonuclear cells. J. of Photochemistry & Photobiology, B: 2019, 196:111511.
33. Arzumanyan G.M., Gurev A.S., Kravtsunova D.E., Mamatkulov K.Z., et al. Micro Raman spectroscopy for NETosis detection. JRS, SPECIAL ISSUE, .2020,1-9.

Description of the proposed research

Focus of the research

Revealing of the physical nature of the anomalous ratio of the anti-Stokes and Stokes components in the SERS spectra in order to formulate the conditions for recording reproducible spectra for biosensors. Studying the structural and spectral characteristics of lipodiscs embedded with proteins. Search for the spectral markers of NETosis and the mechanisms of sterile UV-activation of neutrophils.

Assumptions and hypotheses

Despite the great progress in the applied aspects of SERS, some fundamental issues of this process are still open. One of such unresolved issues is the physical nature of the frequently observed effect of the anomalous ratio of the intensities of aSt / St components in the SERS spectra. Currently, there is no accurate data on the so-called "vibrational, or Raman" pumping, leading to this anomaly. It is well known that in thermodynamic equilibrium condition the ratio of aSt / St is determined by the Boltzmann factor:

$$\frac{I_{as}}{I_s} = \left(\frac{\omega_L + \omega_V}{\omega_L - \omega_V} \right)^4 \exp\left(-\frac{\hbar \omega_V}{kT} \right)$$

where ω_L, ω_V, $\hbar$, k and T are the excitation frequency, vibrational frequency, Planck constant, Boltzmann constant and temperature, respectively.

However, it is not uncommon for cases when this factor is violated in real experiments and the spectrum in the aniStokes part looks like in Fig. 2 below.

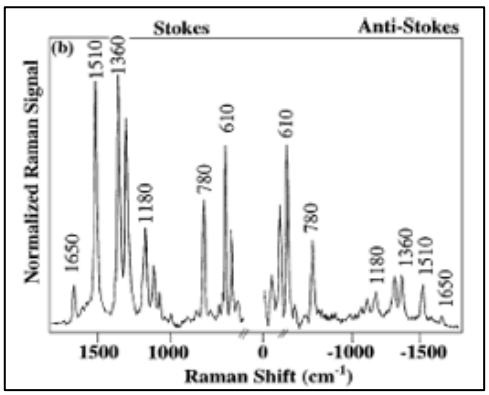


Fig.2. SERS spectrum (aSt and St) of Rhodamine 6G

In addition, in a number of published studies, the authors admitted hypotheses also about the nonlinear, resonant nature of the peak intensities in the anti-Stokes region depending on the pump power. And there can be several such resonances, including plasmon resonance from one isolated nanoparticle, collective plasmon resonance from an ensemble of particles, direct Raman resonance (for example, in the case of dyes), the coupled plasmon resonance state of a molecule, etc. All these resonances can affect Stokes and anti-Stokes intensities in the SERS spectra in different ways, and, therefore, can be a source of various anomalies described in the literature.

Taking into account the above assumptions, in the proposed project it is planned to study the dependence ratio of intensities aSt / St not only on the pump power, but also on the radiation wavelength, and, in our opinion, it is extremely important in the comparative mode with continuous and pulsed (ps) pump modes. We do not currently know of such published works. Namely, the pulsed mode can bring some clarity to the anomalous nature of the measured SERS spectra.

In the part of the project related to UV-activation of netosis, some authors rightly admit that in certain cases ultraviolet radiation can cause not only netosis, but also apoptosis. In order to identify and differentiate these mechanisms of cell death, it is planned to study the activation process: (i) at various radiation doses and (ii) at different radiation wavelengths that fall both in the UV-A region (400-315) nm and in the UV-B region (315-280) nm. This is motivated by the fact that these conventionally two regions of the spectrum of solar UV radiation reach the Earth's surface, although in different proportion.

