Spectroscopy 24 (2010) 525–534
DOI 10.3233/SPE-2010-0474
IOS Press

In situ FTIR studies on mammalian cells

Willem F. Wolkers [a,*] and Harriëtte Oldenhof [b]

[a] *Institute of Multiphase Processes, Leibniz Universität Hannover, Hannover, Germany*
[b] *Clinic for Horses–Unit for Reproductive Medicine, University of Veterinary Medicine Hannover, Hannover, Germany*

Abstract. In this work, we describe the application of FTIR spectroscopy to study lipids and proteins, in cellular systems during heating and cooling. Various spectral analysis methods are described to simultaneously study membrane phase behavior and heat-induced protein denaturation in cells. Membrane phase behavior was studied by following the temperature dependence of the CH_2 stretching vibration bands. Protein denaturation was studied using the amide-I and -III bands. Both amide-I and amide-III band analysis show that heat-induced denaturation of proteins within the cells is typically associated with an increase in extended β-sheet structures and a concomitant decrease in α-helical structures.

Keywords: FTIR, membrane phase behavior, liposomes, erythrocytes, platelets, cryopreservation, cryosurgery

1. Introduction

Fourier transform infrared (FTIR) spectroscopy is emerging as a powerful technique for characterization and structural analysis of biological samples including intact cells and tissues. FTIR studies on intact mammalian cells during freezing or heating provide information on conformational and phase changes of endogenous biomolecules, including membrane lipids and proteins [2,19]. This is relevant for cryopreservation of cells or for thermally-based treatments of diseases such as is done in cryosurgery or by heat treatment. By following the temperature dependence of characteristic peaks in the FTIR spectra of cells, temperature-induced (thermotropic) and dehydration-induced (lyotropic) membrane phase behavior and changes in protein secondary structure can be followed simultaneously [19–21]. FTIR studies are minimally invasive and do not require labeling. Moreover, samples can be measured in any physical state including in hydrated, frozen and dried state.

IR spectroscopy relies on characteristic molecular group vibrations of molecules. The vibrational frequency of a molecular group vibration depends on the mass of the vibrating atoms and on intra- and inter-molecular interactions. Dehydration or changes in temperature affect the intra- and inter-molecular interactions of biomolecules, which may result in conformational or phase changes. This in turn, affects the amount of energy needed for a particular molecular vibration to take place, which is visible as a change in the position and shape of the absorption band in the IR spectrum.

The CH_2 stretching bands arising from the acyl chains of lipids, have been used to detect phase transitions in isolated biological membranes [6,11] as well as in whole cells [7]. Thermotropic membrane phase behavior of native cells gives information about the temperature range over which gel-to-liquid

*Corresponding author: Willem F. Wolkers, Institute of Multiphase Processes, Leibniz Universität Hannover, Callinstrasse 36, 30167 Hannover, Germany. Tel.: +49 511 762 19353; Fax: +49 511 762 19389; E-mail: wolkers@imp.uni-hannover.de.

membrane phase transitions take place [7,14]. Membranes of mammalian cells typically exhibit a gradual increase in conformational disorder during heating with non-cooperative phase transitions. Membrane phase behavior of mammalian cells is strongly influenced by the presence of membrane cholesterol, which masks cooperative phase transitions of membrane lipids. Cooperative phase transitions and an increase in membrane conformational disorder, are usually observed after cholesterol depletion [9]. Mammalian cell membranes exhibit strong lyotropic fluid-to-gel phase transitions during freezing that are governed by the dehydrating effect of freezing on cells [13,19].

The amide-I, -II and -III bands, arising from vibrations of the protein backbone, have been widely used to determine the secondary structure of isolated proteins [8]. Most FTIR studies on isolated proteins rely on the amide-I band for protein secondary structure analysis [17]. When cells or tissues are analyzed the amide bands provide information on the overall protein secondary of the combined composition of endogenous proteins. The amide-I and -II bands are usually visible as pronounced bands in cell pellets. The H_2O band, however, interferes with the amide-I band and to a lesser extent with the amide-II band, which complicates analysis. Nevertheless, the amide-I band can be used to detect heat-induced protein denaturation in cells using difference spectra [4]. This approach yields denaturation profiles that closely resemble those determined by differential scanning calorimetry (DSC) [10]. The amide-II band can also be used to detect protein denaturation in cells, but the different types of secondary structure are less well resolved. Recent studies, have implicated the weaker amide-III band for FTIR protein analysis, because the different types of secondary structure are better resolved, and because this region of the spectrum does not find interference from water and water vapor bands [1,5,18]. The amide-III region can also be used to detect protein denaturation in cells [19] or tissues [15]. It should be noted that the amide-III region, which is located in the fingerprint region of the IR spectrum, is likely to contain contributions from other molecular groups including phosphate. Whether or not the amide-III band can be used for protein analysis depends on the relative abundance of proteins in the cell compared to other biomolecules.

