WITec
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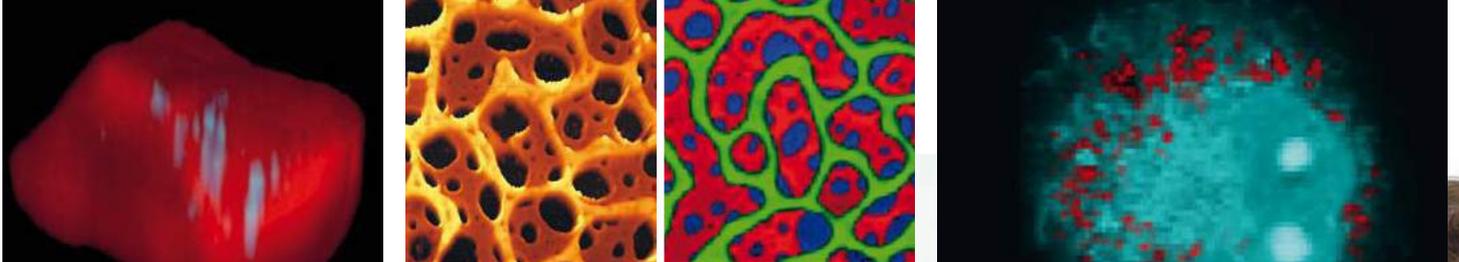# 13th Confocal Raman Imaging Symposium

International Conference for Chemical Characterization & Imaging

September 26-28, 2016 | Ulm, Germany

Abstracts
Invited Talks

Imaging of single endothelial cells –
Raman spectroscopy, AFM and SNOM

Malgorzata Baranska

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Applications of Raman spectroscopy to study *in vitro* endothelial cells are presented. Spectroscopic measurements were combined with Atomic Force Spectroscopy (AFM) and Scanning Near-Field Optical Microscopy (SNOM), and with Infrared and Fluorescence spectroscopy and histochemical staining, if possible.

Endothelium plays an important role in cardiovascular system and regulates vascular homeostasis and its dysfunction is involved in several lifestyle diseases.. The general aim of the research is to find biochemical features of endothelial dysfunction that cannot be found by other methods.

Confocal Raman spectroscopy imaging was used to monitor a molecular composition occurring in a single live human aorta endothelial cell. Based on Raman spectrum and using a chemometric approach it is possible to investigate biochemical changes induced by stress or pharmacological treatment.

Raman spectroscopy applied for cells isolated from the liver tissue enabled their specific characterization. In addition, this methodology can support other approaches for tracking changes at the cellular level due to pathology development. Here, an application of Raman confocal mapping for studies of cells isolated from healthy mice liver is shown. Hepatocytes, HSC, Kupffer and endothelial cells were identified and subjected for the further studies. With the use of chemometric tools we present how to differentiate endothelial cells from the other liver cells.

Atomic Force Microscopy (AFM) and Scanning Near-field Optical Microscopy (SNOM) are techniques providing images of structures (also biological materials) with nanometric optical resolution (about 50-60 nm) and topographic information at the same time. In this work the capabilities of both, AFM and SNOM in transmission configuration, to image endothelial cells are shown.

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Spatially resolved Raman spectroscopy for optomechanics and optoelectronics in graphene and nanotubes

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Optical phonons, in low dimensional sp2 carbon systems, couple easily with different elementary excitations (electron, acoustic phonon, photon), allowing us to be sensitive to various physical phenomena: strain field, mechanical resonances, but also to charge transfer and/or energy transfer.

To illustrate this coupling, I will show how optical phonons can be used to detect mechanical resonances that involved a coupling between acoustic phonon and an optical one and thus be used as a probe of motion and strain in different systems [1,2,3]. I will also show how it can be used to reveal the nature of strain transfer between compressively strained graphene and various substrates. The monolayer geometry, the interfacial properties, and the presence of wrinkles lead to highly non-uniform strain in the graphene, varying from uniaxial to biaxial [4].

To illustrate electron-phonon and phonon-photon coupling, an optical gating of photo-active molecules grafted on an isolated DWNT (graphene) based transistor will be shown [5,6].
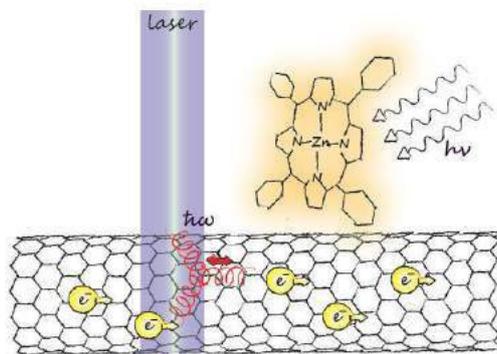

Fig. 1: DWNT hybrid device for optoelectronic application

A huge modification of optical phonons is measured. This photo-induced phenomenon will be elucidated in terms of photo-induced excitation transfer between the grafted molecules and the DWNT component whereas in graphene this effect is different. Finally, by optical gating, this DWNT-hybrid FETs can be used as an optically controlled memory.

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Raman Characterisation of 2D Materials

Niall McEvoy[a,b], Riley Gatensby[a,b], Maria O'Brien[a,b], Kangho Lee[a,b], Chanyoung Yim[a,b], Nina C. Berner[a,b], Conor Cullen[a,b], John McManus[a,b], Toby Hallam[a,c], Georg S. Duesberg[a,b]

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Two-dimensional materials such as graphene and transition metal dichalcogenides (TMDs) have moved to the foreground of the research community owing to their fascinating properties which make them of great interest for both fundamental studies and emerging applications. Thermally assisted conversion (TAC) of predeposited transition metal films and Chemical vapour deposition (CVD) show great promise for the scalable and industry-compatible synthesis of these materials[1]. Here we outline the production of an assortment of TMDs, including period 6 and 10 metals, by TAC and CVD. [2] We demonstrate their high-quality using an array of characterization techniques including Raman spectroscopy, X-ray photoelectron spectroscopy and transmission electron microscopy. This presentation will particular focus and on the use of Raman mapping and low frequency Raman spectroscopy for the characterization of low-dimensional carbon and TMD materials. [3]

[1] *"Controlled Synthesis of Transition Metal Dichalcogenide Thin Films for Electronic Applications"*, Riley Gatensby, Niall McEvoy, Kangho Lee, Toby Hallam, Nina C. Berner, Ehsan Rezvani, Sinéad Winters, Maria O'Brien, Georg S. Duesberg, Applied Surface Science, 297, 139-146, (2014)

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[3] *"Mapping of Low-Frequency Raman Modes in CVD-Grown Transition Metal Dichalcogenides: Layer Number, Stacking Orientation and Resonant Effects"*, Maria O'Brien, Niall McEvoy, Damien Hanlon, Toby Hallam, Jonathan N. Coleman, Georg S. Duesberg, Scientific Reports, 6, 19476, (2016)

Unusual Phase Transformations of Cement Minerals – Raman Spectroscopy and Imaging

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The man-made material most widely used by mankind is concrete, with three tons annually per capita. This poses a huge ecological impact due to consumption of raw materials; emission of approx. 8% of the global CO_2 during cement production; building demolition accounts for around one third of the total volume of waste we produce – around 450 - 500 million tons per year. Concrete is produced by mixing of cement with water and aggregates like sand and gravel. Upon hydration cement hardens while forming hardened cement paste. Its main component, calcium-silicate-hydrates (C-S-H, C = CaO, S = SiO_2, H = H_2O) are the binder in the hardened cement paste and thus responsible for the mechanical and chemical stability of the construction material. The large diversity of their structures makes them on the other hand an ideal object for basic crystallographic and mineralogical research.

Based on comprehensive fundamental knowledge about the large family of C-S-H phases, a new family of cementitious materials was designed at the institute for technical chemistry at KIT, as a promising, environmentally friendly, and cost-effective alternative to Ordinary Portland Cement. Key point in this new technology is a process comprising thermal or tribochemical treatment of hydrothermally synthesized precursor consisting of C-S-H phases with various degree of crystallinity.