Research methods and description of experimental facilities

To implement the proposed project, the following methods and approaches will be used:

1. For each of the selected systems (sample with a SERS active surface + analyte molecule), it is proposed to carry out a complex of both linear (spontaneous Raman and SERS) and nonlinear (CARS, SECARS) spectroscopic studies aimed at elucidating the nature of the ratio aSt / St , reasons for the appearance of resonant and non-resonant contributions to the SERS signals.
2. For the pulsed excitation of SERS process, one out of two picosecond laser radiation output channels installed on a CARS microspectrometer will be used.
3. These studies are planned to be supplemented by atomic force microscopy of the surface morphology of SERS active substrates, and by dynamic light scattering if using colloidal solutions of silver or gold nanoparticles. In experiments, it is planned to use the basic setup of the Raman pectroscopy sector — a CARS microspectrometer with picosecond laser pulses (see further description).
4. To register the anti-Stokes part of the spectrum, filters from Semrock (USA) will be used initially. However, the passband of these filters starts at 350 cm-1, which will not allow detecting low and ultralow wavenumbers either in the SERS spectra or in spontaneous raman in the study of netosis. To solve this extremely key issue of the project, it is planned to purchase a special Raman spectrometer equipped with Bragg filters. This will allow recording spectra in the immediate vicinity of the excitation line of ~ 5 cm^{-1}.
5. For processing and analysis of spectral information, both available algorithms and programs, as well as modified and special software and algorithms (NanoSP, ImageSP, etc.) developed by the CARS microspectrometer supplier will be used.
6. As for the samples with SERS-active surface we intend to use the experience and development of our long-term partner – the Belarusian State University of Informatics and Electronics (BSUIR). These are substrates with silver nanoparticles localized on a mesoporous silicon substrate. In addition, some commercially available GCR substrates (ATO-ID, etc.) will also be used in experiments.
7. For core-shell type nanoparticles, the experience of the synthesis of such particles in the FLNP Raman spectroscopy sector in 2019-2020 will be used for luminescent studies.
8. It is supposed to use the professional experience of partners of the Department of Bioengineering, Faculty of Biology, Moscow State University and the staff of MONIKI, using lipodisc samples and neutrophil cells, respectively.
9. For UV-activation of neutrophils, it is planned to purchase relatively inexpensive diode lasers at 3-4 different wavelengths.

Multimodal optical platform – microscope “CARS”

The laser scanning confocal microscope “CARS” is a laser-optical-mechanical complex-optical platform (Fig.3), developed in a compact configuration and mounted on a vibration-resistant desk SDANDA (Lithuania).

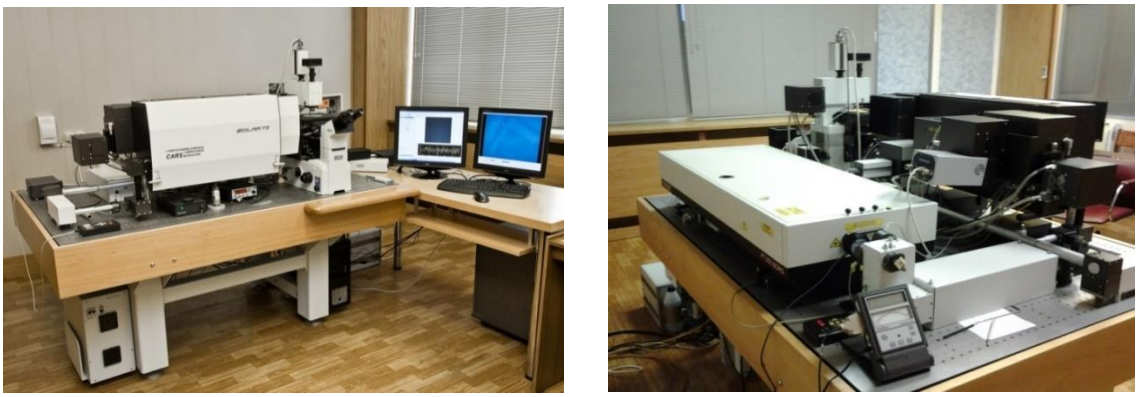


Fig.3. General view of the microscope “CARS”

In 2016, the optical platform was modernized, as a result of which experiments were carried out on the platform for SERS, polarized CARS microscopy (P-CARS), spontaneous Raman spectroscopy at a wavelength of 785 nm with a weak luminescent background for biological samples. Figure 4 shows the upgraded optical scheme of the microscope.