In this work, we describe the application of FTIR to study lipids and proteins in cellular systems during heating. Various spectral analysis methods are described to study membrane phase behavior and heat-induced protein denaturation in cells.

2. Materials and methods

FTIR spectra were recorded using a Perkin Elmer IR-spectrometer equipped with a liquid nitrogen cooled MCT detector. Samples were assayed in transmission mode in a temperature-controlled sample holder. Samples of hydrated cell pellets were loaded between two CaF_2 windows and sealed to avoid dehydration of the sample. As experimental materials were used: red blood cells (RBCs), platelets, human pulmonary endothelial cells (HPMEC), embryonic feeder (3T3) cells and stallion spermatozoa. Human and porcine red blood cells and platelets were isolated as described in detail elsewhere [9,22]. HPMEC and 3T3 cells were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (Biochrom, Berlin, Germany). Cells were trypsinized and centrifuged at 1000g for 10 min, to obtain a pellet for further analysis. Semen was collected from stallions that were held at the National Stud of Lower Saxony in Celle, Germany, as described elsewhere [16]. Gel-free semen was centrifuged for 20 s at 14,000g, the supernatant was removed and the pellet was used for FTIR analysis.

For membrane analysis the spectral region between 3000 and 2800 cm^{-1} was selected, which contain the CH_2 symmetric (νCH_2sy) and asymmetric (νCH_2as) stretching bands at approximately 2850 and

2920 cm^{-1}, respectively. The inverted second derivative spectrum was calculated, and the lipid band was selected and normalized. Band positions were calculated by taking the average of the spectral positions at 80% of the peak height. Wavenumber versus temperature plots were constructed and the phase transition temperature (T_m) was estimated from the νCH_2sy or νCH_2as versus temperature plots.

For protein analysis using the amide-I band, the spectral region between 1700 and 1600 cm^{-1} was selected. Heat denaturation profiles were obtained by subtracting the spectrum recorded at 0°C from the spectrum at a given temperature. The second derivatives of such difference spectra were taken with a 13-point smoothing factor to resolve the different bands more clearly and the area of the bands at 1625 cm^{-1} and 1655 cm^{-1} were determined. Plots were created were the area of these bands, representing extended β-sheet and α-helical structures, were plotted as a function of the temperature, and used to determine the protein denaturation profile. For protein analysis using the amide-III band, the region between 1350 and 1200 cm^{-1} was selected. In this case, the base line corrected area under the original absorbance spectra bands was calculated and plotted as a function of temperature. Bands at approximately 1315 and 1235 cm^{-1} were found to decrease and increase, respectively, upon protein denaturation.

3. Results and discussion

3.1. FTIR analysis of intact cells

Figure 1A shows *in situ* infrared spectra of human red blood cells (RBCs) and platelets. Several bands are visible in the CH-stretching region located between 3000 and 2800 cm^{-1}, mainly arising from CH_3 and CH_2 groups of endogenous lipids and proteins. Characteristic bands arising from cellular proteins are the amide-I band, located between 1600–1700 cm^{-1}, the amide-II band, located between 1500–1600 cm^{-1} and the amide-III band located between 1330–1200 cm^{-1}. In the region below 1500 cm^{-1}, a variety of characteristic IR group frequencies can be observed, but the bands in this region are difficult to assign. The shape of the spectra below 1500 cm^{-1}, however, is highly characteristic and can be used as a 'finger print' of a given cell type. In Fig. 1B examples of the finger print region are shown for different cell types. The amide-III region is visible between 1360 and 1200 cm^{-1}, which usually arises from proteins. It should be noted that this region also contains other vibrations, i.e., PO_4 in lipids or nucleic acids, and it depends on the relative abundance of a biomolecule in a cell or tissue, which type dominates. The region between 1360 and 1200 cm^{-1} can be used to study heat-induced protein denaturation, in a variety of cells. In sperm cells, however, the amide-III region is not sensitive for protein denaturation (data not shown). The characteristic bands at 1225, 1080 and 1050 cm^{-1}, in sperm arise from PO-vibrations of nucleic acids and lipids [3].

