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Analytical applications of Fourier transform-infrared (FT-IR) spectroscopy in microbiology and prion research

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Abstract

A genuine biophysical method, Fourier transform infrared (FT-IR) spectroscopy has become a versatile research tool in biochemistry and biomedicine. Topical applications in microbiology and prion research are impressive illustrations of the vigorous evolution of the technique. FT-IR spectroscopy has established itself as a powerful method for the rapid differentiation and identification of microorganisms, thereby contributing to both clinical medicine and the prevention of bioterrorism. It has also led to considerable progress in various other fields of basic research, not least in prion sciences. In this field, FT-IR spectroscopy has been increasingly applied as a tool for elucidating structural features of the pathological prion protein, and also to study the molecular changes induced by prions in neuronal tissue and blood. This article sets out to give a review of current examples of the analytical potential of FT-IR spectroscopy in microbiology and prion research.

1. Brief introduction to Fourier transform infrared (FT-IR) spectroscopy

The use of infrared (IR) spectroscopy to examine the conformational structure of polypeptides and proteins dates back to 1950. In the 1970s, researchers started to use this analytical technique for measuring the hydrogen-deuterium exchange to probe protein folding and to study the dynamics of protein conformation. The application of IR spectroscopy was gradually also extended to the analysis of nucleic acids and lipids, and the investigation of carbohydrates (Mantsch, 2001).

An IR spectrum of a sample is produced by scanning the intensity of IR radiation before and after passage through the specimen. The infrared region of the electromagnetic spectrum (see Fig. 1a) extends from the visible to microwave, or very short, radar region. IR radiation originates from thermal emission from a hot source. It

is specified by convention by the "wave number", i.e. the number of waves per centimetre (expressed in the unit [cm^{-1}]), extending from ~10 000 cm^{-1} to 10 cm^{-1}.

The IR spectra of most materials show a large number of absorption bands which originate from the interaction (energy exchange) between discrete light quanta and mechanical motions (vibrational and rotational modes) of the molecules excited by the absorption of IR radiation. Since the constituents of biological specimens are normally present in a condensed phase (solids, liquids or solutions), only vibrational modes are observed with IR spectroscopy for such sample materials (Naumann, 2000). With the introduction of Fourier transform infrared (FT-IR) spectroscopy, the intrinsic drawbacks of classic dispersive IR spectroscopy could be circumvented (for review see Naumann, 2000; Naumann, 2001). Other than classic dispersive IR spectroscopy, FT-IR spectroscopy no longer measures one wavelength after the other, but applies an interferometric modulation of radiation. In FT-IR spectrometers (Fig. 1b) the interference patterns of the modulated signals from interferograms are amplified, digitised, electronically stored and finally transformed into a spectrum by the Fast Fourier transform (FTT) algorithm. Therefore, the Fourier transformation can be considered simply as a mathematical means of extracting the individual frequencies from the interferogram for final representation in an IR spectrum.

2. Analytical potential of FT-IR spectroscopy in biochemical and biomedical research

Characteristic frequencies, intensities, and bandwidths in infrared spectra allow the identification of functional groups of molecules (Table 1) and the characterization of conformationally distinct structures in biological molecules (for review see: Fabian and Mäntele, 2002; Naumann, 2000; Naumann, 2001). IR spectroscopy was one of the

earliest analytical methods identified as a powerful tool to gain information on the structural properties of proteins. As early as 1950 it was shown that there is a close correlation between the position of specific bands in the IR spectrum of polypeptides and their secondary structure. This correlation was later refined by linking the frequencies of structure-sensitive amide bands to specific types of secondary structure such as α-helix, β-sheet, or turn (for review see: (Fabian and Mäntele, 2002). There are nine amide bands in the IR spectrum, called amide A, amide B and amides I-VII, according to decreasing frequency. The amide I band, in which different secondary structure elements such as α-helix, triple helix, β-sheet, β-turn, and extended coil absorb IR light of different wavelengths (Krimm and Bandekar, 1986), turned out to be the most useful band for the analysis of secondary protein structure. The amide I mode in IR spectra, which primarily represents the C=O stretching vibration of the amide groups and occurs in the region between 1600-1700 cm^{-1}, is particularly sensitive to β-sheet structures. The position of IR bands associated with these structures is influenced by a variety of factors, including the strength of hydrogen bonds and the packing of β-strands (Surewicz and Mantsch, 1988; Zandomeneghi et al., 2004).