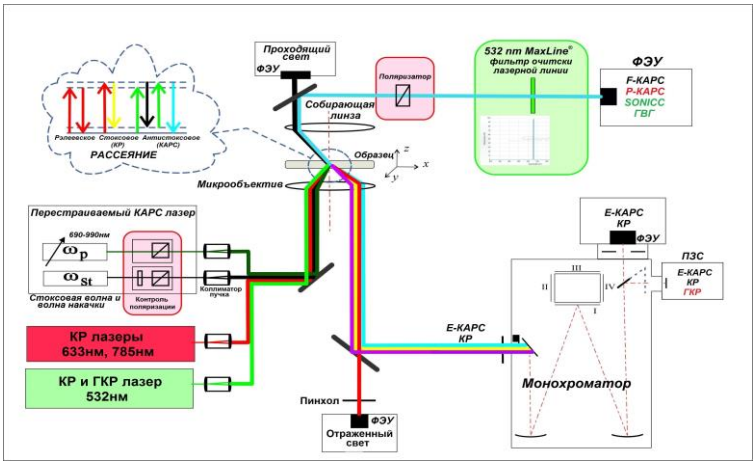


Fig.4. The modernized layout of the optical platform.

Thus, at present, for spontaneous Raman scattering and SERS, three laser sources with wavelengths of 532nm, 633nm, and 785nm are used, depending on the task and the sample. To generate CARS process on the platform a picosecond tunable Nd:YVO_4 laser (model PT257-SOPO, EKSPLA, Lithuania) is installed. The pulse repetition rate is 85 MHz, pulse duration is ~ (5-6) ps, mean power (50-200)mW.

It’s well-known that the pulse duration of several picoseconds is a proper compromise between high intensity and narrow spectral bandwidth necessary for the CARS microscopy. Besides, its intensity is sufficient also for detection of other nonlinear processes, in particular the second and sum harmonic generation. Only a small portion of biologically tolerable laser power is used for CARS and SHG imaging.

The basic laser beam at 1.06μ serves as the source of the Stokes wave (ω_s) in CARS and is simultaneously used to synchronously pump an intracavity-doubled crystal optical parametric oscillator (SOPO). Thereby, the SOPO coherent device provides temporal synchronization with the Nd:YVO_4 and serves as a source of the pump beam (ω_p) which is tunable in the range between (690-990) nm with a maximum output power of 200mW and

linewidth ~(5-7)cm^{-1}. Both excitation picosecond pulse trains are made coincident in time and in space utilizing an computer-controlled optical delay line and a series of dichroic mirrors. For CARS microscopy, we use a water-immersion objective lens with a high numerical aperture (NA=1.2, UPLANAPO-60x, Olympus) to focus the beams tightly. With the tight foci, the phase-matching conditions are relaxed because of the large cone of wave vectors of the excitation beams and the short interaction length.

Using our optical platform, a sample can be analyzed and imaged by utilizing vibration frequencies in the spectral range of (1000−3580) cm^{-1}, which covers all most important vibrational modes of bio-molecules. Five detection channels allow two forward- and three backward- propagated signals to be recorded. The polarization control is adjustable with a half-wave plate in the Stokes beam and the Glan-Taylor polarizer installed on a rotation mount directed to the photomultiplier (Hamamatsu, H6780-01) in the forward direction.