3.2. Temperature dependent FTIR analysis of membrane phase behavior in cells

Figure 2A depicts an enlargement of the 3000–2800 cm^{-1} region of the IR spectrum, of stallion sperm cells at different temperatures. Second derivative analysis shows the different bands in the CH-stretching region more clearly (Fig. 2B). The characteristic asymmetric and symmetric CH_2 stretching vibration bands arising from the lipid acyl chains are visible at 2920 and 2850 cm^{-1}, respectively. CH_3 group vibrations are visible at 2870 and 2950 cm^{-1}. The band position of the CH_2 stretching vibrations shift to higher wavenumber with increasing temperature, indicating an increase in conformational disorder. Wavenumber versus temperature plots of asymmetric and symmetric CH_2 stretching vibrations bands

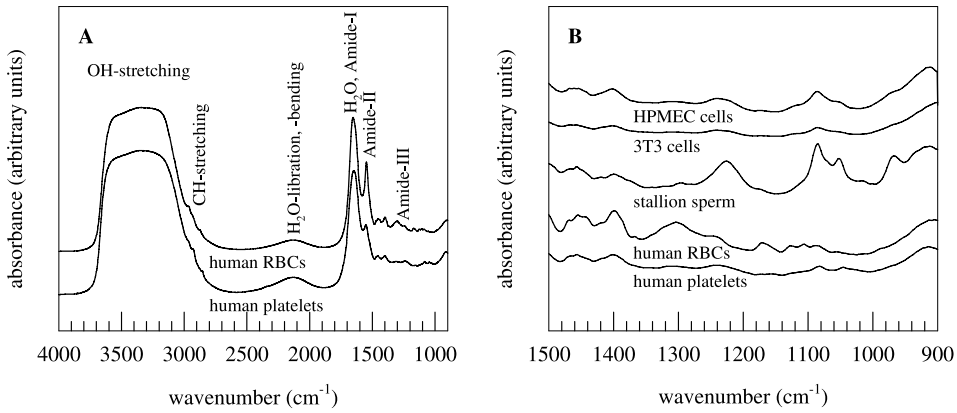

Fig. 1. *In situ* infrared spectra of human red blood cells (RBCs) and platelets are shown in panel A. The characteristic amide-I, -II and -III bands are indicated. The CH-stretching region and the water bands are also indicated. Panel B shows and enlargement of the 1500–900 cm^{-1} region. For comparison spectra of stallion sperm, 3T3 cells and HPMEC cells are also shown to illustrate the cell specific differences in this spectral region.

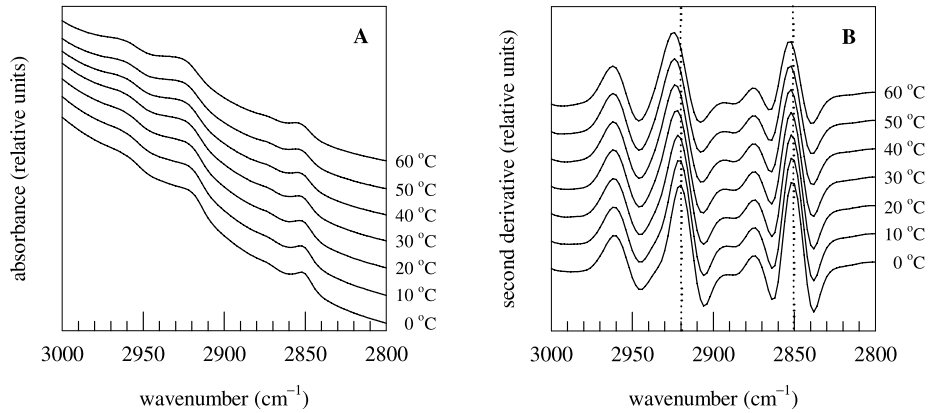

Fig. 2. Infrared absorption spectra (A) and their second derivatives (B) of the CH-stretching region at various temperatures, of stallion sperm cell pellets exposed to heating from 0 to 70°C at a rate of 2°C min^{-1}. The different bands in the CH-stretching region represent the symmetric and asymmetric CH_2 stretching vibration bands at 2850 and 2920 cm^{-1}, and the symmetric and asymmetric CH_3 group vibrations at 2870 and 2950 cm^{-1}.

(νCH_2as and νCH_2sy) reveal the membrane phase behavior of the cells (Fig. 3). Stallion sperm displays a broad gel-to-fluid phase transition from 0 to 30°C. At temperatures above 30°C, the membranes display a monotonous increase in membrane conformational disorder with increasing temperature. Asymmetric and symmetric CH_2 stretching bands show similarly shaped curves, indicating that both can be used to study membrane phase behavior of cells. In the literature, however, νCH_2sy is mostly used to study membrane phase behavior of cells [7,11,12].

