New Worlds

Vibrational Spectroscopy: Movies of protein-bound water and living cells

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For centuries, researchers have endeavoured to make details of life processes irresolvable to the human eye visible to us. Pioneering developments of micro(spectro)scopic techniques have continuously extended the limits of resolution, and every extension of these limits has uncovered new worlds. As these biological procedures are dynamic processes, we do not just want snapshots of individual conditions but would rather be able to record an entire movie at a high spatial and chronological resolution.

The resolution of these processes is however not purely of academic interest. Faults in the processes lead to disease in living creatures, and this is why it is of particular interest to understand the mechanisms in detail. This also permits precise and elegant treatment of the cause of the disease with substances from personalised medicine instead of sujecting the diseased patient with the enormous stresses caused by surgery or chemotherapy.

All diseases are ultimately based on cell malfunction, which in turn is based on defects in protein interaction and the proteins in the cells. Only a few techniques are able to resolve not only living cells but also protein interactions, especially on membranes and individual proteins (Fig. 1). Current approaches of vibrational spectroscopy such as time-resolved FTIR-spectrospcopy and confocal Raman Microscopy however permit precisely this analytical challenge. A number of current results are introduced below.

Infrared and Raman spectroscopy are marker-free detection methods

Every molecule has vibrations as characteristic as a fingerprint, which is why a molecule can be identified on the basis of its characteristic infrared absorption or its Raman emission without any further markers by exciting vibration levels, i.e. the vibrational energy levels of a molecule. This is why we speak of vibrational spectroscopy in both cases. The vibrational levels can be excited with very little energy and, in contrast to fluorescence spectroscopy, do not require specific fluorescence labels with which objects to be examined such as proteins first need to be marked. Complementary to fluorescence spectroscopy, vibrational spectroscopy is able to directly detect the molecules and their biochemical as well as structural properties without markers. In contrast to an x-ray structure analysis, a protein for example does not need to be crystallised for structural clarification but can be observed under physiological conditions instead.

Neurodegenerative disease due to a misfolded protein structure

The prion protein is paradigmatic for proteins which, if misfolded, trigger neurodegenerative diseases such as Alzheimer's. If misfolded it triggers BSE (mad-cow disease) or CJD (Creutzfeldt-Jakob disease). We used time-resolved FTIR-spectroscopy [1] to analyse misfolded native, fully glycosylised prion proteins in native membrane binding, and we also recorded the shift of the amide I bands (Fig. 2). The position of

