Translational Raman Spectroscopic Approaches for Clinical Routine

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The article reviews our latest results on innovative technological and data analysis concepts for bringing Raman spectroscopy closer to clinical use in terms of diagnosing and targeted therapy of infectious diseases and cancer. The first part will report on clinical Raman solutions for rapid diagnosis of infectious diseases - in terms of rapidly identifying the infection causing pathogen its antibiotic resistance pattern and ideally also its immune response - being decisive parameters for a targeted antibiotic administration, which is crucial for the survival of patients. The second part will focus on nonlinear multimodal Raman imaging for a fast and safe precise intraoperative tumor margin control, because reliable tumor margin recognition during an intervention is the key to effective tumor treatment.

Keywords
Raman spectroscopy, Artificial intelligence, Chemometrics, Microbial analysis, Infectious diseases, Cancer, Spectral histopathology

Introduction

Understanding the causes of diseases, recognizing them earlier and treating them more specifically - hopes that are associated with modern biomedicine - requires the determination of diagnostic, prognostic and predictive factors including their comprehensive evaluation in just a few steps or ideally in a single step. In this context, the sharp rise in cancer due to an ageing society and the rapid spread of life-threatening infectious diseases (due to in part unknown pathogens) and antibiotic-resistant germs, which is partly due to an increasing worldwide mobility but also to the ill-considered administration of broad-spectrum antibiotics, should be mentioned in particular. An effective and early diagnosis and personalized therapy of cancer and infections requires new methods of differential diagnosis and represents an outstanding task of medicine. In principle, the following applies to all diseases: the earlier treatment begins, the better the chances of cure. There is therefore a great need for new diagnostic methods for a targeted early diagnosis of diseases in order to be able to use a targeted therapy as early as possible.

Raman spectroscopy plays a key role in the implementation

of these ambitious goals. The application of Raman methods to address biomedical research has grown rapidly over the past ten years and has advanced into a new era due to advances in instrumentation and most importantly due to an enhanced cross-disciplinary dialogue between spectroscopists and clinicians, which e.g. is fostered in Europe by the COST action Raman4clinics (https://www.raman4clinics.eu/).[1, 2]

This contribution will highlight our recent advances in translating Raman approaches towards routine clinical applications with focus on infectious diseases and cancer. Addressing this question requires new Raman instrumentation, which can be applied out of specialized labs in a clinical environment (e.g. operation theatre, bedside or in a doctor's practice).

Point-of-care Raman spectroscopy for a rapid infectious disease diagnosis and treatment

Let's assume the following situation: a patient is at the doctor's office and after a short anamnesis the doctor decides that it is a bacterial infection and prescribes a certain antibiotic. Now one can ask the question, how does the doctor know that it is a bacterial infection, or in case is a bacterial infection, that the causing germs are not resistant to the prescribed antibiotic. While this approach was appropriate a few years ago, nowadays in the course of increasing antibiotic resistance, it would be very beneficial, that prior to prescribing an antibiotic or before taking an antibiotic, a precise diagnosis in terms of rapidly identifying the pathogen and characterizing its resistance profile takes place on which then a targeted therapy follows. In the following the great potential of optical technologies with focus on Raman spectroscopy to successfully address these unmet needs will be shown. At the same time, the hurdles that have to be overcome in order to move from an academic scientific process to a validated and verified product will be outlined.

First the question, what exactly is needed for an optimal and personalized treatment of infectious diseases will be addressed. Here, it is important to know whether the symptoms a patient - the so-called host response - is exhibiting are symptoms of an infectious disease or of another inflammatory disease. In case it is an infection, the next step is to find out what kind of pathogen is causing the infection, i.e. are the infection causing pathogens viruses, bacteria, or fungi? This knowledge is crucial for initiating an effective therapy. In the case of a bacterial infection, it is also necessary to know whether the bacterial pathogen is resistant to certain antibiotics or not.

