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Review article

Raman microscopy as a novel tool to detect endothelial dysfunction

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ABSTRACT

Raman microscopy, a label-free method with high spatial resolution, shows growing potential in various fields of medical diagnostics. Several proof-of-concept studies related to the application of Raman microscopy to detect endothelial dysfunction are summarized in this work. Both *ex vivo* measurements of the tissues in the murine models of endothelial pathologies, as well as *in vitro* investigations of the cell cultures in the context of cellular transport, drug action and inflammation processes are discussed. The future directions in application of Raman spectroscopy-based methods in such studies are also described.
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Introduction

Q2 Infrared absorption spectroscopy and Raman spectroscopy are two classical methods studying vibrations in molecules to provide information on their chemical structures [1–3]. Infrared absorption spectroscopy and Raman spectroscopy are based on different physical phenomena, *i.e.* light absorption and light scattering, respectively. Scattered light can be of the same frequency as the frequency of the excitation source (Rayleigh scattering) or shifted in energy compared to the energy of the incident light due to interactions of the incident photons with the vibrating molecules (Raman scattering). The Raman spectrum shows the dependence between the intensity of the inelastically scattered light and the Raman shift (*i.e.* a difference between the wavenumber of the incident and scattered light). Raman is a complementary technique to infrared spectroscopy, since molecular groups giving intense

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Raman signals show usually infrared bands of low absorbance and *vice versa*. Therefore, one of the biggest advantages of Raman spectroscopy over IR to study biological samples is its ability to measure aqueous solutions (water signals are intense in infrared but not Raman spectra).

Raman mapping spectrometers are often coupled with optical microscopes that transfer both the incident (laser) light and the Raman scattered light. Such techniques are frequently called 'Raman microscopy'.

The original concept of Raman mapping is rather simple. Registration of a dataset of Raman spectra in a chosen area of a sample is followed by spectral analysis that results in determination of the distribution of compounds of interest in the studied volume of the sample. There are diverse methods of sample analysis that can be generally classified as univariate and multivariate. Analysis of the integral intensity of a characteristic band for a given compound in each recorded spectrum is an example of the univariate method, however due to the fact that in complex samples every spectrum contains difficult-to-extract information, multivariate methods of data analysis (*e.g.* cluster analysis) are growing in popularity. Cluster analysis groups the spectra according to their similarity, enabling classification in classes corresponding to the chemical composition of the sample (*e.g.* spectra of lipids and proteins in the tissue can be sorted).

In Raman confocal microscopy, the obtained information is derived from a very small volume of the sample (voxel) limited by the (lateral and depth) resolution of the optical microscope. For confocal microscopes, information from various thin layers of the sample can be recorded and 3D Raman images of components can be reconstructed.

Raman microscopy has various advantages: it provides information simultaneously about multiple sample components, it is highly specific and selective, non-destructive and does not require special sample preparation or labelling. Additionally, the resolving power of the technique is very high for a voxel of the size of subcellular structures: 0.31 μm × 0.31 × μm 0.52 μm (@488 nm exciting laser and the objective of numerical aperture of 0.95) can be resolved. This is the key benefit of Raman microscopy compared to infrared microscopy for which the spatial resolution is wavenumber-dependant and for a high-resolution setup (ATR, germanium crystal, 2000 cm^{-1}, 15×) equals 1.1 μm laterally.

The above-mentioned features make Raman microscopy a perfect technique to analyze *in situ* components and biochemical processes occurring in cells and tissues. In studies of vascular diseases a special interest is focused on endothelial dysfunction. Therefore, in this work, various examples of the application of Raman imaging to study endothelial dysfunction in both cellular models and *ex vivo* tissues are presented. In several cases, illustrations of multimodal imaging, where Raman and fluorescence are detected simultaneously or Raman mapping is accompanied with another mapping technique (atomic force microscopy, AFM), are also shown.

To guide the reader through the presented results, these are divided into two sections. Research related to two models of endothelium dysfunction: diabetes and systemic hypertension are included in *Murine models of vascular pathologies* and studies on cell culture models are included in *Cellular models of endothelial uptake and drug-response*. In Conclusions we summarize presented research and comment on perspectives of application of other Raman spectroscopy based methods to study endothelium dysfunction.

Murine models of vascular pathologies

Diabetes model

Long standing diabetes leads to structural and functional changes in both the micro and the macro vasculature system, particularly in the endothelium. A multiparameter approach based on the combination of Raman confocal 3D imaging with atomic force microscopy (AFM) was applied to analyze the genetically-modified murine model of type 2 diabetes mellitus (db/db [4]) *vs.* control split open (*en face*) aorta [5]. This methodology enabled characterization of the sub-cellular structures of the tissue and direct correlation of their chemical structure (Raman data) and physical properties (AFM). Large differences observed in the Raman and AFM images of the aorta cross-sections of db/db and db+ (control) mice were related to the presence of lipid rafts (LRs) clusters, of which the number drastically increases in samples taken from the mice in the pathological state compared to the control ones (Fig. 1).

The chemical character of LRs was corroborated by their lipid–protein Raman signature with specific bands observed at 780, 725 and 700 cm^{-1} for phosphatidylinositol, phosphatidylcholine/

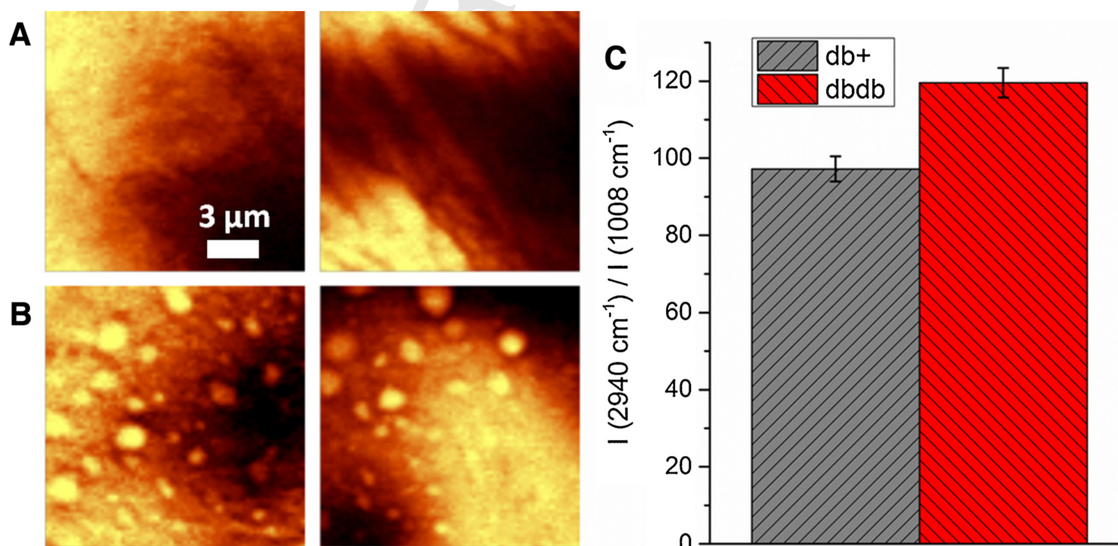

Fig. 1. Comparison of the representative Raman maps (integration: 2800–3100 cm^{-1}) of the *en face* endothelium of db+ (control, A) and db/db (B) mice. The overall endothelium lipid content (defined as the average intensity ratio of the band at 2940 to the band at 1008 cm^{-1} in all measured samples; C).
Figure adapted upon Creative Commons Attribution License from Ref. [6].

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