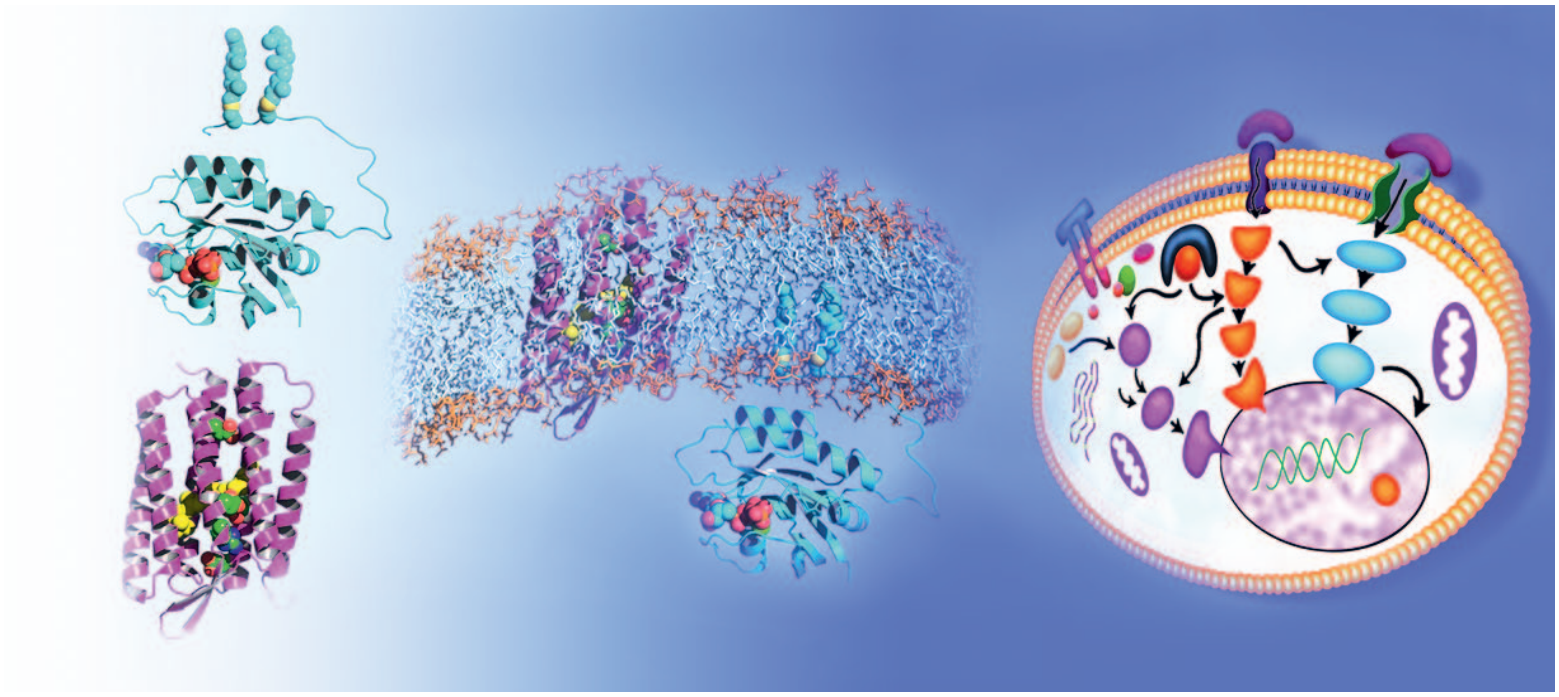

Fig. 1 Vibrational (micro)spectroscopy is used at different scales to analyse protein reaction mechanisms, protein interactions on membranes and on living cells.

Klaus Gerwert, born in Reken/Westphalia, studied Physics in Münster, graduated at the faculty of Chemicals and Pharmacy in Fribourg and completed his post-doctoral studies in Physical Chemistry in Bielefeld. After his doctorate he moved to the Max-Planck Institute in Dortmund, where he developed the time-resolved FTIR difference spectroscopy on proteins. Awarded a Heisenberg scholarship by the DFG, he moved to the Scripps Institute in California. In 1993, at the age of 37 he was appointed the Biophysics chair at the Ruhr University in Bochum. He is founder and speaker of the SFB 642, GTP- und ATP-abhängige Membranprozesse [GTP and ATP –dependent Membrane processes]. In 1992, his work was awarded the Karl-Arnold Prize by the North Rhine-Westphalian Academy of Sciences and Arts, of which he has been a member since 2008. In 2006 he received the Ruhr Innovation Prize from the Minister President of North Rhine-Westphalia. He has a second appointment as Director at the Max-Planck Partner Institute in Shanghai and is also a "Fellow" of the Max-Planck Society. His work is focused on the examination of molecular reaction mechanisms of proteins, especially membrane proteins, for which he specifically developed time-resolved vibrational spectroscopic methods. Since 2010, he has devoted his time to PURE imaging vibrational spectroscopic methods on cells and tissues.

the protein structure's intensive amide I bands, the C=O stretching vibration, depends on the protein's secondary structure within the spectrum. We were able to show that misfolding into the disease-generating condition only takes place during the protein's membrane binding process, i.e. binding is a prerequisite for this misfolding process.

Time-resolved FTIR difference spectroscopy on membrane proteins

Once a protein has been identified as a key component of a biological process, details of the molecular reaction mechanism can be established with the help of time-resolved FTIR difference spectroscopy. Due to the difference formation, only the functionally active groups of the protein are selected from the background absorption of the entire sample. For this the protein reaction needs to be accurately started. All reactions in the protein are then simultaneously recorded at nanosecond time resolution in the infrared spectral range. By comparing site-specific mutants or marking the isotopes, individual IR bands can be uniquely assigned to the protein's molecular groups. Measurements are usually performed on recombinant proteins.