IR spectra of more complex biological specimens such as microorganisms or tissues represent the superposition of all infrared-active vibrational modes of the various molecules present in the sample. Therefore, IR spectroscopy provides information about the chemical constituents (proteins, lipids, nucleic acids, polysaccharides, etc.) of the probed material and their molecular structure. When applied to intact microbial cells the FT-IR technique provides spectral fingerprints of the complex biological structures under investigation (Naumann, 2000; Naumann, 2001). Furthermore, IR spectroscopy delivers information about pathological chemical

alterations in tissues or body fluids resulting from infections with pathogens or from other animal and human diseases. From a diagnostic perspective, FT-IR spectroscopic fingerprints or "signatures" can be used to discriminate between different types of microorganisms, cells, tissues and body fluids, as well as between diseased and normal biological samples (Schaeberle et al., 2001).

When IR spectroscopy is applied to colonies of microorganisms or tissue sections, the chemistry of small areas or even single cells can be studied *in situ* by spatially resolved infrared microspectrometry, which combines infrared spectroscopy and microscopy. FT-IR microspectrometry does not require additional reagents or stains. Since FT-IR microspectrometry can be performed without tissue homogenization or chemical sample modifications, the spectral and chemical integrity of the examined microorganisms or tissues is not compromised. For the generation of spatially resolved chemical IR maps by FT-IR microspectrometry, a high signal-to-noise ratio is required, together with a highly sensitive detector (Wetzel and Levine, 2001). This holds true particularly if secondary protein structure is to be determined within tissue. Such determination requires subcellular spatial resolution and can only be achieved by a synchrotron IR source emitting highly brilliant radiation.

3. Processing and evaluation of FT-IR data

Biological specimens are often of extreme molecular complexity. Therefore, recent breakthroughs in instrumentation and data processing techniques were needed to establish IR spectroscopy as a useful tool in biomedical research, as well as to exploit the analytical and diagnostic options of IR spectroscopy outlined above. The discrimination between microorganisms, different types of biological sample material (e.g., the basic kinds of mammalian tissues such as epithelial, connective, muscular or

nervous tissue, or between grey and white matter in the brain), and between diseased and normal biological samples, requires a thorough evaluation and comparison for spectral similarities and dissimilarities. For this purpose, high-quality IR spectra are only one prerequisite, which needs to be complemented with data compression and pattern recognition techniques. Multivariate statistical analysis (MSA) can use a variety of methods for the pre-treatment, evaluation and representation of huge and complex bodies of spectral data. Among the methods frequently used for pattern recognition in FT-IR spectroscopy are factor analysis, hierarchical clustering and artificial neural networks (ANNs). For further details the reader is referred to reviews on this subject matter published elsewhere (Naumann, 2000; Naumann, 2001).

4. FT-IR spectroscopic characterization of microorganisms

An impressive number of papers published in the last two decades document that FT-IR spectroscopy can be used to rapidly differentiate and identify microorganisms (Fischer et al., 2006; Helm et al., 1991a; Helm et al., 1991b; Kirschner et al., 2001; Kümmerle et al., 1998; Maquelin et al., 2003; Naumann et al., 1991; Naumann, 2000; Ngo Thi et al., 2003; Oberreuter et al., 2002; Rebuffo et al., 2006; Sandt et al., 2003; Tintelnot et al., 2000; Udelhoven et al., 2000; Wenning et al., 2002). As outlined above, FT-IR probes the total composition of a given biological sample, such as a colony of microorganisms, in one single experiment. For FT-IR analysis of microorganisms, standardized experimental protocols including data acquisition and evaluation procedures have been published and these have given reproducible results (Helm et al., 1991b; Helm et al., 1991a; Naumann, 2000). These standardization efforts have arisen from the need to exchange spectral data between different laboratories and to generate validated reference databases for routine

identifications of microorganisms that have been isolated in different laboratories. Microbial samples suitable for FT-IR investigations can be obtained for instance by taking a smear from solid agar plates. Such samples can then be measured as dried films using dedicated commercially available FT-IR instrumentation.