3.3. Temperature dependent FTIR analysis of protein denaturation in cells

Figure 4 depicts FTIR spectra of 3T3 cells recorded during a temperature scan from 0 to 90°C at 1°C min^{-1} in the 1800–1200 cm^{-1} region of the IR spectrum. The amide-I, -II and -III bands are

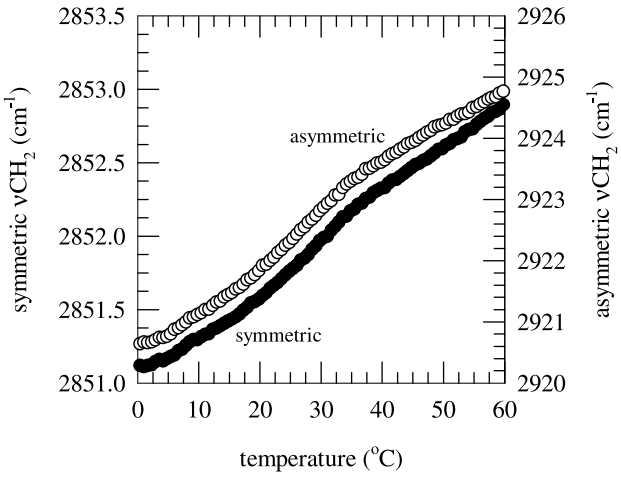

Fig. 3. Membrane phase behavior of stallion sperm cell pellets. The data points reflect the position of the symmetric (open symbols) and asymmetric (closed symbols) CH_2 stretching vibrations bands, as a function of the temperature.

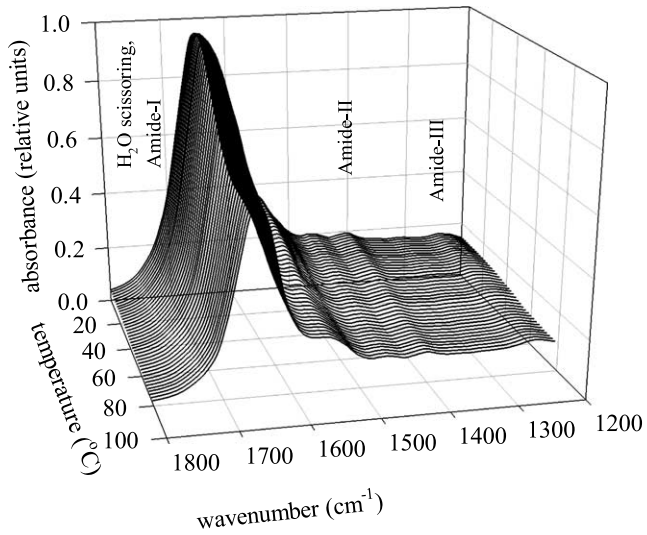

Fig. 4. Three-dimensional representation of IR spectra of HPMEC cells during thermal heating from 0 to 90°C at a rate of 1°C min^{-1}. The amide-I, -II and -III bands are indicated in the figure. The H_2O scissoring band, which overlaps with the amide-I band, is also indicated.

indicated in the figure. The amide-I band and the H_2O scissoring band overlap. In order to analyze changes in the amide-I band during thermal denaturation, difference spectra were calculated from which second derivative spectra were computed for further protein analysis.

Figure 5 illustrates how protein analysis is done using the amide-I region. Panel A depicts the raw absorbance spectra as a function of temperature. Panel B shows difference spectra between spectra at the indicated temperatures and that of the recorded spectrum at 0°C. Not much detail is visible in the difference spectra. By taking the second derivatives of the difference spectra (panel C), however, protein denaturation becomes visible as an abrupt increase of a band at 1625 cm^{-1} (extended β-sheet structures)

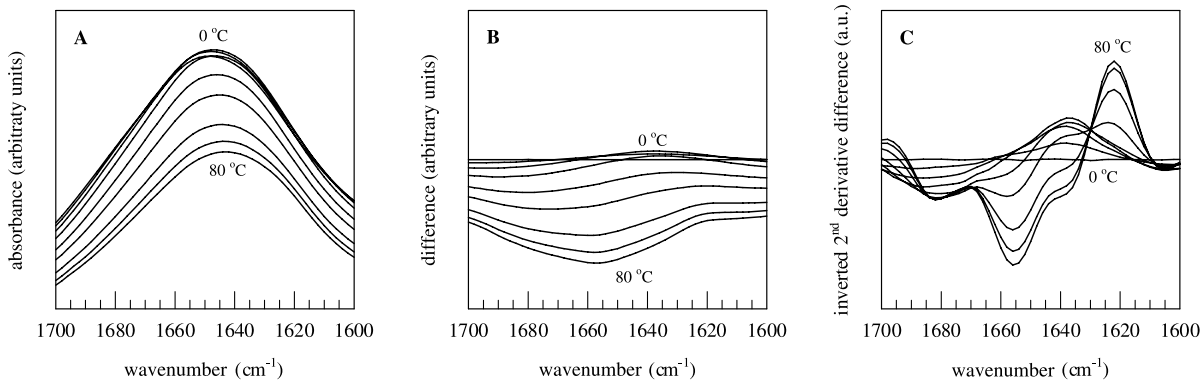

Fig. 5. Absorbance spectra in the amide-I region during heating of 3T3 cells from 0 to 90°C (A). Panel B are difference spectra of the spectrum recorded at 0°C and those at the indicated temperature. Panel C shows second derivative spectra of the difference spectra. Denaturation coincides with a decrease in the band at 1625 cm^{-1} and a decrease in the band at 1655 cm^{-1}.

