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1

2 Analytical applications of Fourier transform-infrared (FT-IR)

3 spectroscopy in microbiology and prion research

4

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6

7 ¹ P24 – Transmissible Spongiform Encephalopathies, ² P25 – Biomedical Spectroscopy

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10 *Running title:* FT-IR spectroscopy in microbiology and prion research

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24 *Key words:* Microorganisms, prion, prion diseases, prion protein, transmissible

25 spongiform encephalopathies, Fourier transform-infrared (FT-IR) spectroscopy

26 **Abstract**

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

39

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

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

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

51 is specified by convention by the “wave number”, i.e. the number of waves per
52 centimetre (expressed in the unit [cm^{-1}]), extending from $\sim 10\,000\text{ cm}^{-1}$ to 10 cm^{-1} .

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

69

70 **2. Analytical potential of FT-IR spectroscopy in biochemical and biomedical** 71 **research**

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

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

93 IR spectra of more complex biological specimens such as microorganisms or
94 tissues represent the superposition of all infrared-active vibrational modes of the
95 various molecules present in the sample. Therefore, IR spectroscopy provides
96 information about the chemical constituents (proteins, lipids, nucleic acids,
97 polysaccharides, etc.) of the probed material and their molecular structure. When
98 applied to intact microbial cells the FT-IR technique provides spectral fingerprints of
99 the complex biological structures under investigation (Naumann, 2000; Naumann,
100 2001). Furthermore, IR spectroscopy delivers information about pathological chemical

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

106 When IR spectroscopy is applied to colonies of microorganisms or tissue
107 sections, the chemistry of small areas or even single cells can be studied *in situ* by
108 spatially resolved infrared microspectrometry, which combines infrared spectroscopy
109 and microscopy. FT-IR microspectrometry does not require additional reagents or
110 stains. Since FT-IR microspectrometry can be performed without tissue
111 homogenization or chemical sample modifications, the spectral and chemical integrity
112 of the examined microorganisms or tissues is not compromised. For the generation of
113 spatially resolved chemical IR maps by FT-IR microspectrometry, a high signal-to-
114 noise ratio is required, together with a highly sensitive detector (Wetzel and Levine,
115 2001). This holds true particularly if secondary protein structure is to be determined
116 within tissue. Such determination requires subcellular spatial resolution and can only
117 be achieved by a synchrotron IR source emitting highly brilliant radiation.

118

119 **3. Processing and evaluation of FT-IR data**

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

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

136

137 **4. FT-IR spectroscopic characterization of microorganisms**

138 An impressive number of papers published in the last two decades document
139 that FT-IR spectroscopy can be used to rapidly differentiate and identify
140 microorganisms (Fischer et al., 2006; Helm et al., 1991a; Helm et al., 1991b;
141 Kirschner et al., 2001; Kümmerle et al., 1998; Maquelin et al., 2003; Naumann et al.,
142 1991; Naumann, 2000; Ngo Thi et al., 2003; Oberreuter et al., 2002; Rebuffo et al.,
143 2006; Sandt et al., 2003; Tintelnot et al., 2000; Udelhoven et al., 2000; Wenning et al.,
144 2002). As outlined above, FT-IR probes the total composition of a given biological
145 sample, such as a colony of microorganisms, in one single experiment. For FT-IR
146 analysis of microorganisms, standardized experimental protocols including data
147 acquisition and evaluation procedures have been published and these have given
148 reproducible results (Helm et al., 1991b; Helm et al., 1991a; Naumann, 2000). These
149 standardization efforts have arisen from the need to exchange spectral data between
150 different laboratories and to generate validated reference databases for routine