A computer-controlled XY galvanometer scanner (GSI-Lumonics VM1000) provided a fast scan of the sample in the lateral focal plane of the objective. In the fast mapping option the signal integration time is 3 μs/pixel which results in a total acquisition time of about 3s. The microobjective is mounted on a computer-controlled Z-axis translation piezostage for scanning through the microscope's optical axis with a minimal scan step of 0.1μm (3D-mapping).

Expected main results upon the project completion.

1. A comparative analysis of the ratio of the intensities of the SERS lines in aSt / St spectral regions depending on the pump radiation power.
2. Determination of the characteristics of the intensity ratio aSt / St depending on the excitation wavelength of analyte molecules.
3. Identification, comparison and characterization of the mechanisms of formation of aSt / St components of SERS spectra in continuous and pulsed modes.
4. A detailed analysis and interpretation of the Raman spectra of lipodiscs with various membrane proteins.
5. Confirmation of the incorporation of membrane protein into lipodisc and determination of the features of its structure.
6. Obtaining new information about the structure of lipodiscs with membrane proteins and “empty” lipodisc.
7. Identification of Raman markers of NETosis in various regions of the Raman spectrum.
8. Determination of the mechanisms of the formation of NETosis under the influence of UV radiation.
9. Gaining experience in ultra-low frequency Raman spectroscopy ~ (5-10) cm^{-1}.

Present experimental background of the proposed project.

In 2020, in the sector of Raman spectroscopy of the FLNP, preliminary study was started on almost all the tasks proposed for the project, and, on netosis, the first paper was published in the rating specialized Journal of Raman Spectroscopy.

1. The spectra of the anti-Stokes and Stokes components were measured for some samples both in the spontaneous mode of Raman scattering and under the conditions of

SERS. The corresponding results are shown in Figures 5 and 6.

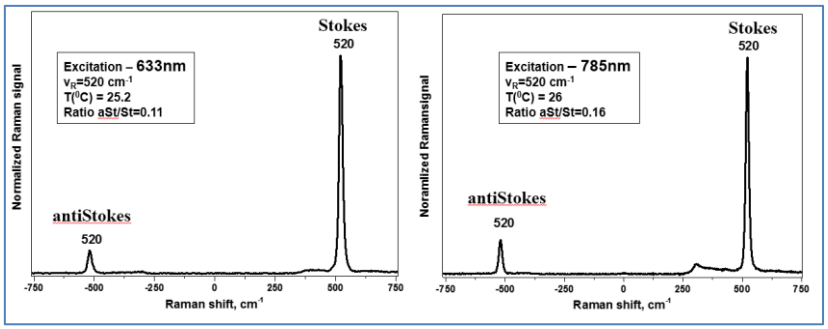


Fig.5. Raman spectra of c-Si in the aSt and St domains

Figure 5 shows that in the spontaneous Raman spectrum no anomaly is observed in the anti-Stokes part of the spectrum and the temperature of the sample corresponds to the expected one, i.e. room temperature. And Fig. 6 shows the SERS spectra obtained from dye analytes of methylene blue and acid DTNB.