In this study we will present *in situ* investigations of the hydrothermal growth of C-S-H phases and their subsequent transformations as a function of temperature, pressure and chemistry. C-S-H tend to a structural disorder, which makes Raman spectroscopy and imaging a very valuable technique.
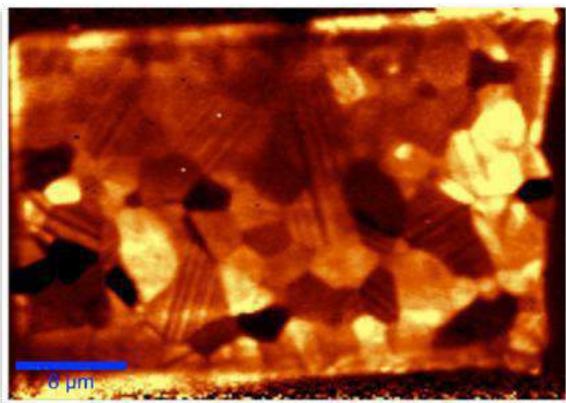

Fig. 1: Raman imaging of β-C_2S twinning

Confocal Raman Microscopy:
Instrumentation, Resolution, Configurations and Correlative Techniques

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Confocal Raman Microscopy is an indispensable tool for the analysis of of chemical species and their spatial distribution either on surfaces or in small 3D volumes. As the name states, two techniques are combined in one instrument.

The confocal microscope provides diffraction limited spatial information, while Raman spectroscopy reveals the chemical composition of the sample. By acquiring a complete Raman spectrum at every image pixel, the chemical information can be linked to the spatial distribution in the sample volume, resulting in nondestructuve imaging of chemical properties without specialized sample preparation. Differences in chemical composition appear in the Raman image, although they are completely invisible in the optical image.

Aim of this contribution is to highlight the instrumental requirements for a high throughput, high resolution Confocal Raman Microscope. Several new developments and their field of application will be presented.
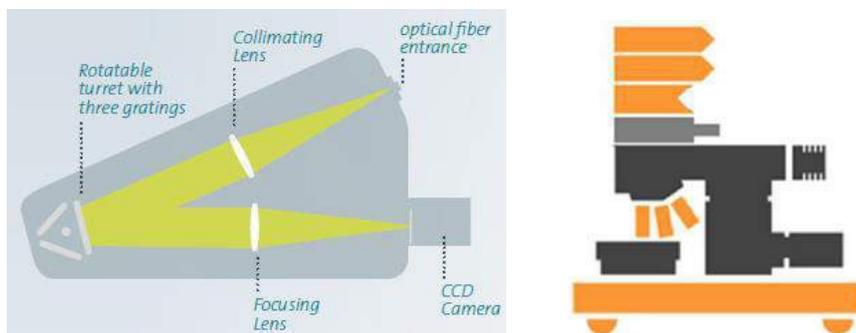

Fig. 1: Confocal Raman microscopy - alpha300 R

Raman Imaging of White Blood Cells – Assessing the Risk of Atherosclerotic Plaques

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Cardiovascular diseases are the leading cause of death worldwide. Atherosclerosis is closely related to the majority of these diseases, as a process of thickening and stiffening of the arterial walls through accumulation of lipids, which is a consequence of aging and life style. Within atherosclerotic plaque depositions monocyte-derived macrophages play a key role in the regulation of lipid metabolism, which can be taken up by various receptor-mediated mechanisms. As phagocytes, macrophages are important for the composition of lipid plaques within arterial walls that contribute to atherosclerosis. Once incorporated into the cytoplasm, lipids are either stored in lipid droplets or are again exported via transporters and acceptor proteins for transport back to the liver. The continuous storage in lipid droplets leads to a foamy appearance of the macrophages. Here we present results of spectroscopic imaging of the uptake of albumin-complexed lipids as well as in the form of low density lipoproteins (LDL) into macrophages. Atherosclerosis affects all people to some extent, but not all arterial plaques will necessarily lead to the complications, such as thrombosis, stroke and heart attack. One of the greatest challenges in the risk assessment of atherosclerotic depositions is the detection and recognition of plaques which are unstable and prone to rupture. It has been hypothesized that the amount of macrophages relates to the overall plaque stability. As phagocytes, macrophages also act as recipients for nanoscale particles or structures. Administered gold nanoparticles are usually rabidly taken up by macrophages residing within arterial walls and can therefore be indirectly detected. A very sensitive strategy for probing gold nanoparticles is by utilizing surface enhanced Raman scattering (SERS).
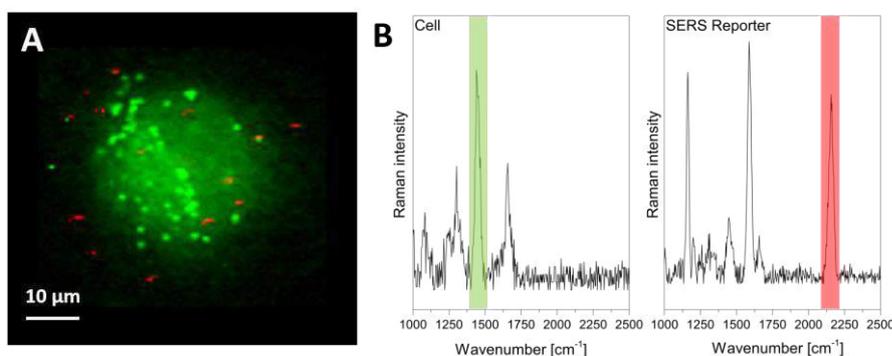

Figure 1: Raman image of a macrophage cell incubated with SERS reporter particles

Multisensor Hyperspectral Imaging:
A Novel Approach to Chemical Structure Determination

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Novel developments in image-based analytical instrumentation allow the acquisition of high-resolution chemical images, using different techniques. However, in many cases analytical problems cannot be clarified by applying only one single technique. Multisensor Hyperspectral Imaging was developed to solve these limitations by allowing a combined statistical analysis of different hyperspectral datasets. After fusion of the single hyperspectral datasets to one combined multisensor datacube [1], a subsequent statistical approach [2] allows a linkage of the different methods to one virtual image with the advantage of chemical structure determination across the limitations of single analytical methods on an image-based approach (Fig. 1).

The concept of Multisensor Hyperspectral Imaging will be demonstrated based on hyperspectral datacubes acquired by a WITec alpha 300 RSA+ Raman Imaging System, a FEI Quanta 200 Electron Microscope and an EDAX EDX Imaging System as well as an IONTOF TOF.SIMS 5 Secondary Ion Mass Spectrometric Imaging System. The application of multivariate statistics to the fused datacubes of different samples, ranging from environmental to industrial and life science applications demonstrates the ability for image-based chemical structure determination and will uncover the overvalue of this approach compared to side-by-side interpretation of separated imaging datasets.
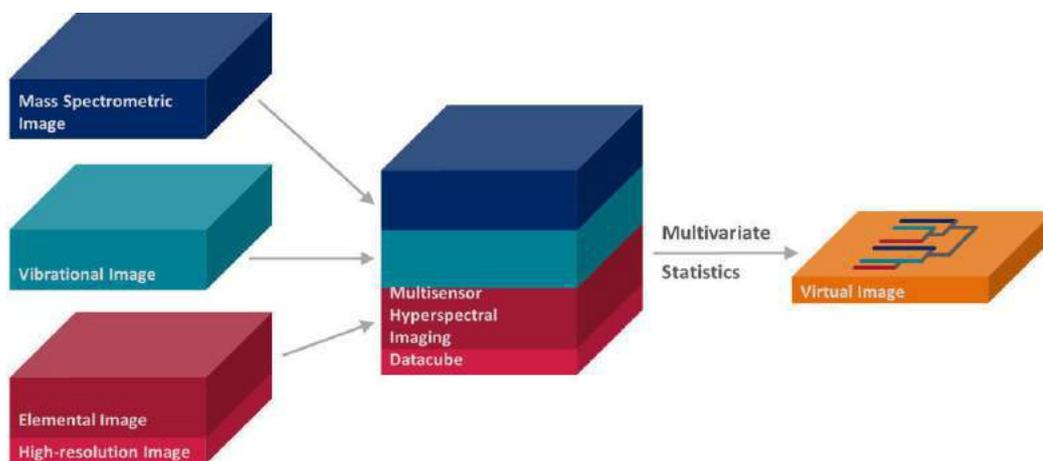

Fig. 1: Concept of Multisensor Hyperspectral Imaging

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Raman Based Chemical Imaging: Recent Applications in Pharmaceutics from Pre-clinical Toxicology Studies to Drug Product Development

Duohai Pan

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Confocal Raman imaging is an emerging platform technology that integrates Raman spectroscopy and conventional confoal optical imaging to provide both spatial and molecular spectral information from the material. This presentation highlights the recent applications of Raman imaging in pharmaceutics from pre-clinical toxicology studies to drug product formulation development.