Bound water molecules conduct protons into a membrane protein

FTIR difference spectroscopy is illustrated here with the light-driven proton pump bacteriorhodopsin as an example. The transport pathway of a proton, starting from the central proton binding site, the protonated Schiff base (PSB) via Asp85 and a protonated water complex, a so-called Eigen cation, could be proven with the help of time-resolved FTIR difference spectroscopy [2]. Figure 3 displays the mechanism in detail. A particular result demonstrated was that the proton donation group behaves like a diode, which is why the term "proton diode" was introduced at the same time [3]. This explains why proton transfer in the protein is directed. In free water, proton transfer follows random directions. To enable the development of a proton gradient at the protein's cell membrane, the protein created an internal, directional process from the undirected process in water by using the laws of physical chemistry. We were able to transfer the results of our observations from classical physical chemistry to today's molecular biology.

The membrane-bound Ras protein

The small, guanosine triphosphate (GTP) binding Ras protein works like a molecular switch, which triggers a growth signal in the cell. Around 30% of all human tumours contain oncogenic mutations influencing the protein's GTPases activity, e.g. K-Ras in intestinal tumours. Time-resolved FTIR experiments were used to start the GTPase reaction with the help of photolabile so-called caged-GTP. A short laser flash splits off the used caged group (o-nitropheny-

lethyl) and starts a GTP hydrolysis reaction of the Ras protein. In the living cell, the hydrolysis is catalysed by so-called GAP proteins (activating GTPases) and thus regulated. Although the Ras protein can be activated in oncological mutations, the hydrolyses' splitting process, especially the catalysis through GAP, is impaired, giving rise to uncontrolled growth signals, which ultimately contribute to the creation of cancer. Figure 4 displays the GTP hydrolysis of the Ras molecule and the acceleration by the GAP. Thus an important intermediate stage for the catalysis could be resolved for the first time [4].

Lipid anchors bind the Ras protein to the cell membrane. Using FTIR with the ATR technique, it was possible to examine Ras in its natural environment on a lipid membrane spectroscopically (measurement with attenuated total reflection) [5]. This now allows the detailed study of the Ras protein's interaction network and the influence of substances on these interactions. If one could influence the interaction network of the oncogenically mutated Ras with small active molecules, this would be a molecular approach within the scope of personalised medicine. The FTIR-spectroscopic ATR technique supplies a marker-free detection system to identify the action mechanisms of possible key substances in personalised medicine [6]. The function of the ATR technique is similar to that of Surface Plasmon Resonance (SPR), but provides biochemical information through vibrational spectroscopy.

Raman imaging on living cells

If one moves further from the in-vitro system, a recombinant protein or a membrane-reconstituted protein to a living cell, vibrational spectroscopy needs to be applied differently: in the case of IR and Raman microscopes, all biochemical properties are each integrally displayed as spatially resolved on all measured image pixel points. Individual proteins, lipids or DNA are no longer resolved but the totality of all analytes is measured instead. It is no longer possible to make detailed statements on individual proteins as was the case in the two previous examples, but one receives an integral image, a spectral biomarker, which reflects the biochemical-structural condition at the measured point of for example a biopsy sample, like a fingerprint (Fig. 5). A database needs to be developed as in fingerprint identification in order to enable the application of the spectral biomarkers as classifiers in the annotation of cell components. In this case, confocal Raman microscopy further permits the recording of various cutting depths, which can then be assembled into a three-dimensional image of the cell (Fig. 6). The individual subcellular components are simultaneously recorded without markers.

The use of infrared microscopes enables the analogue receipt of site-resolved information of tissue sections. With the help of pathologists, these index colour images are then annotated with classically coloured samples by comparison. Multivariate statistics with the use of broad spectral ranges improves the analysis. At the end of this development and with the help of the vibrational spectrums, tissue will be objectively classified with a high degree of sensitivity and specificity. We recently succeeded in this on an automated basis with biopsy samples of intestinal cancer.

The Protein Research Unit Ruhr within Europe (PURE) was founded with the support of the NRW state Government in 2010 to develop these methods and the translation into clinical research.

