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GenRH Application Note 505

Identifying Structural Changes in the Protein Collagen Using Humidity Generation and FT-IR

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Progress in biotechnology in recent years has precipitated renewed interest in the interaction of proteins with each other and the environment. Collagen's function as one of the fundamental structural features of biological systems makes it a protein of prime interest in areas as diverse as trans-dermal drug delivery, pre-cancerous cell diagnosis and bone regeneration. Described herein is an account of preliminary studies of the effect of RH and temperature on the protein and how humidity generation instrumentation combined with FT-IR can be used to quickly demonstrate structural change in proteins.

Introduction

Progress in biotechnology in recent years has renewed interest in the interaction of proteins with each other and their environment. Water vapour can induce changes in the tertiary structure within proteins influencing aggregation behaviour and gelation, in turn affecting formulation stability [1]. Collagen's function as one of the fundamental structural features of biological system makes it a protein of prime interest in areas as diverse as transdermal drug delivery, cancer diagnosis and bone regeneration and so is an ideal protein for study. The purpose of this study is to illustrate how humidity generation instrumentation and spectroscopy can be used to gain understanding on the interaction of proteins with water vapour, and to provide a starting point on the interaction of water vapour with skin and its effect on transdermal delivery mechanisms.

Collagen can be divided in to a number of types depending both on the species and the tissue type and is composed principally of glycine, proline and alanine amino acids, although the exact composition varies between different species and tissue types. The denaturation temperature is dependent principally on the metabolic temperature of the organism from which it comes, there being observable differences between amphibians, fish and mammal collagen, but also the nature of the tissue from which it is derived; skin, bone, lung etcetera [2].

Collagen exists in the form of triple chain helices, which themselves are bundled together to form fibrils. When exposed to temperatures above the denaturation temperature this tertiary structure unravels to leave the collagen molecules in the form of random coil single chains. In solution, this transition is reversible with the rate of reformation of the triple helix dependent on the cooling conditions employed, however in the solid state, denaturation is irreversible.

The degree of hydration of collagen has been shown to have a profound effect on the physical properties of the material, increased hydration increasing the periodic distance of the helices, and also affecting characteristics such as the





degree of piezoelectric effect it exhibits [3]. Its hydration has been shown to be influenced by the relative humidity of the surrounding environment, and so one would expect that its adsorption/desorption behaviour would be greatly influenced by the temperature at which this was studied.

The GenRH is a novel line of humidity generation products. When combined with an environmental control cell (Mcell), designed to fit under a microscope, samples can be observed by FT-IR, Raman or optical microscopic techniques, under controlled humidity conditions. The GenRH-A instrument is an ambient temperature humidity generator that can deliver a steady stream of air over a wide range of flow rates and humidities.

In this paper we describe this technique, demonstrate the correlation of absorbance of spectral bands versus relative humidity plots to adsorption isotherms and follow in situ, by FT-IR spectroscopy, the interactions of the collagen samples with water vapor.

Method

The GenRH-A consists of; a programmable digital controller with full time closed-loop control, temperature monitoring display, temperature and humidity probe, and a rotameter to control flow volume. A picture of the GenRH-A is shown in Figure 1.

Figure 1. Surface Measurement Systems GenRH-A humidity generator.

To use the GenRH-A efficiently with a microscope, Surface Measurement Systems has developed the Mcell. The sample is mounted into the Mcell, which is fixed to the microscope stage as shown in Figure 2. This stage has double-glazed top and bottom windows, allowing both transmission and reflection illumination of the sample while minimizing heat loss through the windows when being used under non-ambient conditions.

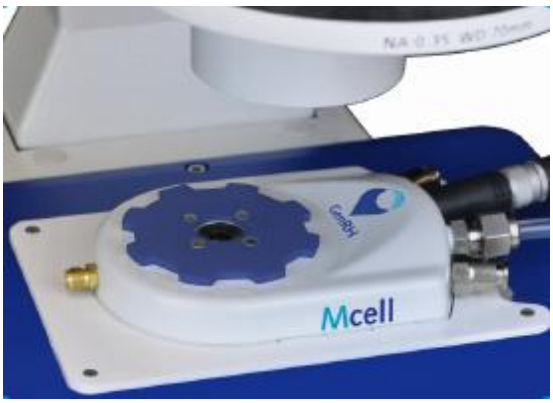


Figure 2. SMS Mcell microscope accessory for the GenRH line of products.