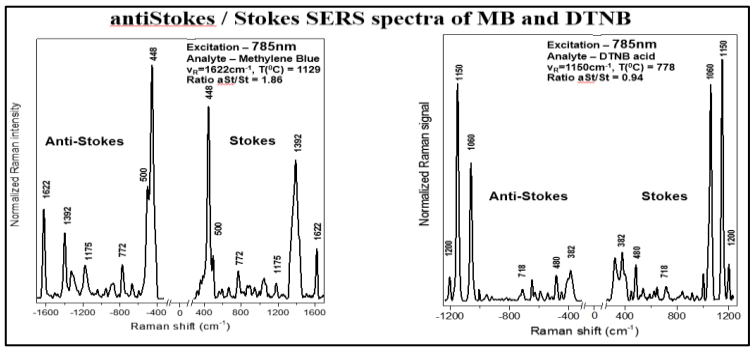
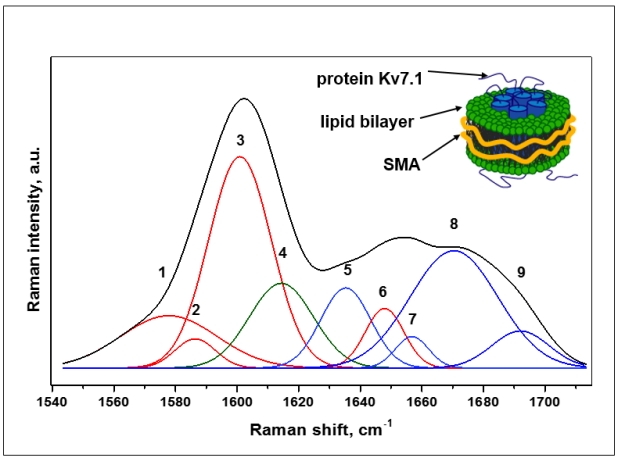


Fig.6. SERS spectra of methylene blue (left)and DTNB (right) in aSt and St parts

Both – the intensities of the peaks in the anti-Stokes part of the spectrum and the temperature of one of the peaks, clearly shows a certain “anomaly” that needs its further explanation.

2. At the CARS microspectrometer, the registration of Raman spectra of lipodiscs with the integrated Kv7.1 protein was tested (Fig. 7).



Область: 1500-1800 см$^{-1}$

№	Raman shift, cm^{-1}	Assignment
1	1578	SMA
2	1586	SMA
3	1601	SMA
4	1614	Tyr, Trp, Phe
5	1635	amide I: unordered
6	1648	SMA
7	1657	amide I: α-helix
8	1670	amide I: β-sheet
9	1692	amide I: β-turn

Fig.7. Approximation (deconvolution) of the Raman spectrum of the Kv7.1 protein built-in lipodisc. Spectrum assignments are on the right

The preliminary result obtained by approximating the Raman spectrum (see the table on the right of Fig. 7) showed the indisputable advantage of vibrational, Raman spectroscopy in identifying the fine chemical structure of these samples. The prospects and innovative nature of these studies will, in our opinion, shed light on lipid-membrane interactions.

3. Another major segment of the proposed project is netosis. It was already noted above that here the experimental groundwork of the Raman spectroscopy sector team reached the level of the first publication in the Journal of Raman Spectroscopy in 2020. A link to this publication is given in the list of references under number 33 on page 10.

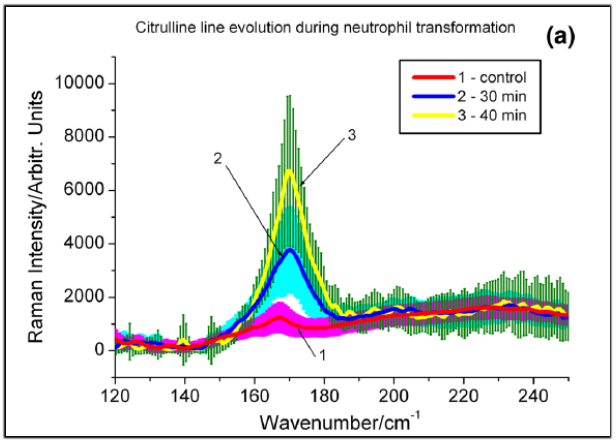


Fig.8. Low-frequency peak of citrulline in the Raman spectrum of neutrophils.

Figure 8 shows the low-frequency Raman peak of citrulline after neutrophil cell activation for 30 and 40 minutes. For comparison, control is also given. Histon citrullination directly indicates the beginning of the netosis process (prenetosis) and can serve as a spectroscopic marker for the early diagnosis of netosis.

Assessment of the human resources and the budget.