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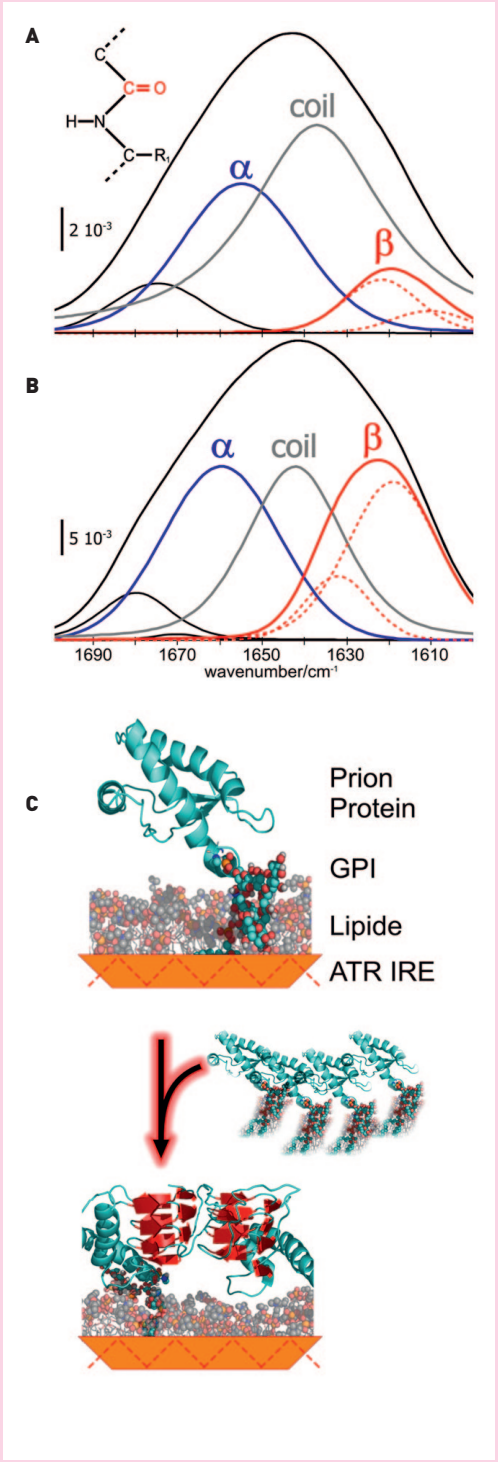

Fig. 2 The conformation-sensitive Amid I band is created by the C=O-stretching vibration of the protein backbone (**A**). The band is divisible into the individual secondary structure elements α-helix, β-pleated sheet and random coil. The structure of native prion proteins bound to membranes with lipid rafts corresponds to the already familiar structure of the anchorless recombinant prion protein in free solution (**A**, **C** above). If an excess of prion protein binds to the membrane, it automatically folds into a Ð-pleated sheet (**B**). One can recognise the start of oligomerisation by the intramolecular Ð-pleated sheet (**B**, **C** below). No misfolding was observed in the solutions used. The high concentration due to membrane binding is therefore significant for the misfolding process.