Spectroscopic information was recorded using a Bruker Hyperion Microscope Spectrophotometer. The humidity profile for spectroscopy measurements at 25°C was an increasing ramp 0-95% RH at 1% RH per minute. The experiment at 37°C included an increasing ramp 0-95 %RH at 1% RH per minute, then held for 10 minutes at 95% RH then reduced at the same rate. Spectra were recorded at 1 minute intervals.

Rat tail Type 1 collagen was used in this study, which denatures at approximately 37°C [2]. The denaturation temperature is known to be dependent on the hydration level of the fibrils. Dynamic Vapor Sorption (DVS) isotherms were first recorded to highlight RH regions of interest. DVS experiments were performed on a DVS-Advantage automated gravimetric vapour sorption analyser (Surface Measurement Systems Ltd., London, UK). The DVS-Advantage measures the uptake and loss of vapour gravimetrically using a recording ultra-microbalance with a mass resolution of 0.1-µg. The relative humidity around the sample was controlled by mixing saturated and dry carrier gas streams using mass flow

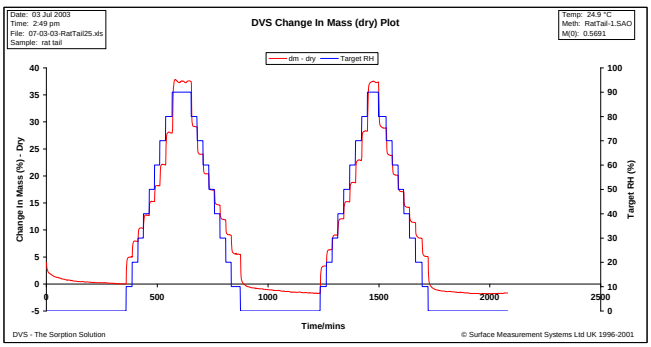
controllers. The temperature was maintained by enclosing the entire system in a temperature-controlled incubator.

Results

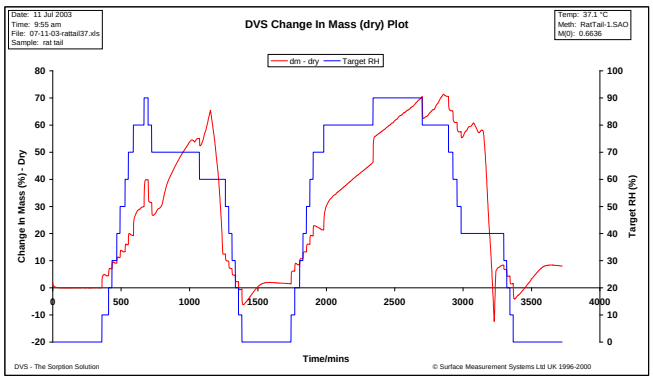
DVS Data

The DVS results are shown in Figures 3a (25°C) and 3b (37°C). Figure 3a shows the adsorption isotherm for the collagen sample at 25°C which is a relatively standard absorption profile, as would be expected from a stable material. The behaviour at 37°C (Figure 3b) is however very different. On the increasing RH stepped program, up to 95% the protein absorbs water in stepwise fashion. However on the decreasing RH program, stepwise adsorption continues at 95% RH, but on reducing the RH initially the amount of adsorbed water decreases, it then starts to rapidly increase, exceeding that adsorbed at higher RH. This pattern is not repeated identically on the second run, suggesting that a phase transition (probably denaturation followed by gelation) has occurred.

Based on these isotherms, IR data was collected at 25 °C with spectra collected every minute for 95 minutes during a 0-95% RH ramp, and then at 37°C with an increasing ramp 0-95%RH, maintained at 95% RH for 10 minutes followed by a decreasing RH ramp 95-0%RH. The rate of change of both ramps was 1% RH per minute.