A few case studies on pharmaceutical development of both small molecule and and protein-based drugs at BMS are presented. (a) In vivo bioaccumulation of drugs which led to precipitation and crystallization in cells and tissues, have been observed in pre-clinical toxicological studies in animals. We have used confocal Raman imaging to identify and analyze the nature of the crystals on tissue surface in support of the assessment of in vivo drug crystallization and the risk in investigational new drug (IND) toxic findings. (b) Confocal Raman imaging was applied to identify the co-crystal formation on the interface of bi-layer tablet during the stability study to support the investigation of the potential drug-drug interactions, which can lead to severe side effects and have resulted in early termination of development. (c) In the drug product process, Raman imaging enabled the rapid identification and continued monitoring of critical quality attributes (CQA), including drug-excipient uniformity distribution in the granules, active pharmaceutical ingredient (API) form and API distribution in drug product intermediate, and the behavior of agglomerated drug substance in the roller compaction process. (d) Confocal Raman imaging was also explored as an orthogonal characterization tool for a comprehensive analysis of biotherapeutics potentially containing silicone oil droplets, air bubbles and proteinaceous particles, especially in the subvisible size range from $1\,\mu m$-$10\ \mu m$. In summary, we demonstrate the power and importance of confocal Raman microscopy to the field of pharmaceutical science throughout drug discovery, drug substance selection, early/late formulation development and drug product process.

Raman spectroscopy in novel two-dimensional (2D) materials

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In recent years, novel graphene-like two-dimensional (2D) structures have appeared into the scientific scene and different systems are now produced as atomically thin structures. Serious efforts are now focused on studying their unique electronic structure. Raman and Resonance Raman spectroscopy are very useful tools to provide many different information in 2D materials such as defects, charges, strain, edges, excitons and their coupling with phonons. In this talk, I will present Raman results in three different types of 2D materials: (i) the transition metal dichalcogenides (MoS_2, WS_2 and WSe_2), (ii) twisted bilayer graphene, and (iii) black phosphorus. The Raman spectra of the transition metal dichalcogenides MoS_2, WS_2 and WSe_2 with one, two, three layers and bulk, were measured using more than 30 different laser lines covering the visible range. Results show that all Raman features are enhanced by resonances with excitons, allowing us to obtain the dependence of their energies with the number of atomic layers [1]. In the case of MoS_2, we observed that the electron-phonon coupling is symmetry dependent, and our results provide evidence of the C exciton, predicted theoretically [2]. I will also present a Raman study of more than 100 samples of twisted bilayer graphene (TBG) with a continuous distribution of twisting angles from 0 to 30°. From the Raman maps of the samples, we could observe giant enhancements of the G band for samples with twisting angles between 9° and 17°. Our results allow us to conclude that the enhancement is due to resonances with van Hove singularities associated with the Moiré pattern, which does not necessarily exhibit a translational symmetry. Finally, I will present results in black phosphorus, which has recently emerged as a new two-dimensional crystal with a peculiar and anisotropic crystalline and electronic band structures. Results show that the unusual angular dependence of the polarized spectra can only be explained by taking into account complex values of the Raman tensor elements. Finally, different black phosphorus edges are experimentally and theoretically studied using Raman spectroscopy and density functional theory calculations. Results show the appearance of new modes at the edges of the sample, and their spectra depend on the atomic structure of the edges (zigzag or armchair). Theoretical simulations confirm that the new modes are due to edge phonon states that are forbidden in the bulk, and originated from the lattice termination rearrangements.

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The Principle of Raman Spectroscopy and its Application in Microscopy

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This lecture gives an introduction into the principles of Raman spectroscopy and its applications in microscopy.

First, both classical and quantum mechanical descriptions of the Raman effect are discussed. The latter (perturbation theory, Kramers-Heisenberg-Dirac dispersion formula) then serves as a starting point for introducing the concept of resonance Raman scattering (RRS). Several examples of RR (from diatomics to proteins) highlight the advantages of this Raman technique.

In addition to the Raman effect, also fundamentals of molecular vibrations and their symmetry (basic group theory) are covered by using the water molecule as an example.

We then make the transition to Raman microscopy, starting with the invention of the first Raman "microprobe" in the 1970s. Also other specialized Raman techniques such as surface-enhanced Raman scattering (SERS) and coherent-anti-Stokes Raman scattering (CARS) microscopy are briefly introduced and their specific advantages over conventional Raman spectroscopy are highlighted.

Finally, quiz questions allow the participants to test their knowledge anonymously in an interactive format (feedback and discussion).

Materials research by Confocal Raman microscopy in the Ural Center for Shared Use "Modern Nanotechnology" Ural Federal University

V. Ya. Shur, P. Zelenovskiy
Ural Federal University, Ekaterinburg, Russia

We present a wide range of researches of various materials by confocal Raman microscopy in the Ural Center for Shared Use "Modern Nanotechnology", Ural Federal University (UCSU MN).

UCSU MN is a leading center for material science and nanotechnologies in the Ural region of Russia and recently it became the Russian leader of the Network Centre for Materials Science and Nanotechnologies of the BRICS countries. For the moment it includes more than hundred equipment units for physical and chemical methods of analysis. The Center collaborates with a number of Russian and foreign academic institutes, universities and industrial enterprises.

Confocal Raman microscopy (CRM) is one of the most demanded tools in UCSU MN. Range of studied materials includes: (i) metal and metal oxide nanoparticles, (ii) nanocarbon and bioorganic materials, (iii) minerals, (iv) ferroelectric and multiferroic domains, (v) composite materials, etc.

Analysis of characteristic lines in Raman spectra used for determination of the chemical composition of nanoparticles created by laser ablation in liquids which used in nanotoxicological research [1]. CRM helped to distinguish single- and multi-layered graphene, carbon nanotubes and fullerenes [2]. It is unique method for characterization of bioorganic materials: peptide nanotubes and nanorods, organic microcrystals and phase transitions therein [3].

Shift of Raman lines allowed us to develop the unique method of visualization with high spatial resolution micro- and nanodomains in the bulk of ferroelectric crystals [4], to measure the residual mechanical stresses at the surface and in the bulk of composite multiferroics [5] and sapphire sensors. High resolution allowed to characterize carbon impurities inside the diamonds [6], single particles of lithium titanate [7] and to determine the accompanying minerals in cokes.