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

155 The FT-IR technique gives results within a few minutes of removing single
156 colonies from a microbial cell culture, and can be uniformly applied to all
157 microorganisms that can be cultivated. To demonstrate this diagnostic potential, Fig.
158 2a shows the dendrogram of a hierarchical cluster analysis performed on 240 FT-IR
159 spectra of more than 30 strains from quite diverse microorganisms, including different
160 species and strains of Gram-positive and Gram-negative bacteria and yeasts.
161 Hierarchical clustering was applied in order to scrutinize spectral similarities, and
162 specific spectral features were selected to achieve a classification scheme that (i)
163 consistently groups the strains of bacteria in correct genus and species clusters, (ii)
164 gives a separation between the Gram-positive and Gram-negative bacteria, and (iii)
165 achieves a distinct clustering of the yeast species. It has to be emphasized that the FT-
166 IR technique is not restricted to the analysis of bacteria, since yeasts and fungi can be
167 separated as well (Fischer et al., 2006; Sandt et al., 2003). As all cell components
168 depend on the expression of smaller or larger parts of the genome, the FT-IR spectra
169 of microorganisms display a specific phenotypic and genetic fingerprint of the cells
170 under study. This is why the specificity of the technique is generally very high,
171 allowing differentiations even down to the subspecies, serogroup/serotype or strain
172 level. The strain-typing capacity of the technique is also demonstrated by the FT-IR
173 based classification of different *Candida albicans* strains shown in Figure 2b.

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

176 Kümmerle et al., 1998; Naumann, 2000; Oberreuter et al., 2002; Rebuffo et al., 2006).
177 It is pertinent that these reference data sets contain representative numbers of spectra
178 covering all relevant spectral types to be identified. Identification is then achieved by
179 comparing the IR-spectrum of an unknown microorganism with all entries in the
180 reference database. A validated algorithm to identify unknown strains has been
181 established, which is based on the calculation of so-called differentiation indices (D-
182 values) (Helm et al., 1991a; Helm et al., 1991b; Naumann, 2000). The spectrum is first
183 subdivided into several spectral windows, selected so that they contain the most
184 discriminative spectral information. The combination of these spectral windows is
185 then used in a stepwise correlation procedure to determine the most similar spectrum
186 contained in the database (Helm et al., 1991a; Kümmerle et al., 1998; Naumann, 2000;
187 Oberreuter et al., 2002). IR-reference databases for routine identifications are already
188 commercially available from a FT-IR spectrometer producing company and from the
189 group led by Scherer (Kümmerle et al., 1998; Oberreuter et al., 2002; Rebuffo et al.,
190 2006; Wenning et al., 2002). Among other entries, these libraries contain spectra of
191 different species and strains of listeria, bacilli, coryneform bacteria, lactic acid bacteria
192 and enterobacteria. FT-IR identification libraries that use search algorithms based on
193 optimised artificial neural net analysis are also available (Udelhoven et al., 2000). At
194 present, these comprise (i) six different bacterial genera, each containing a
195 representative number of species and strains within the genera *Pseudomonas*, *Bacillus*,
196 *Staphylococcus*, *Streptococcus*, *Aeromonas*, *Mycobacteria* and (ii) two subsets for the
197 identification of different *Candida* species and for the differentiation between
198 fluconazole sensitive and resistant *Candida albicans* strains, respectively.

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

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

226 allows differentiations down to subspecies and often even down to the strain level. A
227 particular strength of the FT-IR technique is the ability to perform epidemiological
228 case studies and large screening experiments very quickly. Additional fields of
229 application are the elucidation of infection chains, therapy control, maintenance of
230 strain collections and the differentiation of microorganisms from the environment for
231 which established systems are not yet available. Last but not least, the FT-IR
232 technique requires only small quantities of reagents and other consumables, and is
233 computer compatible. The latter feature greatly promotes a fast electronic exchange of
234 results and databases. Spurred by the advances already made, FT-IR microscopy is
235 now heading towards being used in microbial diagnostics in fully automated systems
236 which combine detection, enumeration, differentiation and identification of
237 microorganisms, and which provide results within one working day (Maquelin et al.,
238 2003).