The FT-IR technique gives results within a few minutes of removing single colonies from a microbial cell culture, and can be uniformly applied to all microorganisms that can be cultivated. To demonstrate this diagnostic potential, Fig. 2a shows the dendrogram of a hierarchical cluster analysis performed on 240 FT-IR spectra of more than 30 strains from quite diverse microorganisms, including different species and strains of Gram-positive and Gram-negative bacteria and yeasts. Hierarchical clustering was applied in order to scrutinize spectral similarities, and specific spectral features were selected to achieve a classification scheme that (i) consistently groups the strains of bacteria in correct genus and species clusters, (ii) gives a separation between the Gram-positive and Gram-negative bacteria, and (iii) achieves a distinct clustering of the yeast species. It has to be emphasized that the FT-IR technique is not restricted to the analysis of bacteria, since yeasts and fungi can be separated as well (Fischer et al., 2006; Sandt et al., 2003). As all cell components depend on the expression of smaller or larger parts of the genome, the FT-IR spectra of microorganisms display a specific phenotypic and genetic fingerprint of the cells under study. This is why the specificity of the technique is generally very high, allowing differentiations even down to the subspecies, serogroup/serotype or strain level. The strain-typing capacity of the technique is also demonstrated by the FT-IR based classification of different *Candida albicans* strains shown in Figure 2b.

Different approaches used to identify unknown microbial strains on the basis of reference databases have been published (Helm et al., 1991a; Helm et al., 1991b;

Kümmerle et al., 1998; Naumann, 2000; Oberreuter et al., 2002; Rebuffo et al., 2006). It is pertinent that these reference data sets contain representative numbers of spectra covering all relevant spectral types to be identified. Identification is then achieved by comparing the IR-spectrum of an unknown microorganism with all entries in the reference database. A validated algorithm to identify unknown strains has been established, which is based on the calculation of so-called differentiation indices (D-values) (Helm et al., 1991a; Helm et al., 1991b; Naumann, 2000). The spectrum is first subdivided into several spectral windows, selected so that they contain the most discriminative spectral information. The combination of these spectral windows is then used in a stepwise correlation procedure to determine the most similar spectrum contained in the database (Helm et al., 1991a; Kümmerle et al., 1998; Naumann, 2000; Oberreuter et al., 2002). IR-reference databases for routine identifications are already commercially available from a FT-IR spectrometer producing company and from the group led by Scherer (Kümmerle et al., 1998; Oberreuter et al., 2002; Rebuffo et al., 2006; Wenning et al., 2002). Among other entries, these libraries contain spectra of different species and strains of listeria, bacilli, coryneform bacteria, lactic acid bacteria and enterobacteria. FT-IR identification libraries that use search algorithms based on optimised artificial neural net analysis are also available (Udelhoven et al., 2000). At present, these comprise (i) six different bacterial genera, each containing a representative number of species and strains within the genera *Pseudomonas, Bacillus, Staphylococcus, Streptococcus, Aeromonas, Mycobacteria* and (ii) two subsets for the identification of different *Candida* species and for the differentiation between fluconazole sensitive and resistant *Candida albicans* strains, respectively.

While it is necessary to isolate pure cultures of the microorganisms in question when they are to be examined using only an FT-IR spectrometer, mixed cultures can

be analysed by measuring microcolonies (50 to 100 µm in diameter) growing separately on solid agar media by means of a light microscope coupled to the FT-IR spectrometer. For such FT-IR microscopic measurements, microcolonies are stamped from the solid agar plates onto an infrared transparent plate. For this purpose, a special replica technique which transfers in a spatially accurate way the first two or three cell layers of the microcolonies can be applied (Naumann, 2000; Ngo Thi et al., 2003; Wenning et al., 2002). The microbial spots transferred to the IR plates can then be measured microspectroscopically using a computer-controlled x,y-stage. This can be either operator- or computer-controlled using video and imaging techniques. The information gained from the light-microscopic data (number of colonies, size, different shapes, etc.) and from the FT-IR spectra of the microcolonies (cell composition, structural data, type-specific FT-IR fingerprints) allows the differentiation and classification of clusters consisting of only a small number of microbial cells even from mixed cultures ($<10^3$ cells per colony spot), and the characterization of colony growth. Figure 3 shows an example of combined light-microscopic and microspectroscopic measurements of a mixed culture containing three different types of microorganisms. Figure 3a shows a light microscopic representation of three different microcolonies obtained with the stamping technique, indicating the presence of three different types of microorganisms in the mixed culture. In a second step, after sampling sufficient numbers of FT-IR spectra from the microcolonies, and after subjecting these spectra to cluster analysis, unequivocal differentiation and identification of the three different types of microorganisms was achieved (Fig. 3b). The main advantages of FT-IR spectroscopy compared to other techniques for the differentiation and identification of microorganisms are its rapidity, its uniform applicability to diverse microorganisms, and a high specificity which