The host response:

In order to find out if the patient suffers from an infection or not it is important to consider the role of immune cells during an infection. When infectious agents enter the body, they interact with immune cells, i.e. among other with white blood cells including neutrophils, monocytes, eosinophils, T cells, B cells or the basophils. The interaction of pathogenic microorganisms, i.e. viruses, bacteria or fungi, with these immune cells leads to a certain cell response, causing molecular changes within the immune cells. Raman spectroscopy offers a very simple approach (requiring only a laser, a microscope, some filters and a spectrometer together with a sensitive camera) to monitor these molecular changes via the Raman spectral fingerprint. Raman spectroscopy requires very simple sample preparation steps. The isolation of white blood cells from whole blood can be achieved very easily via lysis of the red blood cells. After isolation, the white blood cells are placed under the microscope and irradiated with the Raman excitation laser. A notch filter separates the elastically scattered from the inelastically scattered Raman light, which is spectrally dispersed by a monochromator and detected by a camera (e.g. CCD camera). This simple analysis process, which has been established within the EU project Hemospec (https://cordis.europa.eu/project/id/611682), requires just 60 minutes from sampling to the final result. However, in order to achieve this white blood cell analysis two challenges had to be overcome: (1) since the Raman spectra of the different cell types or the activation of the different cells differ only minimally, a simple spectral analysis by eye is not possible and artificial intelligence methods are required to analyze the cellular Raman spectra (see below). (2) a large number of cells need to be examined and commercially available Raman setups are not able to measure several 1000 cells in a very short time. Therefore, a high-throughput Raman (HTR) setup has been developed within Hemospec and put to use in the clinic.[3, 4]

The investigation of the host response started with *in vitro* experiments, where isolated white blood cells here neutrophils from whole blood were spiked with various infectious agents such as bacteria and fungi. The individual cellular Raman signatures were analyzed by a PC-LDA analysis subsequent to a sophisticated data pretreatment.[5] In detail, the Raman spectral data of infected neutrophils have been compared with those of uninfected neutrophils and with an accuracy of 90% the infected could be distinguished from the non-infected neutrophils.[5] The examination of the infected neutrophils revealed that it is possible to distinguish a fungal infection from a bacterial infection with an accuracy of 92%. Finally, neutrophils associated with Gram-positive bacteria such as *Staphylococcus aureus* could be separated from

Gram-negative *E. coli* with an accuracy of 84%, i.e. Raman spectroscopy also allows to distinguish Gram-positive bacteria from Gram-negative bacteria. This *in-vitro* microbial infection diagnostics study[5] (see Figure 1) demonstrates that pathogen-specific activation can be detected with Raman data and opens up new possibilities for clinical diagnostic applications in terms of timely personalized therapy. For this, however, it is necessary to transfer the concept of the *in-vitro* stimulated cells shown in Reference [5] to patient samples.

In a recently published clinical study[4] we were able to show that it is possible to distinguish between inflammation, infection and sepsis by analyzing the Raman spectroscopic fingerprints of leukocytes from the blood of hospitalised patients from an emergency department. Using the aforementioned HTR approach[3], we were able to clearly distinguish patients with viral infection from patients with bacterial infection. This clinical study paves the way for translating this Raman spectroscopic host response approach as a first steps towards a targeted therapy, as it allows to quickly choose the appropriate therapeutic approach based on the host response.[5]

Pathogen diagnostics and antibiotic resistance:

The currently approved microbiological methods to identify the infection-causing pathogens require the cultivation of bacteria and are therefore time-consuming, which means that a targeted antibiotic therapy is usually performed too late. Therefore, broad-spectrum antibiotics are often used empirically before the appropriate antibiotics matched to the infection-causing pathogen are found. However, this approach leads to more and more pathogens becoming resistant to antibiotics. To treat an infection successfully, the physician needs the information about the type of the pathogen and its resistance to antibiotics - as quickly as possible. As mentioned above, standard microbiological methods usually require more than 24 hours due to cultivation, which is often too late.

To overcome this unmet medical need we have developed the so-called RAMANBIOASSAY™ - an approach that allows the identification of the pathogens as well as the determination of the minimum inhibitory concentration (MIC) in a very short time. The unique feature of this RAMANBIOASSAY™ approach is the dramatically reduced diagnostic time: in only 2 to 3.5 hours and with a very small number of microbial pathogens (a few hundred bacteria are sufficient), the pathogens can be clearly identified and their resistance properties can be determined without a prior cultivation step. Based on this information a physician can adapt the therapeutic antibiotic treatment specifically to the pathogen in question.