239

240 **5. FT-IR spectroscopic findings on molecular alterations in pathological prion** 241 **protein, neuronal tissue and blood from prion-infected individuals**

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

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

253

254 **6. Secondary structure analysis of disease-associated misfolded prion protein** 255 **using FT-IR spectroscopy**

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

276 protein from HY- and DY-TME and six other hamster-adapted TSE agents (Safar et
277 al., 1998). FT-IR findings from studies by Thomzig et al. (Thomzig et al., 2004) and
278 Spassov et al. (Spassov et al., 2006) have recently confirmed and expanded on the
279 observations by Caughey et al. (Caughey et al., 1998) with a different set of scrapie
280 strains (263K, ME7-H, 22A-H), and a hamster-adapted BSE isolate (BSE-H). Two of
281 the passaged agents, 22A-H and ME7-H, were found, after different incubation times,
282 to cause TSEs with indistinguishable neurological and behavioural clinical symptoms,
283 indistinguishable lesion profiles, and also electrophoretic mobilities or glycosylation
284 patterns indistinguishable from PrP^{Sc}/PrP27-30. Even when a PET blot analysis of the
285 cerebral PrP^{Sc} deposition or Western blot typing of PrP27-30 after proteinase K (PK)
286 digestion at different pH values were performed, a reliable discrimination between
287 these two strains was not possible. However, all four isolates including ME7-H and
288 22A-H, could be clearly differentiated by FT-IR spectroscopic characterization of their
289 pathological prion protein (Fig. 4, Table 2). The second derivative spectra obtained
290 from D₂O suspensions of PrP27-30 extracted from 263K-, 22A-H-, ME7-H-, or BSE-
291 H-infected hamster brains displayed consistent structural differences, mainly in intra-
292 and intermolecular β -sheets, but also in non β -sheet-related features of secondary
293 structure. These findings demonstrated that phenotypic information of TSE strains is
294 mirrored in β -sheet and other secondary structure elements of PrP^{Sc}/PrP27-30,
295 including in cases where immunobiochemical typing of PrP failed to detect structural
296 differences. Furthermore, they showed that FT-IR characterization of PrP^{Sc}/PrP27-30
297 may provide a versatile tool for molecular strain typing without antibodies and without
298 restrictions to specific TSEs or mammalian species.

299

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

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

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

350 pathogenetic feature in prion and Alzheimer' disease. Binding of metal ions such as
351 copper, zinc, manganese or iron has been suggested as being potentially critical for
352 PrP misfolding and aggregation (Jobling et al., 2001; Kim et al., 2000; Miura et al.,
353 1996; Purdey, 1996a; Purdey, 1996b; Purdey, 2000; Stockel et al., 1998). Therefore,
354 FT-IR microspectroscopic detection of PrP-related and other molecular changes in
355 neurons of tissue sections needs to be correlated with *in situ* measurements of local
356 metal ion concentrations by x-ray fluorescence (XRF) microprobing. Initial
357 experiments have recently demonstrated the feasibility of this approach (Kühbacher et
358 al., 2005; Wang et al., 2005). One of these studies (Wang et al., 2005) showed a co-
359 localization of elevated iron levels and PrP^{Sc} in neurons of DRG sections from
360 hamsters perorally challenged with 263K scrapie.

361

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

363 The identification of molecular markers for prion infections in blood would be
364 of relevance both for a better understanding of TSE pathophysiology and for the
365 development of blood tests for scrapie, BSE or vCJD. TSE infectivity, albeit at low
366 levels, has been detected using bioassays in whole blood or blood fractions from TSE-
367 infected sheep, mice, hamsters, guinea pigs and primates (for review see Brown,
368 2005). Furthermore, three cases of putative iatrogenic vCJD caused by blood
369 transfusion have been reported (Llewelyn et al., 2004; Peden et al., 2004; UK Health
370 Protection Agency, 2006). Infectivity *per se* is a biological, not a molecular entity.
371 However, according to the prion hypothesis and a wealth of experimental findings,
372 prion infectivity is composed essentially – if not entirely – of PrP^{Sc}. Therefore, if TSE
373 infectivity can be detected in blood, PrP^{Sc} (or other infectious forms of misfolded
374 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

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

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

431

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

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

443

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449

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- 636
- 637
- 638

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 →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 ⁻ phosphodiester
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"

640

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

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

643

644

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_2O .