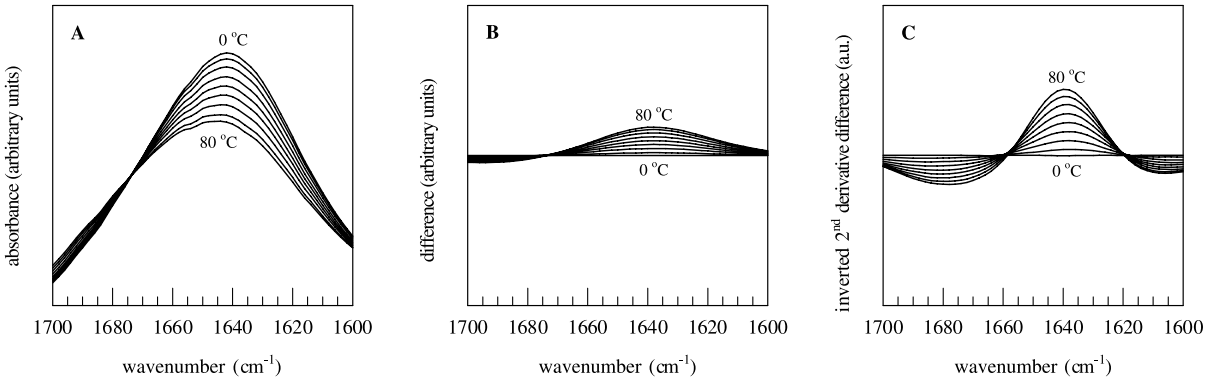

Fig. 6. Absorbance spectra in the amide-I region during heating of buffer from 0 to 90°C (A, control for Fig. 3). Panel B are difference spectra of the spectrum recorded at 0°C and those at the indicated temperature. Panel C shows second derivative spectra of the difference spectra.

and a concomitant decrease of a band at 1655 cm^{-1} (α-helical structures). Because the H_2O band interferes with the amide-I band during heating, we have also analyzed the amide-I region of buffer alone, to show that the observed effects in cell pellets are associated with protein denaturation and not with changes in the water band. Figure 6 shows the thermal analysis of the amide-I region of buffer alone. Gradual changes are observed in the amide-I region during heating. A gradual increasing absorbance at 1640 cm^{-1} becomes visible during heating and a decreased absorbance at 1680 cm^{-1}. These changes are relatively small and do not interfere with the protein denaturation analysis. Therefore, the abrupt increase of the band at 1625 cm^{-1} to protein denaturation can be assigned to protein denaturation. In a previous study on a tumor cell line, we have shown that protein denaturation profiles determined by FTIR closely match those determined by DSC [10].

Figure 7 shows the amide-I band region as a function of the temperature for porcine RBCs and platelets. The three-dimensional representation of the denaturation process shows the clear differences in denaturation profile between the two cell types. In RBCs protein denaturation starts at an onset temperature of 75°C, whereas in platelets denaturation commences just above 45°C. In RBCs the denaturation profile is likely dominated by hemoglobin.