The basis of the RAMANBIOASSAY™ approach is the combination of chip-based Raman microspectroscopy with classical imaging. The smart use of laser light in a Raman setup in combination with a light microscope enables a label-free, non-destructive and culture-independent optical and spectroscopic characterization and identification of bacteria down to the level of single cells. Each molecule generates an individual signature in the Raman spectrum, creating a specific molecular fingerprint for each bacterium. Statistical evaluation algorithms enable automated classification and identification of bacteria and resistance to antibiotics. Furthermore, the additional integration of chip-based enrichment methods into miniaturized structures covers the entire process chain

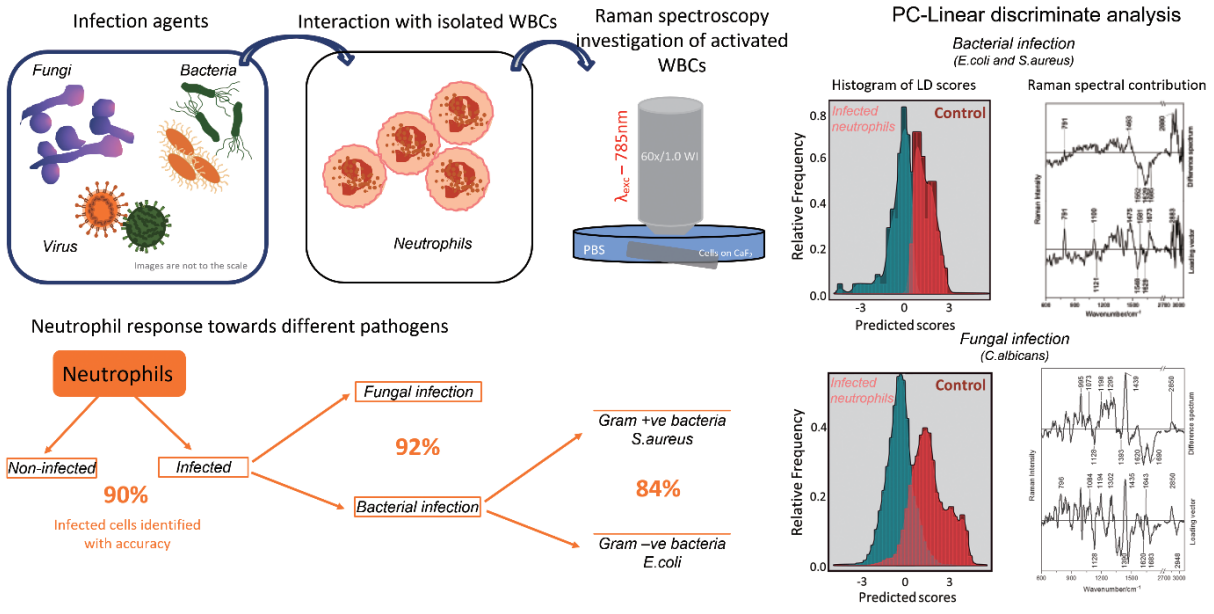

Figure 1 Summary of *in-vitro* Raman study to unravel the host response.[5] For details see text.

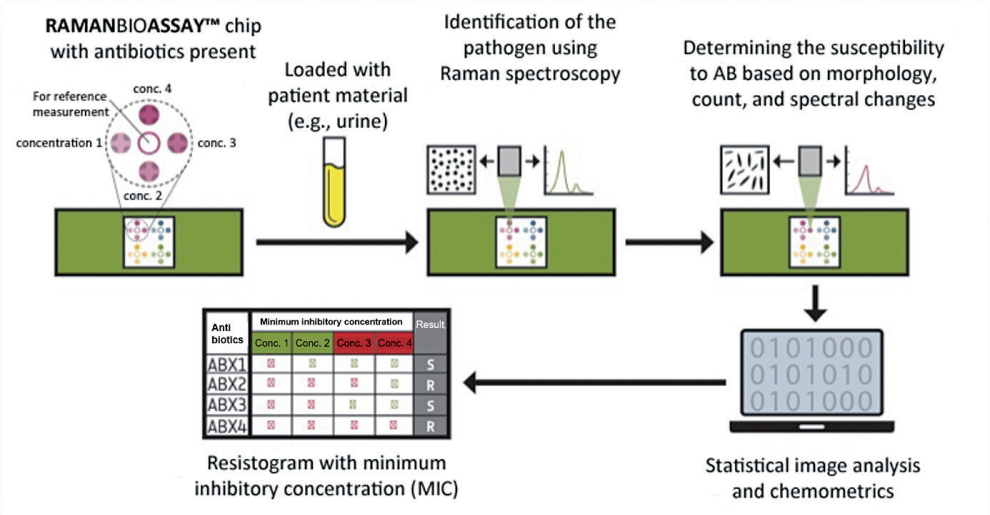

Figure 2 Schematic of the individual steps of the RAMANBIOASSAYTM: The core is a chip onto which the bacteria from a patient sample are applied. Up to four different antibiotics are already present in four concentrations each. This means that the chip is ready for immediate use.

from sampling to final results (see Figure 2).