650

650 **Figure legends:**

651

652 **Fig. 1a: The electromagnetic spectrum**

653

654 **Fig. 1b: FT-IR spectrometer.**

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

661

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

664 (a) Dendrogram of a hierarchical cluster analysis performed on 240 spectra of
665 different strains of Gram-positive, Gram-negative bacteria and yeasts. Cluster analysis
666 was performed using the first derivatives, over the spectral ranges 3000-2800, 1500-
667 1400, and 1200-900 cm^{-1} . Spectral ranges were equally weighted and Ward's
668 algorithm was applied (Helm et al., 1991b; Naumann, 2000). (b) Dendrogram of a
669 hierarchical cluster analysis performed on different strains of *Candida albicans*.
670 Cluster analysis was performed using the first derivatives, involving the spectral
671 ranges 891-859, 761-735, 991-889, and 1401-1369 cm^{-1} . Spectral ranges were equally
672 weighted and Ward's algorithm was applied. The Pearson's correlation coefficient
673 (defined as "D-value", see equation in (Helm et al., 1991b; Naumann, 2000) was used
674 to calculate the distance matrices as input for cluster analysis.

675

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

678 (a) Micrograph (magnification approximately 200x) of three different colony spots
679 deposited on BaF₂ windows by the stamping technique described in the text. (b) FT-IR
680 spectra of the microcolonies shown in (a). (c) Hierarchical cluster analysis of IR
681 measurements of approximately 30 different colony spots. The clusters suggested by
682 hierarchical clustering represent: C₁ = *Staphylococcus aureus* (strain RKI/WG
683 PS42D); C₂ = *Streptococcus faecalis* (strain DSM 20371); C₃ = *Escherichia coli*
684 (strain RKI A139).

685

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

687 FT-IR spectra (second derivatives) in the amide I absorption region obtained from
688 different hamster-adapted scrapie strains (263K, ME7-H, 22A-H) and a hamster-
689 adapted BSE-isolate (BSE-H). Samples were hydrated in D₂O. The overlaid spectra
690 represent measurements of samples produced in independent extraction and
691 purification procedures.

692

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

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

700 root ganglion from a terminally diseased scrapie-hamster. Right picture shows a
701 photomicrograph (scale bar, 50 μm) with the IR mapped area overlaid. The ratio of
702 the β -sheet to α -helical intensities calculated from the absorbance at 1637 cm^{-1} and
703 1657 cm^{-1} , respectively, is shown as a function of pixel location. Red and yellow
704 indicate areas with a relatively higher β -sheet content than spectra from green and
705 blue areas. Examples of amide I band shapes of spectra from areas with relatively
706 higher (red) and lower (green) β -sheet content are shown on the top left (original
707 spectra, normalized between 1400 and 1800 cm^{-1}) and bottom left (corresponding
708 second derivative spectra).

709

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

713 Traces 1-4 have been normalized and are calculated by averaging spectra obtained
714 from 452 BSE-negative (1, 2) or 249 BSE-positive (3, 4) animals, respectively.

715 Trace 5 (blue) represents the difference spectrum of the second derivatives (4, BSE
716 positive; and 2, BSE negative). Traces 6 and 7 are the two standard deviation spectra
717 of the classes BSE positive (6) and BSE negative (7) (which are nearly identical in
718 this case). Note that the y-axis of difference spectrum 5 and the standard deviation
719 spectra (6, 7) is magnified by a factor of 20.