(a.)



(b.)

Figure 3. DVS Sorption data on rat tail collagen at 25 °C (a.) and 37 °C (b.).

IR Spectral Data

The IR spectrum of collagen can be partially assigned as follows: 1655 cm^{-1} is due to the amide I band (C=O), at 1555 cm^{-1} the amide II band (N-H) and at 1235 cm^{-1} the amide III band. The degree to which the denaturation of the protein can be ascertained by the ratio of the intensity of the band at 1235 cm^{-1} to the band at 1450 cm^{-1} (related to the pyrrolidine ring vibrations). A ratio greater than one implies the collagen exists in its triple helical form, less than one and it exists in the random coil form. The bands arising from O-H bending mode at 1620 cm^{-1}, and the amide I band at 1655 cm^{-1} overlap, in this case rendering impossible the correlation of the intensity of this band to water uptake of the collagen.

Spectral Data at 25 °C

The spectra at this temperature are shown in Figures 4-6. The full spectrum is displayed in Figure 4. Enlargements of the spectra in the O-H antisymmetric and symmetric stretch region 2500–4000 cm^{-1} and the fingerprint region are shown in Figures 5ab and 6ab respectively.

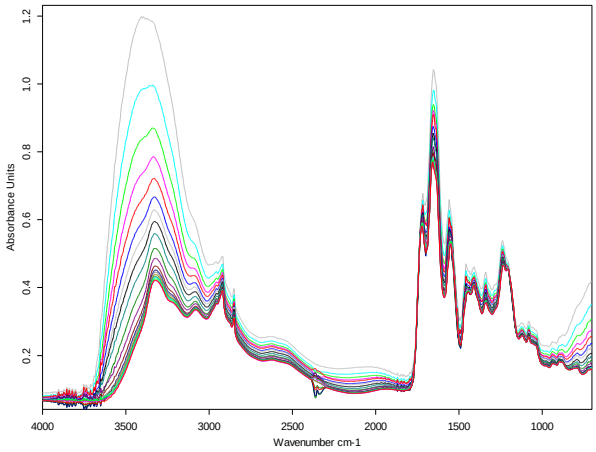
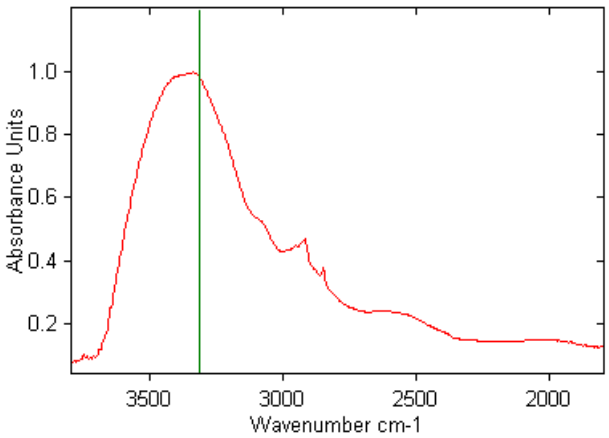
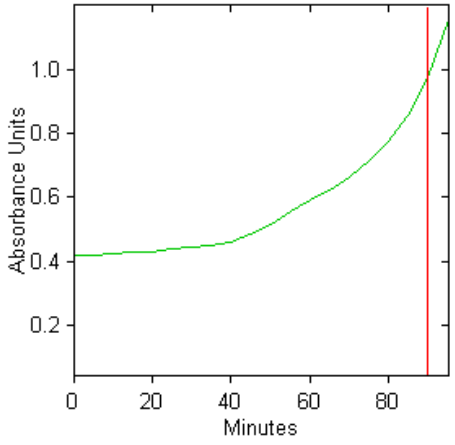


Figure 4. spectra for 25°C adsorption ramp 0-95% RH.