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Abstracts
Contributed Talks

Confocal Raman Applications to Dairy Products

Mark A.E. Auty[1], Zuzana Burdikova[2], Rotislav Vana[3] and J.J. Sheehan[1]

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Confocal Raman microscopy is now established as a powerful technique for spatial molecular mapping of hydrated systems and is an ideal tool for studying food products. This presentation will highlight applications of confocal Raman to dairy product research. Examples will include identifying the cause of "pinking" of natural cheeses and characterization of spray dried milk powders. Natural Cheddar-type cheese can be described simply as a fat-filled protein gel, however the composition is highly complex and contains a rich and active microbial flora. "Pinking" is a reddish discolouration of cheese that negatively affects cheese quality, however despite intense efforts, until now the precise cause was unknown. Confocal Raman analysis of the pink and unaffected areas was performed and Raman spectra of samples from regions of pink discoloration within pink-defect cheeses showed a peak at 1,456 cm^{-1}, characteristic of lycopane (perhydro-transformed carotenoid from lycopene) which was absent from non-pink regions from the same cheese. The pink layer also shows very strong peaks at 877 cm^{-1} and 990 cm^{-1}, which are consistent with a phosphate salt. The localized distribution of prominent Raman peaks at 990 cm^{-1} and 1,456 cm^{-1} (carotenoid-phosphate salt; corresponding to red), 1,441 cm^{-1} and 2,840 to 2,945 cm^{-1} (proteins; corresponding to blue), and 3,060 cm^{-1} (lipids; corresponding to green) is shown in Fig. 1. These results led to the discovery of the bacterium *Thermus* sp. which was the cause of the pinking defect.

In the second study, infant milk base spray-dried powders were characterized using the new RISE confocal-FIB-SEM system which highlighted the major components and showed that free lipids were present on the inner air vacuole wall. This preliminary investigation will require further work to fully characterize the composition based on the Raman spectra, for example using reference spectra from the various ingredients.
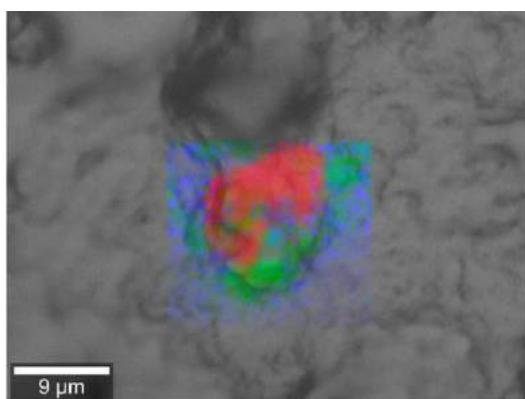

Fig. 1: Overlay of intensity images of the studied cheese matrix (gray) with maps of the chemical composition obtained from local Raman spectral analysis of a pink discoloration region of a defect-associated cheese: red, carotenoid (lycopane); blue, proteins; green, lipids.

Identification of microplastics in marine samples by Raman Microspectroscopy

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K.-J. Eichhorn[a], B. Voit[a,b]

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Microplastics (MP) are micro-sized particles of synthetic polymers in a size range from 5 mm down to 1 µm. They have been observed in marine ecosystems worldwide. Because of their small size MP can be mistaken for food and can be ingested by a variety of organisms. Not only MP themselves, but also embedded additives, adsorbed toxic contaminants or associated potentially pathogenic microorganisms pose a potential risk for the marine foodweb. In the first instance reliable data about the occurrence of MP in marine environments and valid analytical systems are necessary for a risk assessment.

We show the excellent possibilities of Raman Microspectroscopy to identify MP in marine samples distinctly on the basis of the chemical structure. At first the results of the manual step-by-step measurement of larger particles (5 – 0.5 mm) of Baltic Sea samples by Raman Spectroscopy will be shown. Secondly the automated Raman Imaging of marine MP samples (< 0.5 mm) will be presented. For this purpose the particles are extracted from the marine sample, chemically and enzymatically purified and finally filtered on a Si filter [1]. By Raman Imaging we get complete Raman spectra for each measurement "point" of a sample area. The space-resolved Raman Images are generated by choosing a characteristic spectral band range of the synthetic polymers. All particles with a high intensity in this spectral range are coloured, (Fig.1).
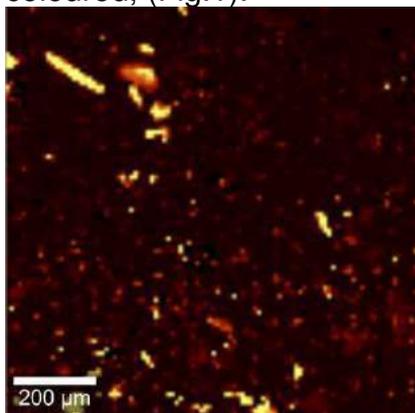

Fig. 1: Raman Image of a marine microplastic sample on a Si filter using the spectral range 2780 – 2980 cm^{-1}

The spectrum of each coloured particle is evaluated and, in case the particle is likely of synthetic nature, the polymer is identified using a spectra database. Furthermore the size of all identified MP can be obtained. Detailed results of our investigations are in [2]. In conclusion Raman Imaging is a good method to detect MP down to 1 µm.

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Easy Imaging of Processed Cheese Microstructure

Geoffrey Smith[1], Keith Gordon[1], Stephen Holroyd[2], David Reid[2]
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Monitoring the microstructure of cheeses is an integral part of product development for dairy manufacturers as it can provide insight into various characteristics of the product, such as its taste, texture, appearance, and stability. To do this routinely, it would be ideal for the imaging technique to be quick and easy to use, with minimal influence over the microstructural properties. Therefore, the utility of Raman microscopy for imaging cheese microstructure was investigated, as it typically meets these criteria.

Controlled processed cheese formulations were made in the lab using commonly used ingredients including trisodium citrate (TSC), sorbic acid, paprika and corn starch. Commercially available cheeses were bought from local supermarkets. Various imaging methods were employed to observe their efficacy for imaging the relative distribution of components including those utilising: low spatial resolution, high spatial resolution, Raman band integrals, and principal component analysis (PCA) scores. Using these methods, the distribution of protein, fat, water, TSC, starch and paprika were imaged. While low spatial resolution images showed that general component distribution can be attained, high spatial resolution was necessary to provide accurate representations of domain sizes and shapes. Furthermore, utilising Raman band integrals was the quickest and easiest method for generating Raman images but PCA was more effective for imaging components with weaker features in the Raman spectrum. Despite this, some additives were not observed in Raman images, indicating that there is still opportunity to improve the methodology. Overall, Raman microscopy proved to be an effective tool for imaging the microstructure of processed cheese, and may potentially be improved further through attempts to image using different multivariate techniques.
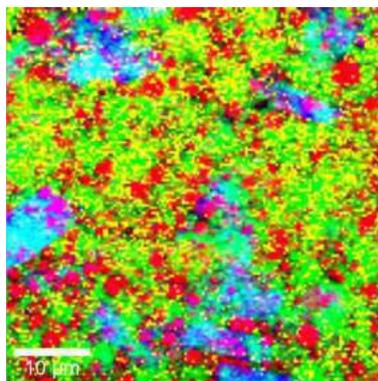

Fig. 1: A multi-component Raman image of processed cheese microstructure. The visible components are fat (red), protein (yellow), water (green), and trisodium citrate (blue). The image is 50 × 50 μm and contains 150 × 150 Raman spectra.

Abstracts
Contributed Posters

Chemical and mineralogical analyses of Antarctic micrometeorites

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Introduction: Antarctic Micrometeorites (AMMs) are submillimeter dust particles originating from asteroids or comets collected in the snow of Antarctica [1]. Three AMMs, recovered near the research station Concordia, were analyzed by Raman spectroscopy to determine their mineralogical compositions and organic constituents.

Methods: The AMMs have sizes of 38 µm x 67 µm x 32 µm; 23 µm x 20 µm x 11 µm and 23 µm x 20 µm x 29 µm. They were placed on gold plates of 1 cm x 1 cm and analyzed by confocal Raman Spectroscopy using a Witec Alpha 3000 microscope. The samples were measured with x100 and x50 objectives using a 532 nm laser and a power between 0.5-1.9 mW. The power was set as low as possible to induce as little damages as possible for potential organic materials.2D mappings with steps of 1 µm x 1 µm were acquired. A layer scan mode was also used to produce a topographic view of the particles with steps between 0.25 µm and 0.8 µm in the lateral dimension and 1µm in depth.

Results: The AMMs are composed of several iron oxides and contain anhydrous silicates like olivine and pyroxene. SO_4 bonds typical of the sulfate minerals were detected, their positions indicate (K,Na) bearing sulfates [2]. Two of the AMMs also show the so-called D and G bands in the Raman spectra that are indicative for the presence of aromatic compounds [3].