Almost all Raman spectroscopy and microscopy related to the proposed project will be performed at the CARS microscope at the JINR FLNP. Over the past 5 years, the staff of the Raman spectroscopy sector has acquired a sufficiently high professional level and skills to successfully cope with the implementation of a new project. This is also confirmed by a number of publications in highly rated journals and participation in major international conferences, including with invited reports, for the past reporting period (list attached). The role of the project partners should also not be underestimated both in terms of supplying unique samples of lipodiscs and neutrophil cells, and in terms of analyzing and discussing the results.

In the sector of Raman spectroscopy, 11 staff members currently work (5 of them are part-time). Seven of them are young scientists and engineers under the age of 35. A nonlinear microspectroscopy group operates within the sector. The entire composition of the Sector will be fully involved in the implementation of the proposed new project.

Thus, the above-described optical platform with its modern characteristics for Raman microspectroscopy and luminescent studies is potentially ready to carry out a new project as part of the extension of the topic and the new project "Biophotonics".

However, the addition of the existing instrument infrastructure with a low-frequency Raman spectrometer is becoming a key position in the new project.

Appendix 3

Form No. 26

Schedule proposal and resources required for the implementation of the Project

"Raman microspectroscopy in biomedical study"

Expenditures, resources, financing sources			Costs (k$) Resource requirements	Proposals of the Laboratory on the distribution of finances and resources		
				1st year	2nd year	3rd year
Expenditures		Main units of equipment, work towards its upgrade, adjustment etc. (ultralow frequency spectrometer, microobjectives, filters, measuring equipment, etc.)	220	85	70	65
		Materials	50	10	20	20
Financing sources	Budgetary resources	Budget costs, including foreign currency	270	95	90	85
	External resources	Contributions by collaborators. Grants. Contributions by sponsors. Contracts. Other financial resources, etc.	-	-	-	-

PROJECT LEADERS G.M. Arzumanyan

N. Kučerka

Appendix 4

Form No. 29

Estimated expenditures for the Project
"Raman microspectroscopy in biomedical study"

#	Expenditure items	Total cost, k$	Expenditures per year (k$)		
			1st year	2nd year	3rd year
5.	Materials	50	10	20	20
6.	Equipment	220	85	70	65
9.	Payments for agreement-based research	26	9	9	8
10.	Travel allowance, including:	69	23	23	23
	a) non-rouble zone countries		17	17	17
	b) rouble zone countries		6	6	6
	c) protocol-based		-	-	-
	Total direct costs:	**365**	**127**	**122**	**116**

PROJECT LEADERS G.M. Arzumanyan

N. Kučerka

LABORATORY DIRECTOR V.N. Shvetsov

LABORATORY CHIEF ENGINEER-ECONOMIST L.S. Ovsjannikova

Schedule proposal for the years 2021-2023

Activities		Plans per year		
		2021	2022	2023
1.	Study of the features of the ratio of intensities aSt / St in SERS spectra depending on the power and pump wavelength.			
2.	Identification of mechanisms of formation of aSt / St spectra in continuous wave and pulsed modes.			
3.	Systematic experiments aimed at formulating the conditions for recording reproducible SERS spectra for biosensorics.			
4.	Development of a technique for recording and analyzing Raman spectra of lipodiscs without and with embedded membrane proteins.			
5.	Study of the influence of the lipid environment on the structure of the membrane protein.			
6.	Obtaining new spectroscopic information on the structure of lipodiscs with membrane protein.			
7.	Search for spectral / Raman markers of NETosis.			
8.	Investigation of the mechanisms of sterile activation of NETosis under UV radiation.			
9.	Ultra-Low Frequency Raman spectroscopy ~ (5-10) cm^{-1}			