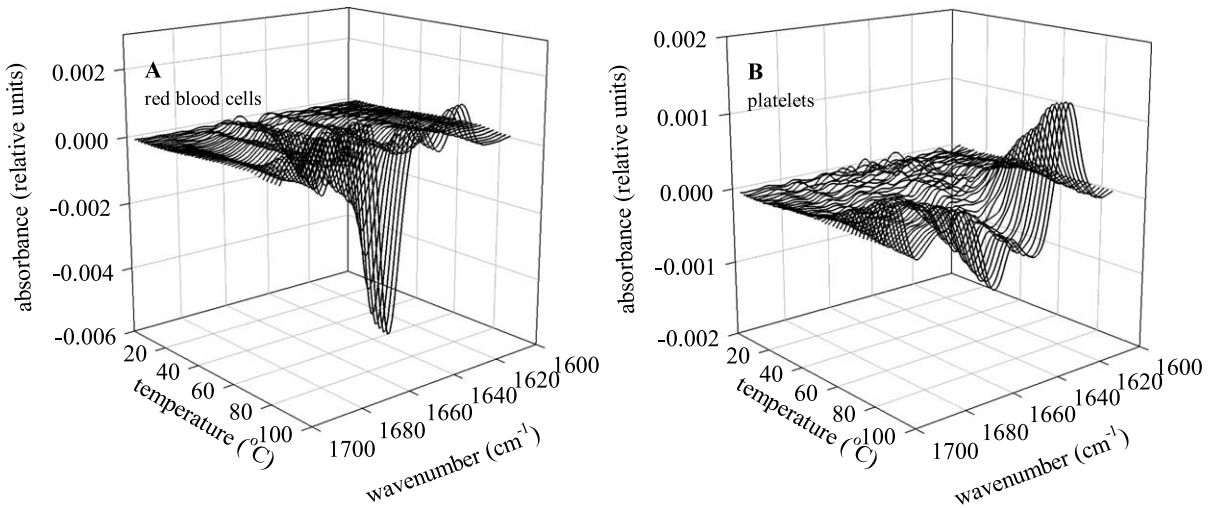

Fig. 7. Three-dimensional representation of second derivative difference spectra of red blood cells (RBCs) (A) and platelets (B) illustrating the differences in denaturation profile between the two cell types. The amide-I, -II and -III bands are indicated in the figure. The H_2O scissoring band, which overlaps with the amide-I band, is also indicated.

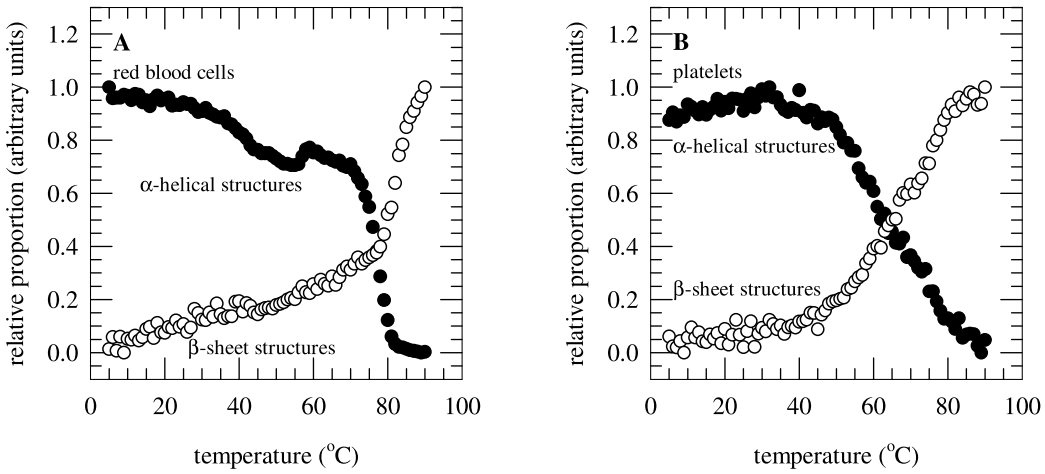

Fig. 8. Protein denaturation based on amide-I band analysis for red blood cells (RBCs) (A) and platelets (B). The data points reflect the relative area of the band at 1625 and 1655 cm^{-1}, assigned to β-sheet and α-helical structures, respectively.

Figure 8 shows the quantitative denaturation analysis for platelets and RBCs. Denaturation is visible as an abrupt increase in β-sheet structures coinciding with a decrease in α-helical structures. This is typically observed during heat denaturation of cells [4,10,19].

The amide-III band can also be used for protein analysis of cells. Figure 9 shows the 1360–1200 cm^{-1} region as a function of the temperature, containing the amide-III band. The intensity of the band at 1330 cm^{-1} decreases with increasing temperature, whereas the band at 1240 cm^{-1} increases in intensity. The band areas of the bands at 1240 and 1330 cm^{-1} have been calculated and plotted as a function of temperature (Fig. 10A). There is an abrupt increase in the area of the band at 1240 cm^{-1} just above 45°C, which likely indicates the formation of extended β-sheet structures associated with protein denaturation.

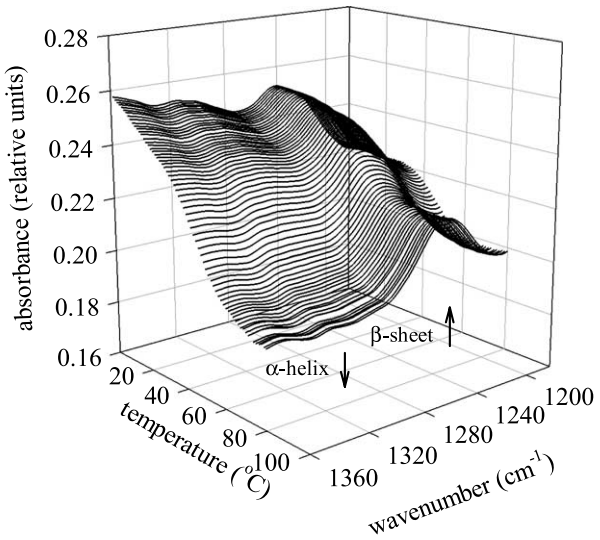

Fig. 9. Three-dimensional representation of the amide-I region during thermal denaturation of HPMEC cells.