Discussion: In order to determine the chemical composition of the minerals, the exact peak positions of the mineral features were measured. Using the relations between chemical compositions and Raman peak positions from [2,4,5], we estimated a chemical composition from $Fo_{37\pm19}$ to $Fo_{99\pm16}$ for the olivine inclusions in one of the particles and compositions of $En_{97.5}Fs_{2.5}$ to $En_{70}Fs_{30}$, $En_{50}Fs_{50}$ to $En_{90}Fs_{10}$ and $En_{45}Fs_{04}Wo_{50}$ for the pyroxene inclusions in the three particles. Sulfates have not been previously identified in Concordia micrometeorites, for which exposition to liquid water is limited to a few hours during the collection of the particles. These sulfates might be produced by interaction with the terrestrial atmosphere, like was observed in meteorites like Orgueil [6]. The organic material is not spread all over the particles but located in specific regions. The Raman properties of the organic D and G bands i.e. width, intensity and position, are affected by thermal metamorphism [3]. For the two AMMs analyzed, they indicate a mild thermal alteration.

Conclusion: Raman spectroscopy has been performed on three micrometeorites to study their mineral and organic constituents. These studies show that they contain anhydrous silicates, as well as other minerals and organic matter showing different stage of thermal and aqueous alteration.

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In vivo Raman spectroscopic study of iron influence on the pigment composition in the marine diatom *Stephanopyxis turris*

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The influence of different supplied iron concentrations in the culture medium upon diatom pigment composition was investigated here by the nondestructive Raman spectroscopy for the marine diatom species *Stephanopyxis turris* (*S. turris*). Marine diatoms are unicellular, photosynthetic eukaryotes creating intricate, species-specific, micro- and nanostructured cell walls. These so-called frustules consist mainly of silicon dioxide (SiO_2) with minor amounts of chemical elements like iron. Iron is known to be an essential micronutrient in the metabolism of diatoms. Genomic studies indicate a connection between silicon and iron metabolic pathways in diatoms, but the physiological role of iron in the biosilification process and the reason for iron incorporation into biosilica are still unknown (Mock *et al.* 2008).

We recently examined the form and amount of iron incorporation into the biosilica of *S. turris*: Iron is mainly clustered and finely distributed within the siliceous cell walls. Only a minor fraction of iron is dispersed (i.e., occupying silicon sites) (Kaden *et al.* 2016). Quantitative investigations of the isolated biosilica revealed a low amount of incorporated iron, hence, the major fraction of supplied iron is located within the cell (e.g. stored in the vacuole). Since iron plays a major role in photosynthesis (Raven 2013, Kuczynska *et al.* 2015) it may influence photosynthetic pathways and pigment composition. *In vivo* Raman spectroscopic measurements of *S. turris* in the exponential growth phase during exposure to various iron concentrations in the culture medium was applied to examine the influence of iron. Least-squares regression of cell spectra including spectra of the pure diatom pigments chlorophyll *a*, *ß*-carotene and fucoxanthin allowed for the detection of related changes in the pigment composition of *S. turris*.

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The binding of tyrosine kinase inhibitors to EGF receptors by Raman micro-spectroscopy: drug distribution and metabolism in cancer cells

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Spontaneous Raman and coherent anti-Stokes Raman scattering (CARS) are well-established, non-invasive imaging techniques for characterization of both cells and tissues. The Raman spectra are used for detection and differentiation between cellular and tissue components. We have used a combination of Raman, CARS, fluorescence, and cluster analysis to study cellular response to anti-cancer agents and monitoring the distribution and metabolism of drugs in cancer cells.

Cancer is one of the leading causes of death worldwide. It involves abnormal cell growth and uncontrolled proliferation with the possibility of metastasis. The epidermal growth factor receptor (ERGR) and HER2 which belong to the same tyrosine kinase receptor superfamily are overexpressed in many tumors. Therefore, tyrosine kinases (TKs) play a critical role in targeted cancer therapy. Erlotinib and neratinib are examples of first and second generation tyrosine kinase inhibitors, respectively, which block the activation of the signaling pathways that would promote tumor cell growth and proliferation.

In this study, we were able to monitor the effect of erlotinib and neratinib on cellular components on different cancer cell lines using Raman spectroscopy. Raman results also indicated that neratinib has a strong C≡N stretching vibration (2208 cm^{-1}), which is located in a Raman silent region of cells. Thus, it can be used as a label-free marker band for monitoring neratinib. We have used both Raman and CARS to monitor the neratinib distribution in cancer cells. Furthermore, the Raman results indicated that neratinib is metabolized in cells.

RAMAN SPECTROSCOPY AND IMAGING OF BILAYER GRAPHENE FILM PREPARED ON COMMERCIAL DILUTE COPPER FOIL

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This study reports the Raman spectroscopy and imaging of bilayer graphene films prepared on commercial dilute Cu(0.5 at% Ni) foils using atmospheric pressure chemical vapour deposition (AP-CVD). In the Raman optical microscope images, a wafer-scale monolayer and large-area bilayer graphene films were distinguished and confirmed with Raman spectra and mapping (Fig. 1). The Raman spectroscopy imaging of a large-area bilayer graphene film suggested a Bernal stacking order in prepared bilayer graphene film. Nonetheless, Raman spectroscopy/imaging is a well-known powerful and noninvasive technique to determine, among others, the number of graphene layers, the stacking order and the interlayer interactions in few layers graphene sample. A four-point probe sheet resistance of graphene films confirmed a bilayer graphene film sheet resistance distinguished from that of monolayer graphene. The large-area part of bilayer graphene film obtained was assisted by Ni surface segregation since Ni has higher methane decomposition rate compare to Cu. A relatively higher Ni surface concentration in Cu(0.5 at% Ni) foil was confirmed with time-of-flight secondary ion mass spectrometry.
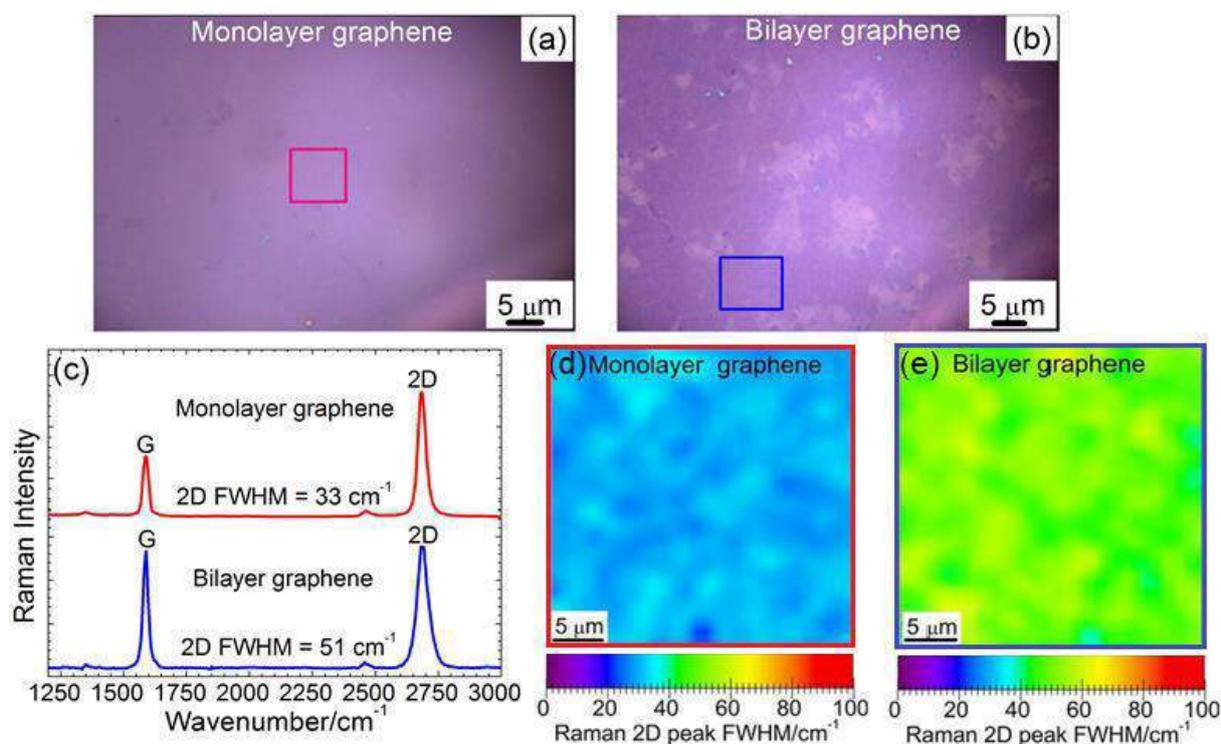

Fig. 1: The Raman optical microscope images of (a) mono and (b) bilayer graphene films. (c) The average Raman spectra obtained from 30 μm^2 areas of mono and bilayer graphene films shown in (a) and (b) with square boxes and the corresponding (d)-(e) 2D peaks FWHM mapping.