allows differentiations down to subspecies and often even down to the strain level. A particular strength of the FT-IR technique is the ability to perform epidemiological case studies and large screening experiments very quickly. Additional fields of application are the elucidation of infection chains, therapy control, maintenance of strain collections and the differentiation of microorganisms from the environment for which established systems are not yet available. Last but not least, the FT-IR technique requires only small quantities of reagents and other consumables, and is computer compatible. The latter feature greatly promotes a fast electronic exchange of results and databases. Spurred by the advances already made, FT-IR microscopy is now heading towards being used in microbial diagnostics in fully automated systems which combine detection, enumeration, differentiation and identification of microorganisms, and which provide results within one working day (Maquelin et al., 2003).

5. FT-IR spectroscopic findings on molecular alterations in pathological prion protein, neuronal tissue and blood from prion-infected individuals

Prions, the causative agents of transmissible spongiform encephalopathies (TSEs), trigger a pathogenic conversion of cellular prion protein (PrP^{C}) into a pathologically misfolded form (PrP^{Sc}) which is associated with infectivity. During the course of infection, they cause pathological changes such as vacuolation, glial activation and neuronal loss to the central nervous system (CNS). It can be assumed that any alteration in affected tissue caused by prions is accompanied by TSE-specific compositional and structural modifications at the molecular level. FT-IR spectroscopy provides a promising biophysical tool for probing such molecular alterations in pathological prion protein, neuronal tissue and blood from TSE-infected mammalians,

and can thereby contribute to further elucidating the complex pathogenesis of prion diseases.

6. Secondary structure analysis of disease-associated misfolded prion protein using FT-IR spectroscopy

The "protein-only model" of the prion hypothesis postulates that TSE agents replicate through a molecular mechanism in which abnormally folded pathological prion protein (PrP^{Sc}) acts as a catalyst or template nucleus which recruits normal prion protein (PrP^{C}) and transforms the cellular prion protein into its own "infectious" spatial structure (Prusiner, 1982; Prusiner, 1998). Furthermore, the prion hypothesis holds that phenotypic features of TSE strains are encoded in the secondary, tertiary, or quaternary structure of PrP^{Sc}, or in its specific glycosylation. FT-IR spectroscopy had a significant impact on the evolution of this aetiological concept. Although it had previously been shown that the amino acid sequences of PrP^{Sc} and PrP^{C} are identical (for review see Prusiner, 1998), FT-IR spectroscopic analyses revealed that the two protein isoforms differ in their spatial structure. While PrP^{C} contains about 42% α-helix and only 3% β-sheet, PrP^{Sc} has 30% α-helix and 43% β-sheet (Pan et al., 1993). For PrP27-30, the protease-resistant core of PrP^{Sc}, a content of 47% β-sheet and 17% α-helix was reported by Caughey et al. (Caughey et al., 1991), while Gasset et al., (Gasset et al., 1993) found 54% β-sheet and 25% α-helix. Subsequently, Caughey et al. (Caughey et al., 1998) demonstrated through IR spectroscopy that PrP^{Sc} extracts from two distinct strains of hamster-adapted transmissible mink encephalopathy (TME), Hyper (HY)- and Drowsy (DY)-TME, exhibit different β-sheet structures. This is consistent with conformational differences revealed by a conformation-dependent immunoassay (CDI), which was able to differentiate pathological prion

protein from HY- and DY-TME and six other hamster-adapted TSE agents (Safar et al., 1998). FT-IR findings from studies by Thomzig et al. (Thomzig et al., 2004) and Spassov et al. (Spassov et al., 2006) have recently confirmed and expanded on the observations by Caughey et al. (Caughey et al., 1998) with a different set of scrapie strains (263K, ME7-H, 22A-H), and a hamster-adapted BSE isolate (BSE-H). Two of the passaged agents, 22A-H and ME7-H, were found, after different incubation times, to cause TSEs with indistinguishable neurological and behavioural clinical symptoms, indistinguishable lesion profiles, and also electrophoretic mobilities or glycosylation patterns indistinguishable from PrP27-30. Even when a PET blot analysis of the cerebral PrP^{Sc} deposition or Western blot typing of PrP27-30 after proteinase K (PK) digestion at different pH values were performed, a reliable discrimination between these two strains was not possible. However, all four isolates including ME7-H and 22A-H, could be clearly differentiated by FT-IR spectroscopic characterization of their pathological prion protein (Fig. 4, Table 2). The second derivative spectra obtained from D_2O suspensions of PrP27-30 extracted from 263K-, 22A-H-, ME7-H-, or BSE-H-infected hamster brains displayed consistent structural differences, mainly in intra- and intermolecular β-sheets, but also in non β-sheet-related features of secondary structure. These findings demonstrated that phenotypic information of TSE strains is mirrored in β-sheet and other secondary structure elements of PrP^{Sc}/PrP27-30, including in cases where immunobiochemical typing of PrP failed to detect structural differences. Furthermore, they showed that FT-IR characterization of PrP^{Sc}/PrP27-30 may provide a versatile tool for molecular strain typing without antibodies and without restrictions to specific TSEs or mammalian species.