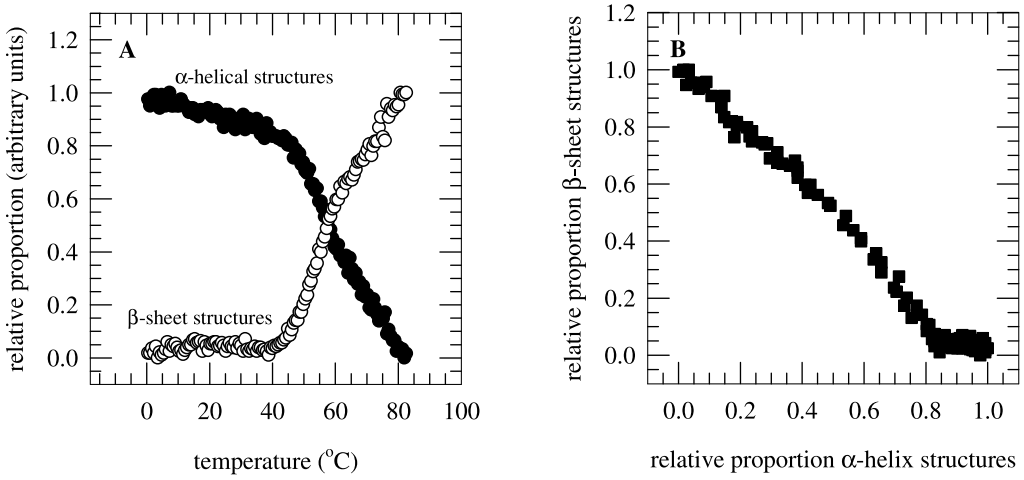

Fig. 10. Protein denaturation based on amide-III band analysis. The data points reflect the relative area of the band at 1315 and at 1240 cm^{-1}, respectively assigned to α-helical and β-sheet structures (A). Panel B shows a correlation plot of the relative proportions of α-helical and β-sheet structures.

The decrease of the band at 1315 cm^{-1} reflects a decrease in α-helical structures. Figure 10B shows that the increase in β-sheet structures correlates with the decrease in α-helical structures. Amide-III analysis can be used for a variety of mammalian cells. It should be noted that the amide-III region is located in the fingerprint region of the IR spectrum, and thus likely contains contributions from other molecular groups including phosphate. For sperm cells, the amide-III region could not be used for protein analysis. This is likely due to the relatively high DNA content in sperm causing interference of the DNA PO_4 band with the protein amide-III band. Thus, whether or not the amide-III band can be used for protein analysis depends on the relative abundance of proteins in the cell compared to other biomolecules.

4. Conclusion

FTIR spectroscopy can be used to simultaneously study membrane phase behavior and protein denaturation in cellular systems. Membrane phase behavior can be studied by following the temperature dependence of the CH_2 stretching vibrations. Data analysis is done by taking the second derivative of the absorbance spectra and by plotting the band positions of the symmetric or asymmetric CH_2 stretching vibrations as a function of temperature. Protein denaturation can be studied using the amide-I and -III bands. Protein denaturation using the amide-I band analysis is relatively complicated and requires taking difference spectra followed by second derivative analysis. This method, however, allows studying protein denaturation in cells without subtraction of the water band. Amide-III band analysis is relatively simple and can be done by computing the baseline corrected band area of bands at 1240 or 1330 cm^{-1}. This works for a variety of cells. Amide-III band analysis, however, cannot be used with cells that have low protein contents or relatively high nucleic acid or lipid contents. Both amide-I and amide-III band analysis show that heat-induced protein denaturation of cells is typically visible as an increase in extended β-sheet structures and a concomitant decrease in α-helical structures.

Acknowledgement

This work is supported by funding from the German Research Foundation (DFG, Deutsche Forschungsgemeinschaft) for the Cluster of Excellence "From Regenerative Biology to Reconstructive Therapy" (REBIRTH).