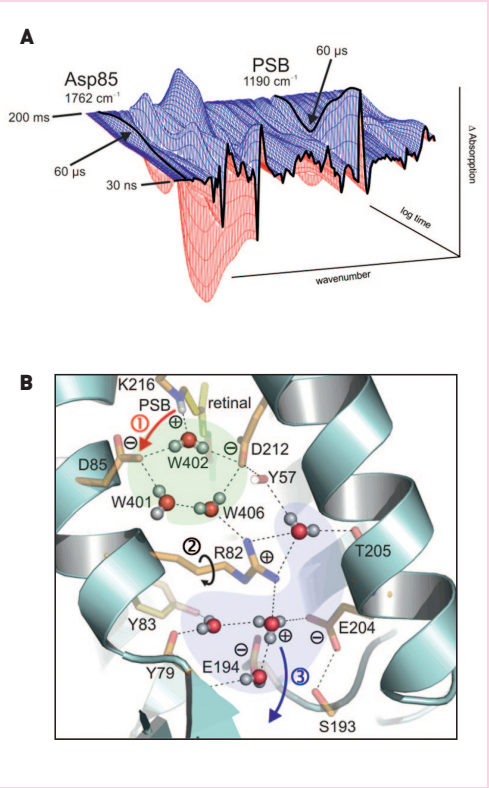

Fig. 3 A The time-dependent modifications of the infrared absorptions during the bR-photo cycle of 30 ns to 200 ms in logarithmic display simultaneously recorded at 4 cm^{-1} spectral resolution between 1800 cm^{-1} and 1000 cm^{-1}. Protonation of Asp85 (band at 1762 cm^{-1}) and deprotonation or reprotonation of the Schiff base (PSB) (band at 1190 cm^{-1}) are marked. In 60 µs, a proton is transferred from the PSB to Asp85 (**B**), which is clarified in the spectrum by the reduction of 1190 cm^{-1} absorption and the increase at 1762 cm^{-1}.

Fig. 3 B Due to light-induced isomerisation of the chromophore Retinal, the strong H-bridge of the water 402 to PSB is broken up and about half of the light energy is stored in the protein. As the free OH group of water 401 (dangling water) to isomerisation is hydrogen-bridge bound, water 401 no longer stabilises the negative charge of Asp85 and the pK value is increased. The proton migrates from the central proteon binding site PSB to the counterion Asp85. Due to the neutralisation of Asp85, the saline bridge to Arg82 is broken and Arg82 turns towards the protonated water complex. The movement of the positively charged guanidinium group of the Arg82 destabilised the protonated water complex near the protein surface. The protonated water cluster (shaded blue) stores a proton in a so-called Eigen cation ($H^+(H_2O)_3$). In contrast to a random Grotthuss proton transfer in water, the protonated water complex in the protein is deprotonated via a targeted movement of the Arg82. In the protein, the proton is stabilised in the second hydration shell via stationary amino acids instead of via flexible water molecules. The protein has used the physicochemical properties of the water to enable a fast and targeted transfer of the proton.

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Protein Research Unit Ruhr within Europe (PURE)

Recognising diseases before they become dangerous

A single blood test, a urine sample and all important preventative checkups are complete – this may still be very far off but PURE (Protein Research Unit Ruhr within Europe) is working at it. PURE's mission is the search for disease-indicating proteins, so-called biomarkers, for diseases such as bladder cancer, liver cancer, Alzheimer's or Parkinson's. Just like we humans all have unique fingerprints, every disease also has its own characteristic protein profile. If we detect diseases such as cancer or Alzheimer's at their early, symptomless stage, we could provide a treatment which is much gentler and more successful than today. PURE also develops the most modern bioanalytical techniques, especially protein analytics and vibrational spectroscopic image methods.

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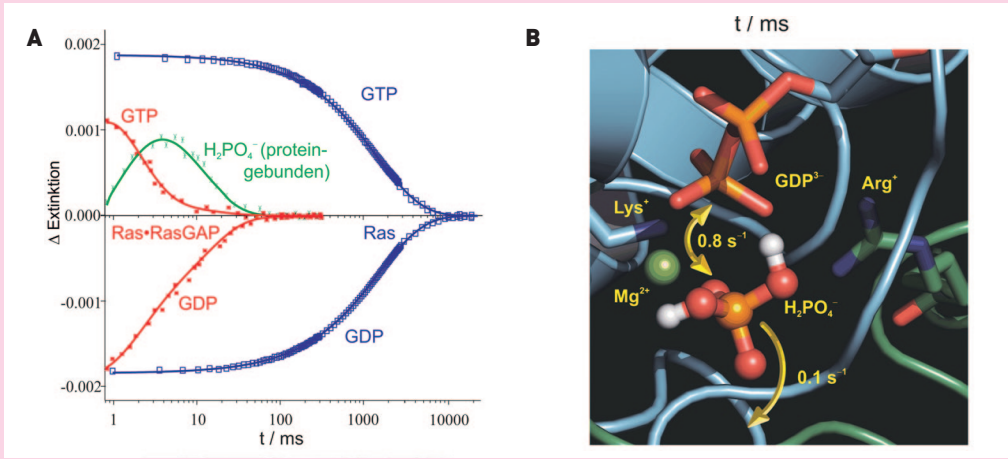

Fig. 4 A) The kinetics of selected marker bands for the GTPase reaction of the Ras protein (blue) and of the Ras·RasGAP (red). The protein-protein interaction of the Ras with the GAP leads to an acceleration of the reaction by a magnitude of 5. Furthermore, the Ras·RasGAP catalysing reaction does not create the product GDP+Pi directly but an intermediate step (green) occurs instead. The structure displayed in B) could be assigned to this due to its spectrum.