In the O-H stretch region (Figure 5a), the absorption RH plot shows the increase in water content consistent with the DVS data. Within the finger print region there are no bands which move significantly, however the overall intensity both of each band and the base line increases suggesting an increase in the path length. As there are no significant changes in band position or ratio of intensities, these observations suggest that water adsorption at this temperature has not induced significant change within the fundamental structure of collagen, but rather the protein has simply swelled.

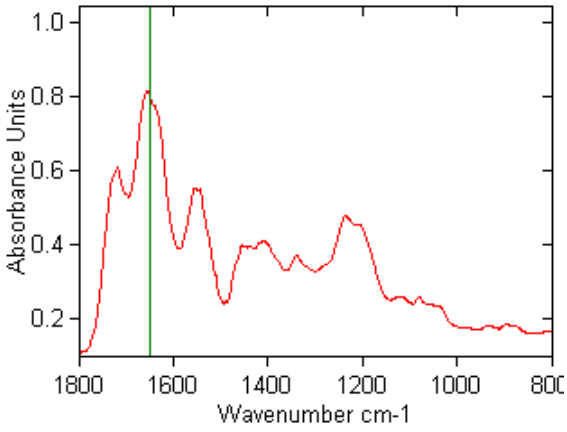


(a.)

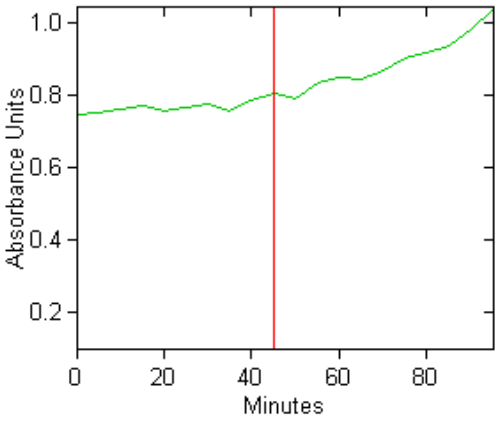


(b.)

Figure 5. spectra for band arising from O-H symmetric and asymmetric stretches (a.) and absorbance/RH plot (b.).



(a.)



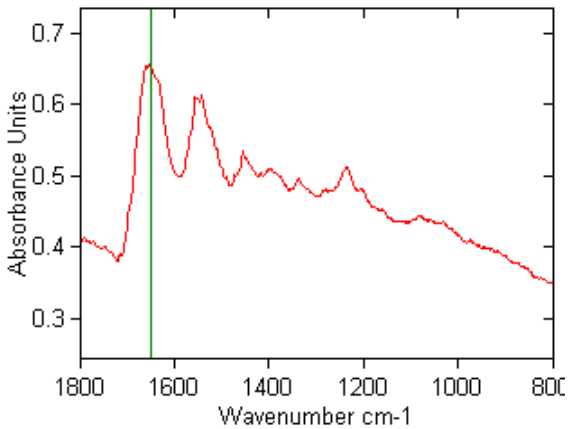
(b.)

Figure 6. spectra for fingerprint region, amide I band (a.) and absorbance/RH plot (b.).