7. TSE-induced molecular alterations in neuronal tissue revealed in situ by FT-IR microspectroscopy

FT-IR microspectrometry offers a relatively new approach for detecting spatially resolved TSE-induced compositional and structural changes in tissue of the nervous system that has been pursued in order to add biochemical information to known neuropathological parameters (Kretlow et al., 2006). The first report on a spatially resolved *in situ* FT-IR spectroscopic examination of TSE-infected brain sections was published a few years ago by Kneipp et al. (Kneipp et al., 2000). In this study, FT-IR microspectroscopy was performed on brain specimens from hamsters clinically affected with 263K scrapie, and the assignment of individual spectra from spectral maps to specific cerebellar tissue structures allowed the comparison of FT-IR spectra from topologically corresponding brain substructures of scrapie-infected and uninfected hamsters. This turned out to be crucial for the analysis, since it emerged in the study that spectral differences between distinct cerebellar substructures were generally much larger than those identified between identical cerebellar tissue structures in normal and scrapie specimens. The FT-IR microspectroscopic comparison of identical cerebellar tissue structures in scrapie-infected and control brains revealed consistent alterations in membrane state-of-order, protein composition, and carbohydrate as well as nucleic acid constituents in cerebellar samples from intraperitoneally challenged scrapie hamsters. The newly established FT-IR microspectroscopic methodology was subsequently used by Kneipp et al. (Kneipp et al., 2002) for scrutinizing the initial sites of PrP^{Sc} deposition, i.e. the dorsal motor nucleus of the vagus nerve (DMNV) and the commissural solitary tract nucleus (SN) (Beekes et al., 1998; McBride et al., 2001). These studies on brain stem sections from preclinically infected hamsters perorally challenged with scrapie showed spectral

differences between normal and infected animals as early as 90 days after infection (i. e. at ~ 60% of the incubation period), long before the onset of clinical symptoms. The spectral alterations indicated changes in carbohydrates, nucleic acids and membrane constituents early in pathogenesis, and were found to provide a new biophysical parameter, based on molecular tissue changes occurring in addition to PrP^{Sc} deposition, that reflected the spread of cerebral scrapie pathology starting in the DMNV and SN. While the two FT-IR microspectrometric studies discussed above found consistent spectral changes in TSE-affected nervous tissue, they were not able to detect a PrP^{Sc}-associated increase of the β-sheet content in protein structure *in situ*. However, this could be accomplished when synchrotron-based IR microspectroscopic imaging was performed on cryo-sections from dorsal root ganglia (DRGs; Fig. 5) of terminally ill hamsters perorally challenged with 263K scrapie (Kneipp et al., 2003; Kretlow et al., 2006). It had previously been shown by FT-IR spectroscopy and circular dichroism, that PrP^{Sc} or PrP27-30 extracted from tissue has a significantly increased proportion of β-sheet structure compared to PrP^{C} (Baldwin et al., 1994; Caughey et al., 1998; Caughey et al., 1991; Gasset et al., 1993; Pan et al., 1993). By generating IR maps of intact sections from scrapie-infected DRGs based on the shift in the frequency of the amide I band, it became possible to monitor, in a spatially resolved manner, protein conformational changes across the sample. This confirmed *in situ* a decreased α-helical and an elevated β-sheet content in subcellular areas of neurons with prominent PrP^{Sc} deposition. Synchrotron FT-IR maps of brain sections from patients with Alzheimer's disease had revealed similar spectral shifts, indicating increased β-sheet structure in classic amyloid plaques (Choo et al., 1996). Taken together, these biophysical data provide direct molecular evidence showing that protein misfolding involving the formation of β-sheets *in situ* is a common