Modification of mineral composition is an integrative part of crustacean cuticle morphogenesis: application of Raman spectroscopy

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Studying the formation of mineralized biological structures is methodologically and interpretatively feasible by the integration of expertise from different scientific fields. Tissue structure as well as its organic and mineral composition must be considered in the context of the organism development. Exoskeletal cuticle of crustaceans is an epidermal chitinous matrix, incorporating calcite, amorphous calcium carbonate and amorphous calcium phosphate (ACP) in adult animals. It is a highly organized structure, secreted and constantly reorganized by cells and displaying spatially non-homogenous distribution of its constituents. Raman spectroscopy was used here to characterize calcification of the cuticle during larval development in the invertebrate model organism *Porcellio scaber* (Crustacea, Isopoda).

Calcium mineral forms were determined in the cuticle of three sequential larval stages: newly hatched larva, advanced early larva and mid-stage larva, and compared to those in the cuticle of adults. Raman spectroscopy showed that mineral composition of the cuticle differs between these larval stages and progressively approximates the mineral composition in adults (Fig. 1). We observed differences in the presence and in the relative intensities of individual peaks characteristic for particular calcium minerals, which were the most pronounced for calcite. In our preceding research we demonstrated that in newly hatched larvae cuticle formation is initiated and calcium is sequestrated in the cuticular matrix as revealed by TEM and SEM-EDS. Cuticle ultrastructure and elemental composition in the advanced larval stages already resembles the cuticle of adults (Mrak et al. 2014). Collectively, all these results together show that mineral composition of the cuticle is modified synchronously with changes in cuticle ultrastructure and elemental composition during *P. scaber* larval development.

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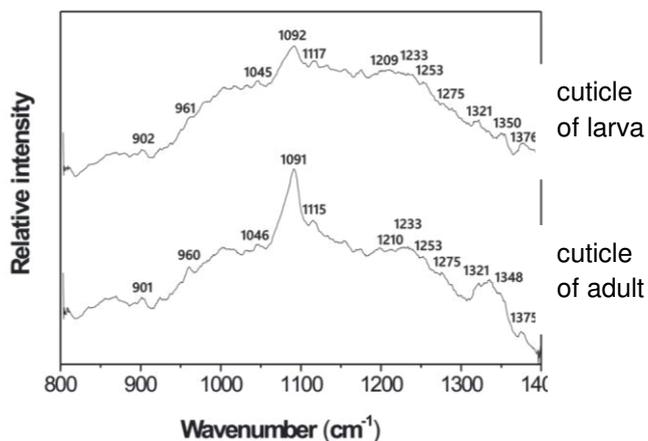

Fig. 1: Raman spectra obtained from the cuticle of adults and the cuticle of advanced early larva of isopod P. scaber. Both spectra show peaks characteristic for carbonate (1091/1092 cm^{-1}) and for ACP (960/961 cm^{-1}), with lower intensities in the spectra obtained from larval cuticle.

Identification of microplastics in marine samples by Raman Microspectroscopy

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Microplastics (MP) are micro-sized particles of synthetic polymers in a size range from 5 mm down to 1 µm. They have been observed in marine ecosystems worldwide. Because of their small size MP can be mistaken for food and can be ingested by a variety of organisms. Not only MP themselves, but also embedded additives, adsorbed toxic contaminants or associated potentially pathogenic microorganisms pose a potential risk for the marine foodweb. In the first instance reliable data about the occurrence of MP in marine environments and valid analytical systems are necessary for a risk assessment.

We show the excellent possibilities of Raman Microspectroscopy to identify MP in marine samples distinctly on the basis of the chemical structure. At first the results of the manual step-by-step measurement of larger particles (5 – 0.5 mm) of Baltic Sea samples by Raman Spectroscopy will be shown. Secondly the automated Raman Imaging of marine MP samples (< 0.5 mm) will be presented. For this purpose the particles are extracted from the marine sample, chemically and enzymatically purified and finally filtered on a Si filter [1]. By Raman Imaging we get complete Raman spectra for each measurement "point" of a sample area. The space-resolved Raman Images are generated by choosing a characteristic spectral band range of the synthetic polymers. All particles with a high intensity in this spectral range are coloured, (Fig.1).
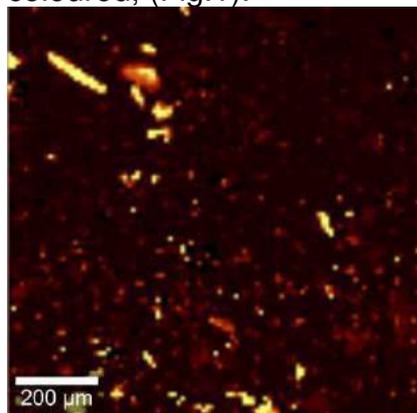

Fig. 1: Raman Image of a marine microplastic sample on a Si filter using the spectral range 2780 – 2980 cm^{-1}

The spectrum of each coloured particle is evaluated and, in case the particle is likely of synthetic nature, the polymer is identified using a spectra database. Furthermore the size of all identified MP can be obtained. Detailed results of our investigations are in [2]. In conclusion Raman Imaging is a good method to detect MP down to 1 µm.

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Detection of Explosives' Inclusions in Fingerprints Using Raman Chemical Imaging

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Confocal Raman Imaging (CRI) is a promising technique for the detection and identification of inclusions in fingerprints, including those of materials like explosives and abuse drugs.

In this work we have studied the contamination of human fingerprints with trace amounts of 2,2-dinitroethene-1,1-diamine (FOX-7) on aluminium-coated microscope slides (Figure 1). The explosive was not distinguishable at the optical microscope using the same magnification conditions.

The same approach has been successfully applied to the detection of other explosives in blind samples containing 0, 1 or 2 different compounds, with a highly favorable statistics.
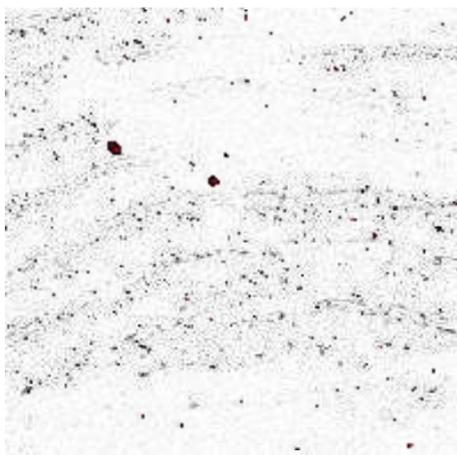

Figure 1 – *Raman image of FOX-7 inclusions (red) superimposed with the optical image of a fingerprint.*

Understanding the process of bone formation using Raman spectroscopy

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Bone tissue is a composite material, comprising of a matrix of organic and inorganic constituents. The primary inorganic component of bone is hydroxyapatite mineral, which is a form of calcium phosphate. The growth and structure of this mineral is controlled by the organic matrix upon which it is grown. The major constituents of the organic matrix such as collagen, osteocalcin and osteopontin are formed by cells called osteoblasts. These cells are therefore critical to the formation of bone.