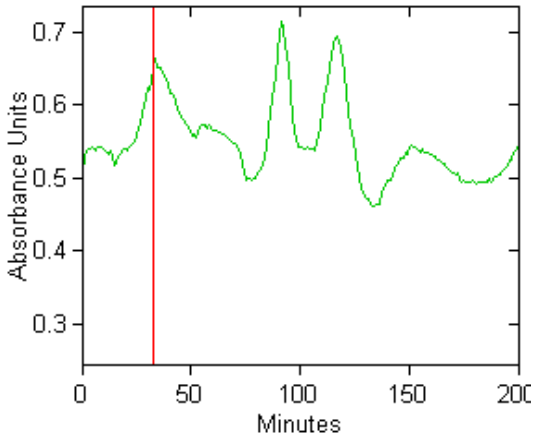
Spectral Data at 37 °C

Based on the DVS data, the RH profile consisted of an increasing RH ramp 0-95% at a rate of 1% per minute, followed by constant 95% RH for 10 minutes and a decreasing RH ramp 95-0%RH, also at a rate of 1% per minute. Spectra were collected at 1 minute intervals for the entire 200 minute experiment. The complete spectral data set has not been shown here as it is extremely complex, however all spectra exhibited the similar band shape and pattern, the principle variations being that of the intensity of the O-H stretching region and a change in the base line absorbance depending on the conditions. No significant change in any of the band positions was observed during the experiment; there are however, 2 significant differences between the spectra observed at 25°C and those observed at 37°C. The first is the loss of the band at 1720 cm^{-1}, whilst the second is the decrease to below one of the ratio of the band intensity at 1235 cm^{-1} to that at 1450 cm^{-1} on increasing the temperature to 37°C. This latter observation implies the collagen is denatured at this higher temperature, which in turn suggests the band at 1720 cm^{-1} is also linked to the triple helical structure.

Figures 7 and 8 show a typical fingerprint and O-H stretch spectra for the sample at 37°C, with the graph to the right in each case showing the variation of absorbance of the band highlighted in green with time (and so RH). In both cases the absorbance/RH plot exhibits a similar graph which correlates approximately with the DVS data.

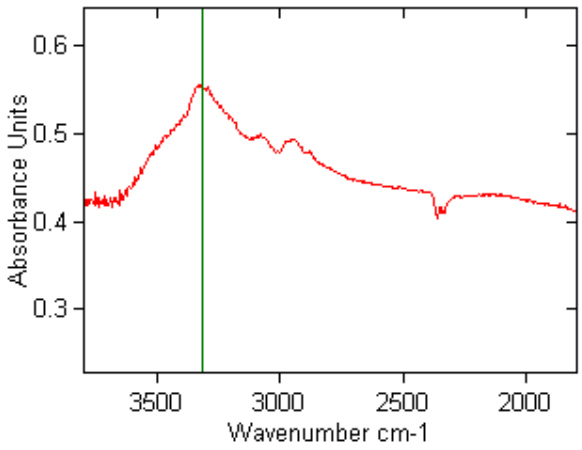


(a.)

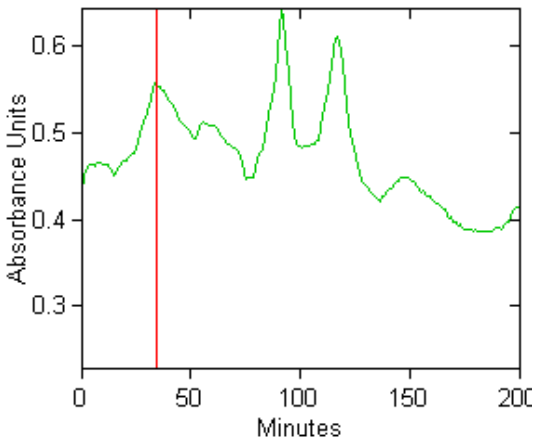


(b.)

Figure 7. IR spectra at 37 °C, fingerprint region, amide I band (a.) and absorbance/RH plot (b.).



(a.)

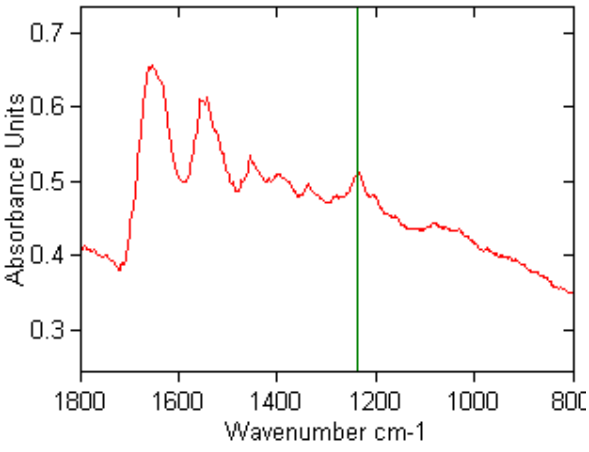


(b.)