pathogenetic feature in prion and Alzheimer' disease. Binding of metal ions such as copper, zinc, manganese or iron has been suggested as being potentially critical for PrP misfolding and aggregation (Jobling et al., 2001; Kim et al., 2000; Miura et al., 1996; Purdey, 1996a; Purdey, 1996b; Purdey, 2000; Stockel et al., 1998). Therefore, FT-IR microspectroscopic detection of PrP-related and other molecular changes in neurons of tissue sections needs to be correlated with *in situ* measurements of local metal ion concentrations by x-ray fluorescence (XRF) microprobing. Initial experiments have recently demonstrated the feasibility of this approach (Kühbacher et al., 2005; Wang et al., 2005). One of these studies (Wang et al., 2005) showed a co-localization of elevated iron levels and PrP^{Sc} in neurons of DRG sections from hamsters perorally challenged with 263K scrapie.

8. IR spectroscopic search for molecular markers of TSE infection in blood

The identification of molecular markers for prion infections in blood would be of relevance both for a better understanding of TSE pathophysiology and for the development of blood tests for scrapie, BSE or vCJD. TSE infectivity, albeit at low levels, has been detected using bioassays in whole blood or blood fractions from TSE-infected sheep, mice, hamsters, guinea pigs and primates (for review see Brown, 2005). Furthermore, three cases of putative iatrogenic vCJD caused by blood transfusion have been reported (Llewelyn et al., 2004; Peden et al., 2004; UK Health Protection Agency, 2006). Infectivity *per se* is a biological, not a molecular entity. However, according to the prion hypothesis and a wealth of experimental findings, prion infectivity is composed essentially – if not entirely – of PrP^{Sc}. Therefore, if TSE infectivity can be detected in blood, PrP^{Sc} (or other infectious forms of misfolded prion protein) should also be present. Indeed, the detection of pathological prion

375 protein in the blood of scrapie-infected hamsters by protein misfolding cyclic
376 amplification (PMCA) has been reported recently (Castilla et al., 2005; Saa et al.,
377 2006).

378 Further TSE-associated molecular alterations in blood may result from a host
379 defense response, or from pathological effects of the infection on the organism. Such
380 changes would provide haematological surrogate markers which may, or may not, be
381 specific to prion diseases.

382 The presence of chemical markers for TSE infection in serum has also been
383 examined by FT-IR spectroscopy. Serum samples from scrapie-infected and control
384 hamsters were analysed with FT-IR spectroscopy and artificial neural networks for
385 differences in their spectral fingerprints or "signatures" (Schmitt et al., 2002). This
386 revealed that the terminal stage of scrapie is associated with compositional and/or
387 structural alterations of serum constituents, independently from whether the hamsters
388 were infected via the intracerebral, intraperitoneal or peroral route. With an analytical
389 test sensitivity and specificity of 97% and 100% respectively, the findings
390 demonstrated that FT-IR spectroscopy and artificial neural network (ANN) analysis
391 may provide a new approach for the identification of scrapie and other TSEs from
392 blood. Subsequent investigations have confirmed that the technique can also achieve
393 high sensitivities and specificities when applied to BSE testing of field serum samples
394 from cattle. Lasch et al. (2003; Fig. 6) reported a test sensitivity and specificity of 96%
395 and 92% respectively, after having examined bovine sera from more than 800 animals
396 (including BSE-positives, healthy controls and animals suffering from classic viral or
397 bacterial infections). Martin et al. (Martin et al., 2004) achieved a test accuracy of ~
398 94% with sera from 84 BSE cases and 76 control cattle. Thus, FT-IR spectroscopic
399 pattern recognition in bovine sera has been established independently in different