In this study, osteoblasts were grown on fused silica substrates and the mineralization process was analyzed by using Raman spectroscopy. After nine days of differentiation, extracellular aggregates were seen to be heterogeneously distributed across the sample. Raman spectra from these aggregates and a detailed analysis of the spectra will be presented. For instance, the presence of amino acids such as tyrosine, phenylalanine, proline and hydroxyproline in the aggregates can be assigned based on the spectra. Moreover, the peaks in the Amide III band between 1200 and 1300 cm^{-1} indicate that the proteins mostly have an α-helical structure rather than β-sheet, which is expected since the most abundant protein, collagen, is a triple helix consisting of 3 α-helices. By the 16th day of differentiation, a distinctive sharp peak from phosphate at around 960cm^{-1} can be seen to dominate the spectra of the aggregates, thus confirming that mineralization begins in these organic aggregates. Over time, these aggregates grow in size and the intensity of the phosphate peak increases as mineralization progresses across the sample. Lastly, the effects on the process of mineralization caused by altering the cell culture medium using additives such as growth factors or binding sugars will also be shown.

SERS-Investigation of the Diatom *Stephanopyxis turris*

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Diatoms are unicellular algae which are responsible for about 20 % of the worldwide CO_2 fixation. They live in sea as well as in sweet water and their size varies between 5 and 500 µm. Their cell walls exhibit species-specific and highly structured nano- and micropatterns made from silica. The industry applies these cell walls as filters, abrasives, or as carrier material for catalysts. There are, however, numerous open questions with respect to the biological and biochemical processes underlying biosilica synthesis. This makes diatoms attractive for fundamental research, but also for materials science.

The Raman spectra of the diatom *Stephanopyxis turris* are usually dominated by pigment bands which prevents the detection of other substances in integer cells. Using SERS, we were able to collect Raman spectra from integer cells containing a multitude of new signals. First analyses of these spectra indicate the presence of organic material like amino acids, monosaccharides and fatty acids in *S. turris*.

We generated the SERS-effect by incubation of *S. turris* in a gold-containing growth medium. Scanning electron and transmission electron microscopy proved that the diatom cells produced gold nanoparticles *in-vivo* by reducing the gold salt of the cultivation medium. These *in-vivo* synthesized nanoparticles induced the observed SERS effect and enabled us to establish a new method for *in-vivo* investigation of diatom cells. This procedure could make an important contribution to the elucidation of intracellular processes in diatoms.
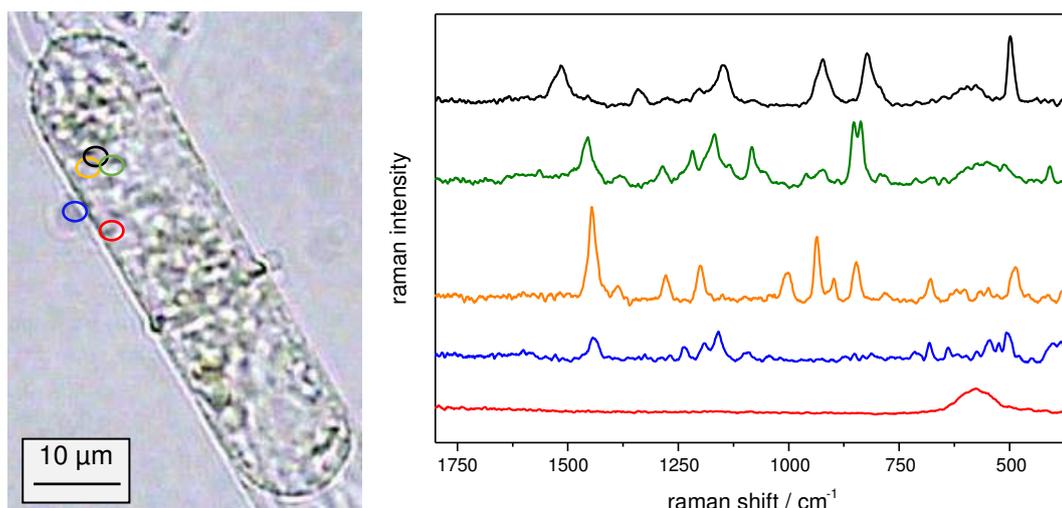

Fig. 1: Cell of S. turris and gathered SERS spectra of this cell. The colors indicate the location of the detected spectra in the cell.

Raman microscopy to study the differentiation pathways of human mesenchymal stem cells

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Mesenchymal stem cells (MSC) are adult stem cells that have the capacity to renew themselves or commit to become specific tissue such as bone, cartilage or fat depending upon the stimuli provided by the environment. These cells have thus become vital in understanding the formation and repair of skeletal tissues in the body. Conventional methods used to study the differentiation of MSCs are usually destructive, time-consuming techniques requiring extensive sample preparation. Raman microscopy, on the other hand is a non-destructive, label-free technique that requires minimal sample preparation. In this study, we have used WITec's Raman microscope alpha 300S to study three differentiation processes of human MSCs (hMSCs): osteogenesis (bone), chondrogenesis (cartilage) and adipogenesis (fat). Osteogenesis and chondrogenesis involve the formation of aggregates outside the cells while adipogenesis primarily involves the formation of spherical droplets inside the cell. Raman spectra and mapping of the cells show the distinctive chemical composition of the different lineages. Firstly, the formation of bone is shown to start with protein-rich aggregates outside the cells upon which phosphate crystals are seen to grow. Secondly, the formation of cartilage is shown by the presence of proteins and glycosaminoglycans in the aggregates. Finally, for the fat cells, excess glucose in the medium is taken into the cells, converted to lipid droplets and stored in vesicles inside the cells.
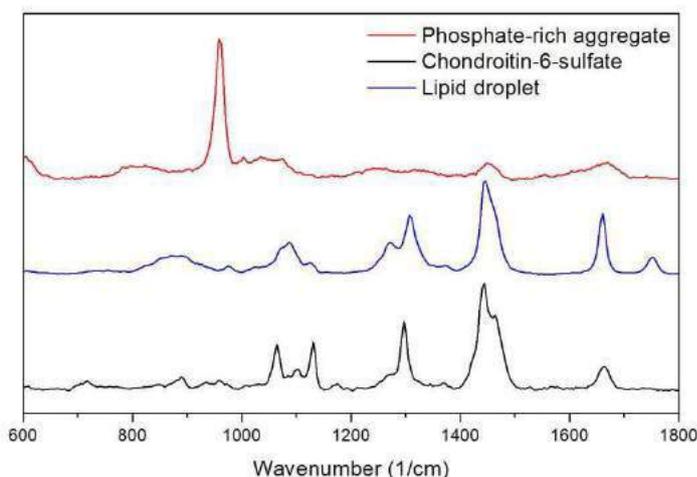

Fig 1: Raman spectra showing differentiation of hMSCs to bone, cartilage and fat cells.

Raman Spectroscopy of Ice XII and XIV

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By including a minimum of 17 crystalline and 3 amorphous phases the phase diagram of ice/water is one of the most complex known one- component- phase diagrams. Raman spectroscopy is not only a quiet simple way to detect transition points, but also to assess the temperature dependence of stiffnesses and lenghts of inter- and intramolecular OH- bounds. Thus changes within these phases and possible reasons for transitions might be determined.

Ice XII is a metastable ice- phase, which can be synthesized at 1.2 GPa and 180 K. Whereas the positions of oxygen- atoms are clearly defined within ice XII, that ones of the hydrogen- atoms are, up to a certain point, arbitrary. After cooling below 100 K, a transition to the hydrogen- ordered ice XIV takes place. The system of ice XII/XIV can be stored at ambient pressure up to temperatures of maximal 135 K. However, once the transition from ice XIV to ice XII takes place, complete hydrogen ordere can not be achieved by simply cooling down, as it is possible in different hydrogen- order/disorder- transitions of ice.