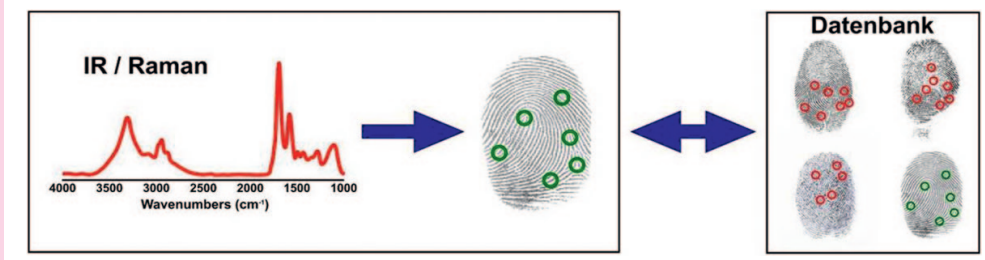

Fig. 5 The vibrational spectrum of a sample (protein, tissue, body fluids, etc.) reflects the biochemical condition. This makes it as characteristic as a fingerprint and allows identification based on a database with a suitable comparison algorithm.

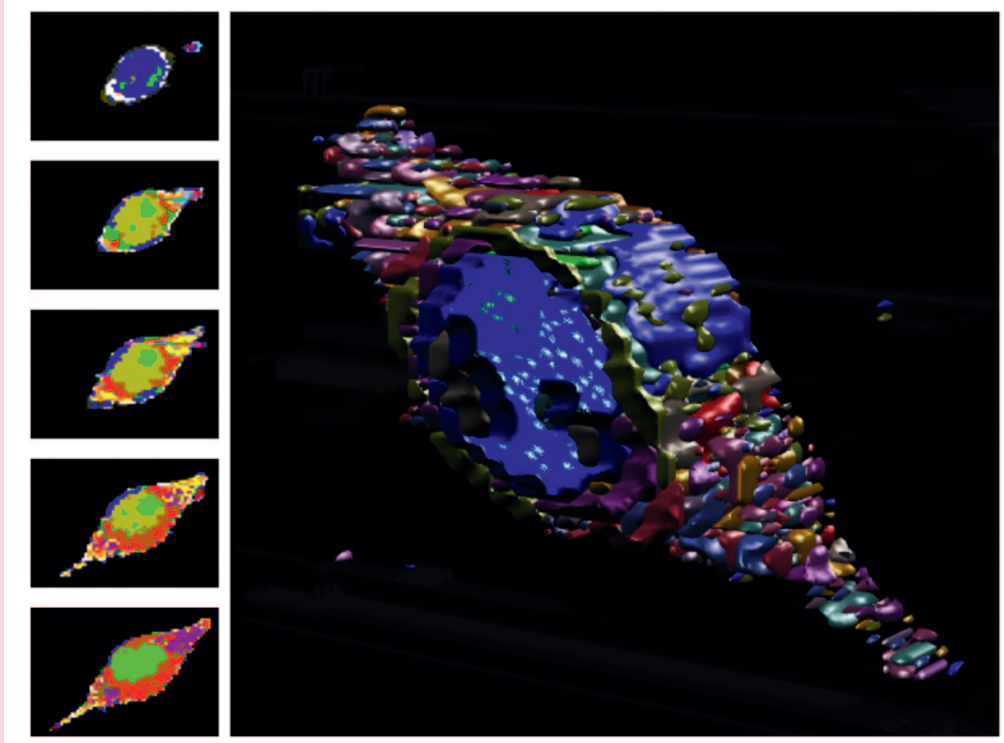

Fig. 6 Left: Confocal Raman microspectrostropic marker-free index images of a living cell. These can be reconstructed into a three-dimension index image of the cell (right).