laboratories as a method that is able to identify BSE-related molecular changes in bovine blood. Recently, the FT-IR spectroscopic approach was also used in a time-course study with hamsters which were perorally inoculated with the 263K scrapie agent, or mock infected with normal hamster brain homogenate (Lasch et al., 2006). Sera from these animals were obtained at preclinical stages of incubation after 70, 100 and 130 days post infection (dpi), and at the terminal stage of scrapie (160 ± 10 dpi). The analysis of these sera using FT-IR spectroscopy and ANNs confirmed the earlier findings reported by Schmitt et al. (Schmitt et al., 2002) and revealed subtle, but reproducible, spectral variations that allowed the detection of preclinical scrapie infection at 100 dpi or later, but not yet at 70 dpi. Interestingly, the IR spectral features characteristic for preclinical stages of infection differed from those observed in sera from terminally ill donors. The approach by Lasch et al. (Lasch et al., 2006) yielded an accuracy of 93% which also confirmed the presence of scrapie-associated molecular changes in serum for preclinical stages of incubation. Taken together, the experimental results of this study suggested that the composition of serum proteins, or polypeptides, is characteristically altered in hamsters preclinically infected with scrapie. When Carmona et al. (Carmona et al., 2005) examined membranous fractions from leucocytes of scrapie-infected sheep and non-infected control animals using infrared spectroscopy, they were similarly able to identify clinically and preclinically infected animals. However, other than in the study by Lasch et al. (Lasch et al., 2006) spectral bands generated by β-sheets significantly contributed to the differentiation between infected and control animals. The molecular basis of this finding remains to be established since the analytical sensitivity of IR spectroscopy appears rather unlikely to allow detection of the low levels of PrP^{Sc} to be expected in blood or blood fractions (Lasch et al., 2006). However, these analyses and those undertaken by and Carmona et

al. (Carmona et al., 2005) were performed on different animal species, which may account for the differences observed in the two studies. In any case, the identification of TSE-associated molecular markers in blood constitutes an ongoing challenging task which could be successfully addressed in the future by a combination of biochemical separation techniques such as high performance liquid chromatography (HPLC) and biophysical approaches such as mass spectroscopy.

9. Outlook: FT-IR spectroscopic applications in prion research

FT-IR spectroscopy has established itself as a versatile biophysical research tool for the investigation and detection of TSE-induced molecular changes in neuronal tissue, blood and pathological prion protein from TSE-infected individuals. However, the data obtained from the FT-IR approaches described above have raised a variety of questions to be addressed in future studies. The concerted use, in various combinations, of biochemical and immunohistological PrP analysis, FT-IR (micro)spectroscopy, and other biophysical techniques such as XRF microprobing and mass spectroscopy will be helpful for achieving further insights into the complex etiopathogenesis of TSEs – a group of diseases that still seems to be puzzling in in a number of respects.

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638 **Table 1: Tentative assignment of some bands frequently found in biological FT-**

639 **IR spectra.**

Frequency (cm^{-1})	Assignment
~ 3500	O-H str of hydroxyl groups
~ 3200	N-H str (amide A) of proteins
2959	C-H str (asym) of $-CH_3$
2934	C-H str (asym) of $>CH_2$
2921	C-H str (asym) of $>CH_2$ in fatty acids
2898	C-H str of $\rightarrow$C-H methine
2872	C-H str (sym) of $-CH_3$
2852	C-H str (sym) of $>CH_2$ in fatty acids
1741-1715	>C=O str of esters >C=O str of carbonic acids, nucleic acids
~ 1695	Amide I band components
~ 1685	resulting from antiparallel pleated sheets and
~ 1675	β-turns of proteins
~ 1655	Amide I of α-helical structures
~ 1637	Amide I of β-pleated sheet structures
1548	Amide II
1515	"Tyrosine" band
1468	C-H def of $>CH_2$
~ 1400	C=O str (sym) of COO^-
1310-1240	Amide III band components of proteins
1250-1220	P=O str (asym) of $>PO_2^-$ phospodiesters
1200-900	C-O-C, C-O dominated by ring vibrations of carbohydrates C-O-P, P-O-P
1085	P=O str (sym) of $>PO_2^-$
720	C-H rocking of $>CH_2$
900-600	"Fingerprint region"

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641 (Peak frequencies have been obtained from second derivative spectra). Abbreviations:

642 asym = asymmetric; sym = symmetric; str = stretching; def = deformation

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644 **Table 2: Comparison of secondary structure characteristics observed with FT-IR**
645 **spectroscopy of PrP27-30 from brains of hamsters infected with 263K, ME7-H,**
646 **22A-H, and BSE-H agent.**
647

Strain	Structural components peak positions in cm^{-1}					
	β-sheet (low frequency)		Unassigned structure	α- Helix	Turns	Turns / β-sheet (high frequency)
263K	1620-21	1637	-	1656	1671	-
ME7-H	1620-21	1634	-	1658	1671	1679
22A-H	1620	1630	1642	1657	1670	-
BSE-H	1620	1632	1647	1659	1670	1677

648
649 Findings for hydrated stage in D_20.
650

Figure legends:

Fig. 1a: The electromagnetic spectrum

Fig. 1b: FT-IR spectrometer.