The Raman spectroscopic identification of the pathogen can be completed after 35 minutes. At the same time, the morphological changes of the pathogens in a short-term culture (on the RAMANBIOASSAY™ chip) together with the Raman spectra are used to determine to which antibiotic agent resistance exists and what the MIC is. This information is extracted from the morphological images and the Raman spectra using computer-assisted statistical image data analysis and chemometrics. By doing so the complete resistogram is thus already available after 2 to 3.5 hours and the physician can react much earlier than currently and does not have to resort to the application of a broad-spectrum antibiotics.

The potential of the RAMANBIOASSAY™ approach was demonstrated using the example of urinary tract infections with an identification of the pathogens, their resistance to antibiotics and their MIC from patient material.[6-11]

The uniqueness of the RAMANBIOASSAY™ approach lies in its independence from culture and the scalability of the approach. The independence from culture allows the analysis time to be reduced to 2 to approximately 3.5 hours after sample collection. The scalability enables universal use on the one hand in clinical microbiology in combination with pipetting robot compatible Raman chip platforms, which allows a high degree of automation in the clinical microbiology workflow, and on the other hand in the form of a closed cartridge-based approach with a miniaturized Raman reader for direct use in a physician's office or in a hospital without its own microbiology unit. Figure 3 shows the current status using the RAMANBIOASSAY™ chip together with the BioParticle Explorer (a manual Raman spectroscopy system together with a microscope for morphological analysis) as well as future developments of the RAMANBIOASSAY™ for clinical microbiology or use in a physician's office.

Equally important as the development of clinically usable Raman devices (see Figure 3) is the development of tailored Raman spectroscopic analysis routines. As mentioned above the analysis of bacterial Raman spectra or changes within induced by e.g. antibiotics treatment cannot be analyzed by naked eye and require sophisticated artificial intelligence based spectral analysis routines.

In general, the success of Raman spectroscopy for medical diagnosis and therapy (and also other applications like e.g. in life sciences, process analytics, pharmacy or environmental analysis) is inherently connected with the development of customized Raman data evaluation algorithms for translating Raman measurement data (spectral data sets, image data, etc.) into qualitatively and quantitatively usable information for end users. In this context we have developed a universally applicable Raman data analysis software called RAMANMETRIX

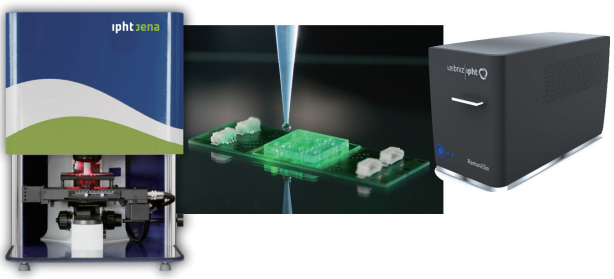

Figure 3 Photograph of RAMANBIOASSAY™ chip (middle) together with the BioParticle Explorer(left) and future developments towards a cartridge-based system with miniaturized reader for doctor's office and clinics without clinical microbiology (right).

(see: https://docs.ramanmetrix.eu/). This software allows for a one-click data analysis of Raman spectroscopic data in a robust and reliable way.

Non-linear spectroscopic multimodal instrumentation for intraoperative tumor identification

The diagnostic gold standard in most surgeries is the extraction of biopsies and their histopathological examination to confirm the tumor and the tumor borders. The histopathological examination is carried out by means of rapid sections of non-contrasted tissue sections during the operation and definitively also on tissue sections of fixed material. Depending on the number of removed rapid sections, the rapid section diagnostic takes about 20-30 minutes. Then the surgeon is typically informed by telephone about the result and can decide on his/her further approach. If, for example, a sample in the border region of the tumor still shows tumor tissue, the surgeon will resection here and, if necessary, provide a new border section for rapid section diagnostics until the rapid sections indicate complete tumor resection.