PROJECT LEADERS G.M. Arzumanyan

N. Kučerka

Main publications

1. Arzumanyan G.M., Mamatkulov K.Z., Fabelinsky V.I., et al., “Surface-enhanced micro-CARS mapping of a nanostructured cerium dioxide/aluminum film surface with gold nanoparticle-bound organic molecules”, J. Raman Spectr., 49, 2018, 7(2), pp. 1145-1154.
2. Arzumanyan G.M., Mudrii A.V., Dymshits O.S., et al., “Photoluminescence and structural characteristics of glass ceramics containing zinc oxide nanocrystals and europium ions”, Proceedings of the SSP-2018 Conference, September 24-28. 2018, Vol. 2, p. 254-257
3. Poimanova O.Yu., Radio S.V., Arzumanyan G.M., et al., “Hexakis (dimethylsulfoxide-O)-cobalt(II) hexatungstate, [Co(C2H6OS)6][W6O19]: synthesis from aqueous di ethylsulfoxide solution, crystal structure determination, FT-IR and Raman spectroscopy analysis, and surface micromorphology”, J. Coord. Chem., 71(3), 2018, pp. 1-19.
4. Sarychev A.K., Arzumanyan G.M., Mamatkulov K.Z., et al., “Optical properties of mesoscopic, multiscale silver films: surface plasmon localization and giant SERS”, Proceedings of SPIE-2018, Paper No.: OP101-87.
5. Poimanova O.Yu., Medved A.O., Arzumanyan G.M. et al., “Nickel Decavungstate [Ni (C2H6SO) 5 (H2O)] 2 [W10O32]: Synthesis from a Water-Dimethyl Sulfoxide Solution, Determination of the Crystal Structure, IR and Raman Spectroscopic Analysis, Surface Micromorphology”, J. Struct. Chemistry, 59 (1), 2018, p. 149-157.
6. Arzumanyan G.M., Linnik D.S., Mamatkulov K.Z., Glazunova V.S., “Synthesis of up-conversion SrF2 Nanoparticles: Yb, Er @ SrF2”, Proceedings of III Int. conf. “Donetsk Readings-2018”, October 2018, Vol. 2, p. 122-124
7. Arzumanyan G.M., Mamatkulov K.Z., Fabelinsky V.I., et al., “Laser intensity limits in surface-enhanced linear and nonlinear Raman micro-spectroscopy of organic molecule/ Au-nanoparticle conjugates”, J. Raman Spectr., 2019; 50,
8. Arzumanyan G.M., Linnik D.S., Mamatkulov K.Z., Vorobeva M.Yu., et al., “Synthesis of NaYF4:Yb,Er@SiO2@Ag core-shell nanoparticles for plasmon-enhanced upconversion luminescence in bio-applications”, Annals of Biomedical Science and Engineering, 2019, 3, pp 013-019.
9. Zavatski Sergey, Khinevich Nadia, Arzumanyan Grigory, Bandarenka Hanna et al., “Surface Enhanced Raman Spectroscopy of Lactoferrin Adsorbed on Silvered Porous Silicon Covered with Graphene”, Biosensors, 2019, 9, 34, pp. 1-19.
10. Arzumanyan G.M., Marchenko A.S., Mamatkulov K.Z., Vereshagin K.A., Volkov A.Yu., "On the spectral identification of activated and non-activated neutrophils by Raman scattering". Proceedings of the 61st All-Russian Scientific Conference MIPT, Moscow, 2019, pp. 27-28.
11. Arzumanyan G.M., Mamatkulov K.Z., Fabelinsky V.I., et al., “Micro-SECARS Studies of Organic Molecules on Randomly -Nanostructured SERS-Active Surfaces”, Proceedings of the 18-th European conference on non-linear optical spectroscopy (ECONOS), 2019, April 7-10, Rouen, France, p.74, (Microspectrometer “CARS”).
12. Yakimchuk D.V., Kaniukov E.Yu., Arzumanyan G.M., et al., "Self-organized spatially separated silver 3D dendrites as efficient plasmonic nanostructures for Surface-enhanced Raman spectroscopy applications", J. Appl. Phys, 2019, 126