To control temperature, the sample has to be stored in a nitrogen- cryostat including a heater and temperature- control during the whole measurement. The deposition of ice from aerial water- vapour is prevented by a vacuum of 8 – 40 mbar. For the resulting big free working distance of this setup, high times of measurements are necessary, as 4x 400 sec for measurements at 300 cm^{-1} or 2x 120 sec at 3300 cm^{-1}.
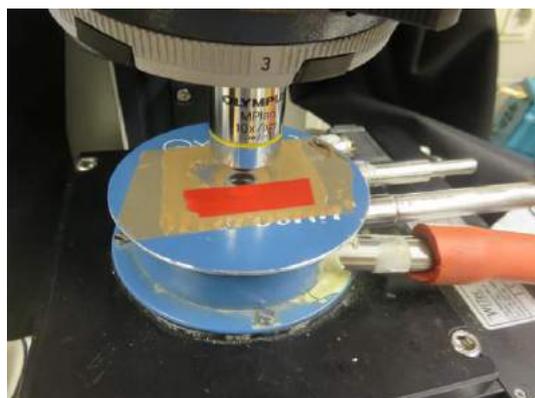

Fig. 1: The nitrogen- cryostat.

Early Diagnosis of Breast Cancer Based on Surface Enhanced Raman Spectroscopy

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Successful and effective treatment of cancer can be achieved by early diagnosis of cancer. Nowadays, oncologists have been focusing on finding a noninvasive method for early detection of cancer and also for the monitoring of its progress during the disease. Among different types of bio-recognition elements which can be used for cancer diagnosis, circulating cancer cells can be good candidates for the early diagnosis and therapy of cancer.

Since a very small number of CTC cells normally present in body fluids to be used as a type of an early diagnostic marker, development of highly sensitive technique is necessary to circumvent this issue. Different techniques for the detection of CTCs have been developed including polymerase chain reaction, (PCR)-based methods, cytometric methods and methods for the cell-enrichment. Surface Enhanced Raman spectroscopy (SERS) has been widely developed as an analytical method for biosensing purpose.

As a result, the main idea of this research is the development of a new technique based on SERS to isolate and identify breast cancer cells simply and specifically based on the biomarker which is overexpressed on the surface of them.
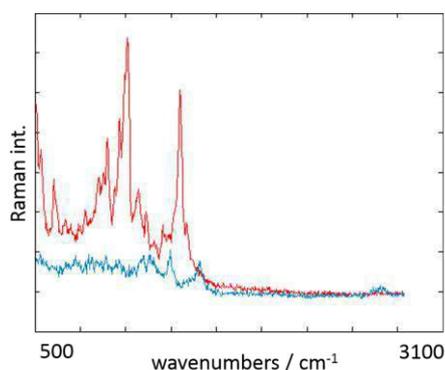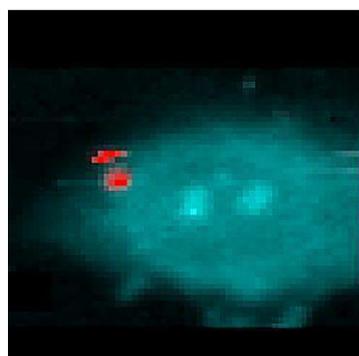

Figure 1: Raman image and associated spectral information of an MCF-7 cell incubated with SERS labeled receptor sensors.

Quantitative analysis of the small fluid inclusion using FIB-XCT technique and Raman microscopy

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Fluid inclusions in high-grade metamorphic rocks are occasionally small in size up to several microns, and have difficulty to perform quantitative analysis, though physical and chemical properties of them provide several important information about geodynamics. Recently, we established a powerful method for detailed descriptive studies of such small inclusions using a combination of a focused ion beam (FIB) system, synchrotron X-ray computed tomography (XCT), conventional microthermometry, and Raman microscopy (Yoshida et al., 2016). We carried out detailed morphological studies on a prograde-stage fluid inclusion (H_2O-NaCl-CH_4), which is observed in a metamorphic quartz vein collected from the Sanbagawa metamorphic belt, southwest Japan (Yoshida and Hirajima, 2012). The fluid inclusion was picked up as a "house-shaped" particle with about 20 µm size by using a FIB system. Then, it was investigated by synchrotron radiation-based high-resolution XCT using a Fresnel zone plate at BL47XU in SPring-8. XCT observation provides a detailed volumetric properties of bubble and liquid phases of the fluid inclusions. On the other hand, we can estimate vapor pressure of the bubble (mainly consists of CH_4) by Raman microscopy and the salinity of liquid phase by microthermometry. Combining these data together, bulk chemistry and density can be quantitatively estimated.

We performed another mass-balance calculation to testify these results. The bulk chemistry and density of fluid inclusions in H_2O-NaCl-CH_4 system can be also estimated by using a combination of Raman microscopy and microthermometry (Bakker et al., 2010). The calculated result agrees well with those estimated from the CT data, which indicates powerful potential of FIB-XCT technique for quantitative analysis of small inclusions.
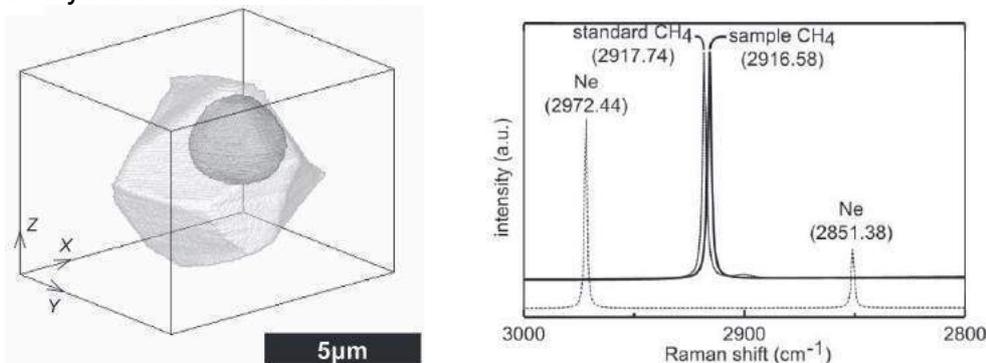

Fig. 1: Left: A bird's eye view of the fluid inclusion obtained by synchrotron XCT. Right: CH_4 Raman spectra used for pressure estimation.

Kinetics of polymorphic phase transformations in glycine crystals

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Investigation of two types of polymorphic phase transformations in organic ferroelectric β-glycine by confocal Raman microscopy (CRM) and piezoresponse force microscopy (PFM) allowed to suggest a microscopic description of the transformations.

Glycine (NH_2CH_2COOH) is the simplest amino acid and a model material for investigation of polymorphic phase transformations in organic crystals. Under normal conditions glycine forms three polymorphes: α, β and γ [1]. Ferroelectric β-glycine is an advanced material for development of functional biomedical devices [2]. Being metastable phase β-glycine requires detail investigation of phase transformations. Up to now existed publications deal with the final products of crystallization only [3]. In this work we present *in situ* investigation of β-γ and β-α transformations with high spatial resolution by CRM and PFM.

PFM was realized using scanning probe microscope Ntegra Therma (NT-MDT, Russia) with Ti/Pt probe. Raman spectra with spectral resolution 3 cm^{-1} were measured in backscattering geometry by Alpha 300 AR (WITec GmbH, Germany) with 488 nm solid state laser.

Detailed analysis of kinetics of β-γ transformation was performed by PFM. We found that transformation of β-phase into γ-phase is determined by the relative humidity (RH). The velocity of the transformation depends on RH and phase boundary is very rough and moves inhomogeneously along the polar axis of the initial β-phase. CRM measurements allowed to suggest the microscopic mechanism of the process.

Kinetics of β-α transformation was studied *in situ* by CRM during glycine crystallization from saturated water solution. β-phase formed at the initial stages of the crystallization in accordance with Ostwald rule spontaneously transforms into α-phase. Analysis of the temporal behavior of certain Raman lines allowed to reveal the main stages of the transformation.

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