This procedure is time-consuming, investigator-dependent, and dependent on the size, number and quality of the removed tissue samples, which are, of course, limited. Furthermore, since the quality of intraoperatively prepared frozen sections is not comparable to that of embedded tissue, the results of frozen section diagnostics often differs from those of an examination on embedded sections, which is why subsequent diagnostic confirmation using embedded sections is still necessary. For example, in head and neck surgery, depending on tumor size and location, the incidence of residual tumor (R1), i.e., subsequent finding of tumor cells in the incision margin, is approximately 7.5 - 10%. For such cases, the patient has to undergo a new operation and often also postoperative radiotherapy, which is an enormous burden for the patient. Therefore, even within the primary surgery, where possible, more tissue is removed than would actually be necessary to reach the tumor limit. However, the more tissue that is removed, the greater the impact on the patient's healing process. Thus, new methods and approaches are urgently needed for a fast and reliable intraoperative diagnosis.

Over the last few years, we have been investigating a multimodal nonlinear imaging approach that has the potential to reliably assess tissue and the success of surgery or endoscopy directly in the operating theatre or endoscopy room. The approach combines three different nonlinear imaging techniques namely two-photon excited autofluorescence (TPEF), second harmonic generation (SHG) and coherent anti-Stokes Raman scattering (CARS) displaying vibrations in the CH-stretch wavenumber region. The combination of these three modalities allows to determine the morphological and chemical composition (morphochemistry) of unfixed tissue sections in a label-free manner.[12] In order to translate the morphochemical information encoded in the multimodal spectroscopic images into medical relevant information photonic data science, i.e. machine and deep learning approaches are necessary.[13-16]

In the following we will briefly highlight the potential of multimodal nonlinear imaging in combination with innovative image analysis routines as a powerful tool for computer based spectral histopathology allowing for an automatic prediction of tissue types / disease and thus offering great potential to fulfill the aforementioned unmet medical needs in terms of reducing the time in an operative theatre due to instant feedback and smaller workload due to automatization.

In a recent study we investigated head and neck squamous cell carcinomas using the multimodal combination of CARS, TPEF and SHG. The analysis of the images by a machine learning classification model features a 90% accuracy compared to gold standard diagnosis of a blinded pathologist.[14, 15, 17, 18]

Furthermore, we could show that the utilization of deep learning approaches also allows for a pseudo-staining of multimodal images. The deep learning generated pseudo H&E images nicely represent the real H&E images and show that the combination of the three label-free non-linear imaging modalities CARS, TPEF and SHG yields information that can be translated into computational pseudo hematoxylin and eosin (HE) images.[13,16] For a clinical application compact and easy to use devices are needed. Thus, we have transferred the presented CARS/SHG/TPEF approach into a compact microscope suitable for clinical use in terms of a rapid *ex-vivo* tissue analysis.[12]

In order to further extend the applicability of this multimodal microscopy approach for *in vivo* tissue screening, various endoscopic probe concepts were also realized.[19-23] The core of all these setups are robust and alignment-free fiber laser concepts. In the following two concepts of endoscopic probes for multimodal nonlinear imaging, which have been researched or are still currently under investigation will be briefly summarized. The first is a rigid needle endoscope for neurosurgical applications.[21] This probe provides very good imaging in a compact design, there are no moving or electric parts in the probe head, but it is not flexible (which is fine for neurosurgery and some other fields of application, e.g., head and neck).

For applications requiring flexible endoscopes, a unique endoscopy concept using a double-core double-clad fiber and focus-combining micro-optical concept allowing for a background free, low-loss, high peak power laser delivery, and an efficient signal collection in backward direction has been successfully realized.[23]

Conclusion

In conclusion this short review highlights the great potential of Raman spectroscopy in combination with innovative photonic data science concepts for clinical diagnosis and therapy.

In the first part of this contribution, we report on the culture-free isolation and identification of pathogens, their host-response and their antibiotic resistance by using a combination of Raman spectroscopy, chip-based sampling strategies as well as chemometric spectroscopic data analysis methods. The main advantages of this rather simple approach compared to conventional microbiological analysis methods is that the analysis result is available in just a few hours. This represents a major step forward, as antibiotic therapy can be started promptly and specifically tailored to the pathogen. It will be shown how this approach was transferred into an automated clinically applicable system the RAMANBIOASSAY™

The second part of this presentation reports on multimodal non-linear imaging solutions in the field of pathological cancer diagnostics. Here, the combination of CARS with SHG and TPEF imaging in combination with adapted image analysis methods represents a powerful *ex-vivo* and *in-vivo* approach for a labelfree clinical intraoperative tissue diagnostic for tumor margin detection in terms of computer based spectral histopathology.

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