References

[1] G. Anderle and R. Mendelsohn, Thermal denaturation of globular proteins. Fourier transform-infrared studies of the amide III spectral region, *Biophysical Journal* **52** (1987), 69–74.
[2] S.K. Balasubramanian, W.F. Wolkers and J.C. Bischof, Membrane hydration correlates to cellular biophysics during freezing in mammalian cells, *Biochimica et Biophysica Acta* **1788** (2009), 945–953.
[3] O. Barčot, M. Balarin, O. Gamulin, D. Ježek, P. Romac and J. Brnjas-Kraljevic, Investigation of spermatozoa and seminal plasma by Fourier transform infrared spectroscopy, *Applied Spectroscopy* **61** (2007), 309–313.
[4] J.C. Bischof, W.F. Wolkers, N.M. Tsvetkova, A.E. Oliver and J.H. Crowe, Lipid and protein changes due to freezing in dunning AT-1 cells, *Cryobiology* **45** (2002), 22–32.
[5] S. Cai and B.R. Singh, A distinct utility of the amide III infrared band for secondary structure estimation of aqueous protein solutions using partial least squares methods, *Biochemistry* **43** (2004), 2541–2549.
[6] D.G. Cameron, A. Martin and H.H. Mantsch, Membrane isolation alters the gel to liquid crystal transition of *Acholeplasma laidlawii* B, *Science* **219** (1983), 180–182.
[7] J.H. Crowe, F.A. Hoekstra, L.M. Crowe, T.J. Anchordoguy and E. Drobnis, Lipid phase transitions measured in intact cells with Fourier transform infrared spectroscopy, *Cryobiology* **26** (1989), 76–84.
[8] E. Goormaghtigh, V. Cabiaux and J.M. Ruysschaert, Determination of soluble and membrane protein structure by Fourier transform infrared spectroscopy. I. Assignments and model compounds, *Subcellular Biochemistry* **23** (1994), 329–362.
[9] K. Gousset, W.F. Wolkers, N.M. Tsvetkova, A.E. Oliver, C.L. Field, N.J. Walker, J.H. Crowe and F. Tablin, Evidence for a physiological role for membrane rafts in human platelets, *Journal of Cellular Physiology* **190** (2002), 117–128.
[10] X. He, W.F. Wolkers, J.H. Crowe, D.J. Swanlund and J.C. Bischof, *In situ* thermal denaturation of proteins in Dunning AT-1 prostate cancer cells: implication for hyperthermic cell injury, *Annals of Biomedical Engineering* **32** (2004), 1384–1398.
[11] H.H. Mantsch and R.N. McElhaney, Phospholipid phase transitions in model and biological membranes as studied by infrared spectroscopy, *Chemistry and Physics of Lipids* **57** (1991), 213–226.
[12] D.J. Moore and R. Mendelsohn, Adaptation to altered growth temperatures in *Acholeplasma laidlawii* B: Fourier transform infrared studies of acyl chain conformational order in live cells, *Biochemistry* **33** (1994), 4080–4085.

[13] H. Oldenhof, K. Friedel, H. Sieme, B. Glasmacher and W.F. Wolkers, Membrane permeability parameters for freezing of stallion sperm as determined by Fourier transform infrared spectroscopy, *Cryobiology* (2010), to appear, doi: 10.1016/j.cryobiol.2010.06.002.
[14] H. Oldenhof, W.F. Wolkers, F. Fonseca, S. Passot and M. Marin, Effect of sucrose and maltodextrin on the physical properties and survival of air-dried *Lactobacillus bulgaricus*: an *in situ* Fourier transform infrared spectroscopy study, *Biotechnology Progress* **21** (2005), 885–892.
[15] N.B. Shah, W.F. Wolkers, M.S. Morrissey, W.Q. Sun and J.C. Bischof, Fourier transform infrared spectroscopy investigation of native tissue matrix modifications using a gamma irradiation process, *Tissue Engineering Part A* **15** (2009), 33–40.
[16] H. Sieme, G. Martinsson, H. Rauterberg, K. Walter, C. Aurich, P. Petzoldt and E. Klug, Application of techniques for sperm selection in fresh and frozen-thawed stallion semen, *Reproduction in Domestic Animals* **38** (2003), 134–140.
[17] W.K. Surewicz, H.H. Mantsch and D. Chapman, Determination of protein secondary structure by Fourier transform infrared spectroscopy: A critical assessment, *Biochemistry* **32** (1993), 389–394.
[18] M. van de Weert, P.I. Haris, W.E. Hennink and D.J.A. Crommelin, Fourier transform infrared spectrometric analysis of protein conformation: effect of sampling method and stress factors, *Analytical Biochemistry* **297** (2001), 160–169.
[19] W.F. Wolkers, S.K. Balasubramanian, E.L. Ongstad and J.C. Bischof, Effects of freezing on membranes and proteins in LNCaP prostate tumor cells, *Biochimica et Biophysica Acta* **1768** (2007), 728–736.
[20] W.F. Wolkers and F.A. Hoekstra, *In situ* FTIR assessment of desiccation-tolerant tissues, *Spectroscopy* **17** (2003), 297–313.
[21] W.F. Wolkers, H. Oldenhof and B. Glasmacher, Dehydrating phospholipid vesicles measured in real-time using ATR Fourier transform infrared spectroscopy, *Cryobiology* (2010), to appear, doi: 10.1016/j.cryobiol.2010.06.001.
[22] W.F. Wolkers, N.J. Walker, Y. Tamari, F. Tablin and J.H. Crowe, Towards a clinical application of freeze-dried human platelets, *Cell Preservation Technology* **1** (2003), 175–188.