13. Arzumanyan G.M., Gur'ev A.S., Mamatkulov K.Z., et al. "Micro Raman spectroscopy for NETosis detection", J. Raman Spectr., 2020, 1-10

Conferences

1. Arzumanyan G.M., Mamatkulov K.Z., Fabelinsky V.I., Int. conference "Actual Problems of Raman Scattering – 90 years of Raman effect", 28 May – 01 June 2018 г., Novosibirsk, Russia. Invited report
2. Arzumanyan G.M., "Polarization-sensitive CARS imaging and surface-enhanced micro-CARS of organic molecules", BIT's 2nd International Biotechnology Congress-2018, October 14-16, 2018, Fukuoka, Japan. Oral report.
3. Doroshkevich N.V. "IC-ANMBES 2018", 23-25 May, 2018, Brasov, Romania. Poster
4. Sarychev A.K., Arzumanyan G.M., Mamatkulov K.Z., et al., Int. conf. SPIE-2018, 19-23 August, 2018, San Diego, California, United States. Oral report.
5. Mudrii A.A., Arzumanyan G.M., «Photoluminescence and structural characteristics of glass-ceramics". VIII International conference SSP-2018. 24 – 28 September 2018, Minsk, Belarus. Oral report
6. Bandarenka H.V., Arzumanyan G.M., Mamatkulov K.Z., et al., 02–05 July, 2018, ETH Zurich, Switzerland. Oral report.
7. Arzumanyan G.M., Mamatkulov K.Z., Fabelinsky V.I., "First International Conference on Molecular Modeling and Spectroscopy, 2019, 19-22 February, Cairo, Egypt. Keynote report
8. Arzumanyan G.M., Mamatkulov K.Z., Fabelinsky V.I.,2019, 08-10 July, Prague, Czech Republic. Keynote report
9. Arzumanyan G.M., "Modern Trends in Raman Microspectroscopy", International School of Nuclear Physics «JINR Days in Bulgaria», 2019, 13 -17 May, Borovets, Bulgaria. Oral report
10. Mamatkulov K.Z, Vorobeva M.Yu., Bandarenko H.V., Arzumanyan G.M. ICNBME-2019, September 18-21, 2019, Chisinau, Moldova. Oral report
11. Vereshchagin K.A. Volkov A.Yu., Gurev A.S., Kravtsunova D.E., Mamatkulov K.Z., Marchenko A.S., Arzumanyan G.M., "18-th European conference on non-linear optical spectroscopy (ECONOS), 2019, 7-10 April, Rouen, France. Oral report
12. Rudnikh S.K., Vorobyeva M.Yu., Mamatkulov K.Z., Arzumanyan G.M., Openbio-2019, Novosibirsk Region, Koltsovo, 22-25 October, 2019 г. Oral report
13. Bandarenka Hanna, Khinevich Nadia, Zavatski Sergey, Mamatkulov Kahramon, Vorobyeva Maria and Grigory Arzumanyan, VI International Caparica Conference on Analytical Proteomics 2019, 08 – 11 July 2019, Caparica, Portugal. Oral report
14. Marchenko A.S., Arzumanyan G.M., 26-st scientific-practical conference of students and young specialists of Dubna state university, Dubna, 18-19 April 2019. Oral report
15. Marchenko A.S., Vereshchagin K.A., Volkov A.Yu., Mamatkulov K.Z., Arzumanyan G.M., International Conference on Radiation Applications (RAP 2019), September 16-19, 2019, Serbia, Belgrade. Poster

16. Vorobeva M.Yu., Mamatkulov K.Z., Bandarenka H.V., Arzumanyan G.M., EuroSciCon Conference on Nanotechnology and Smart Materials, 2019, 08-10 July, Prague, Czech Republic, Oral report
17. Arzumanyan G.M., Bandarenko H.V., Mamatkulov K.Z., 2020, Feb. 21-22, EuroSciCon-2020, Amsterdam, Netherlands, Keynote report