(a) Schematic representation of the basic components of an FT-IR spectrometer. (b) Working principle of a Michelson interferometer consisting of a light source, beam splitter, fixed mirror, moving mirror, detector and a sample (upper panel). A single frequency light source (central panel, left) is modulated to a sinusoidal signal recorded by the detector (central panel, right). A white-light source is transformed to the interferogram (lower panel). Reproduced from Naumann (2000).

Fig. 2: Typical classification schemes obtained with FT-IR spectroscopic analysis of intact microbial cells.

(a) Dendrogram of a hierarchical cluster analysis performed on 240 spectra of different strains of Gram-positive, Gram-negative bacteria and yeasts. Cluster analysis was performed using the first derivatives, over the spectral ranges 3000-2800, 1500-1400, and 1200-900 cm^{-1}. Spectral ranges were equally weighted and Ward's algorithm was applied (Helm et al., 1991b; Naumann, 2000). (b) Dendrogram of a hierarchical cluster analysis performed on different strains of *Candida albicans*. Cluster analysis was performed using the first derivatives, involving the spectral ranges 891-859, 761-735, 991-889, and 1401-1369 cm^{-1}. Spectral ranges were equally weighted and Ward's algorithm was applied. The Pearson's correlation coefficient (defined as "D-value", see equation in (Helm et al., 1991b; Naumann, 2000) was used to calculate the distance matrices as input for cluster analysis.

Fig. 3: Detection and FT-IR microspectroscopic classification of different microbial microcolonies.

(a) Micrograph (magnification approximately 200x) of three different colony spots deposited on BaF_2 windows by the stamping technique described in the text. (b) FT-IR spectra of the microcolonies shown in (a). (c) Hierarchical cluster analysis of IR measurements of approximately 30 different colony spots. The clusters suggested by hierarchical clustering represent: C_1 = *Staphylococcus aureus* (strain RKI/WG PS42D); C_2 = *Streptococcus faecalis* (strain DSM 20371); C_3 = *Escherichia coli* (strain RKI A139).

Fig. 4: FT-IR spectroscopic characterization of PrP27-30.

FT-IR spectra (second derivatives) in the amide I absorption region obtained from different hamster-adapted scrapie strains (263K, ME7-H, 22A-H) and a hamster-adapted BSE-isolate (BSE-H). Samples were hydrated in D_2O. The overlaid spectra represent measurements of samples produced in independent extraction and purification procedures.

Fig. 5: *In situ* identification of structural protein changes in prion-infected tissue.

(a) Sections of dorsal root ganglia from a scrapie-infected (A) and a control (B) hamster stained with the PrP specific antibody 3F4. Arrows indicate areas with a high PrP^{Sc} deposition. The pathological prion protein can be detected as dark brown dots in most, but not all, of the cells (see asterisked cells). Since 3F4 stains both PrP^{Sc} and PrP^{C}, B shows a 3F4-stained control slide as an example of the PrP^{C} distribution (brown colour) (scale bars, 50 μm). (b) IR map and spectra of a dorsal

root ganglion from a terminally diseased scrapie-hamster. Right picture shows a photomicrograph (scale bar, 50 μm) with the IR mapped area overlaid. The ratio of the β-sheet to α-helical intensities calculated from the absorbance at 1637 cm^{-1} and 1657 cm^{-1}, respectively, is shown as a function of pixel location. Red and yellow indicate areas with a relatively higher β-sheet content than spectra from green and blue areas. Examples of amide I band shapes of spectra from areas with relatively higher (red) and lower (green) β-sheet content are shown on the top left (original spectra, normalized between 1400 and 1800 cm^{-1}) and bottom left (corresponding second derivative spectra).

Fig. 6: Typical infrared absorbance spectra (1, 3) and the corresponding second derivatives (2, 4) of sera originating from BSE-negative and BSE-positive animals.

Traces 1-4 have been normalized and are calculated by averaging spectra obtained from 452 BSE-negative (1, 2) or 249 BSE-positive (3, 4) animals, respectively. Trace 5 (blue) represents the difference spectrum of the second derivatives (4, BSE positive; and 2, BSE negative). Traces 6 and 7 are the two standard deviation spectra of the classes BSE positive (6) and BSE negative (7) (which are nearly identical in this case). Note that the y-axis of difference spectrum 5 and the standard deviation spectra (6, 7) is magnified by a factor of 20.