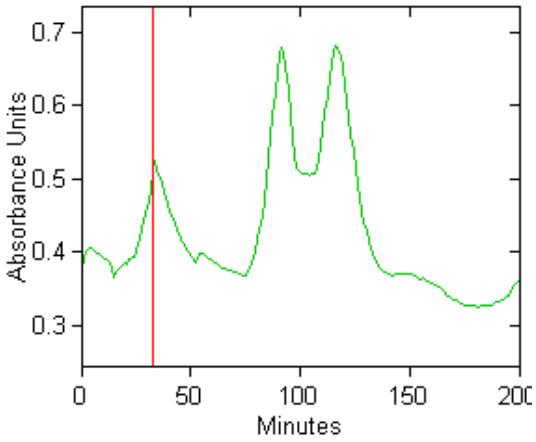
Figure 8. IR spectra at 37 °C, O-H symmetric and asymmetric stretch (a.) and absorbance/RH plot (b.).

Principally, the variation in absorbance shows features based around 40,55, 95, 120, and 150 minutes. Those at 40, 55 and 150 minutes, corresponding to 40, 55(increasing RH), and 55 (decreasing RH) %RH respectively correspond to an increase in the base line absorbance, implying increased sample length, and reflecting swelling of the collagen material with water uptake. However the features at 95 and 120 minutes (95% and 80% RH respectively) persist even after normalisation (set to zero absorbance within the region 1800 – 1850 cm^{-1}) of the spectra and are due to an increase in the absorbance of the bands at 3350, 1655 and 1555 cm^{-1}. These features reflect a considerable change in the gel phase of the denatured protein.

Figure 9 provides the absorbance/RH plot for the amide III band at 1235cm^{-1}, the profile of which is slightly different to that of the amide I and II bands, whilst Figure 10 also provides the shape of the fingerprint region at 95% RH for completeness.



(a.)



(b.)

Figure 9. IR spectra at 37 °C, amid III band (a.) and absorbance/RH plot (b.).

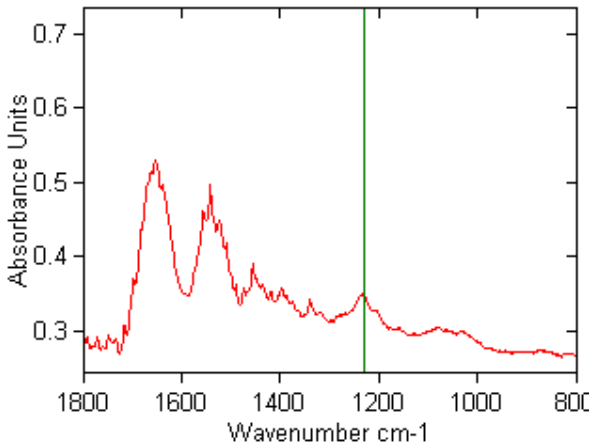


Figure 10. Finger print region of the spectrum at 95% RH.

Conclusion

This paper has clearly demonstrated the ability of humidity generation in combination with FT-IR to show differences in the behaviour of collagen to both temperature and humidity, providing an obvious test for denaturation of the protein, and showing that potentially valuable information can be gained. Further work could include the variation of humidity below the denaturation temperature to determine the effect of hydration on denaturation temperature, in addition to repeat the experiment whilst keeping collagen in contact with a drug formulation, to investigate the effect of humidity on drug absorption properties of the collagen. In addition a similar study on denatured protein could provide an insight into the absorption of drug formulations on skin scarred by heat or chemical burns.

Acknowledgement:

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References

[1] Shamblin,S., Hancock, B., Zografi, G., European Journal of Pharmaceutics and Biopharmaceutics., 45, 3, 1998, 239.

[2] Traube, W., Piez, K., Adv. Prot. Chem., 25, (1971), 243-352.

[3] da Cruz, A., Goes, J., Figueiro, S., Feitosa, J., Ricardo, N., Sombra, A., European Polymer Journal, 39, (2003), 1267-1272.

[4] Sionkowska, A., Kaminska, A., Int. J. Biol. Macromol., 24, (1999), 4, 337-340.

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