# IN-SITU SPECTROSCOPIC INVESTIGATION OF UNFOLDING AND AGGREGATION OF INSULIN UNDER ULTRASONIC EXCITATION

## *An Ultrasonic Actuator for FTIR-spectrometry on Biomatter*

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Abstract: It is well-known that fibrillogenesis of proteins can be influenced by diverse external parameters, such as temperature, pressure, agitation or chemical agents. This paper presents a newly developed ultrasonic actuator cell and a corresponding first feasibility study shows that also ultrasonic excitation at moderate intensities has a clear influence on the unfolding and aggregation behaviour of insulin. Irradiation with an average sound intensity of about 180 mW/cm$^2$ leads to a decrease of the unfolding and aggregation temperature up to 7 K.

# 1 INTRODUCTION

The main topic of this paper is the spectroscopic detection and interpretation of the *in-situ* molecular response of biomolecules on ultrasonic excitation. The novelty of this approach is the monitoring of empirical and molecular parameters during the application of ultrasound rather than after treatment, as in many other studies. To the best of our knowledge, there is almost no literature on in situ or even on time resolved methods, and this is due to a number of inherent difficulties to combine ultrasonic excitation and spectroscopic detection. As a relevant example for a biological process to study, fibrillogenesis was selected. The aggregation of globular proteins, sometimes leading to amyloid fibrils, is of great importance for biology, medicine and also industrial processes. In the medical field, there is a strong link between fibrillogenesis, and a number of neurodegenerative diseases, such as the Alzheimer disease (Dobson, 2003). Preliminary investigations in the framework of this research project showed that even very small sound velocity intensities (range of μW/cm$^2$) have a stimulating influence on the aggregation behaviour of synthetic polymers (publication under preparation). In the work presented here, the impact of ultrasonic excitation on insulin fibrillogenesis was monitored by infrared spectroscopy (Bouchard, Zurdo et al., 2000). This approach enables the quantitative determination of the varying population of secondary structures that are characteristic for the unfolding and aggregation process, i.e. the transformation of a dominant α-helix population into the β-sheet conformation (β-strands) as well as the appearance of side bands that indicate aggregation.

# 2 MATERIALS AND METHODS

## 2.1 Ultrasonic Excitation System

The infrared sample holder was equipped with a home-made ultrasonic actuator (Figure 1). It consists of two $CaF_2$ plates (10x20mm) that are separated by a polytetrafluorethen spacer (50 μm thickness) with a centred square opening of 5 x 5 mm. At one plate, two identical piezoceramic lead zirconate titanate



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(PZT) transducers (with wrapped-around electrode, provided by Meggit/InSensor®, Denmark) were attached by epoxy resin adhesive, and the nickel electrodes were wired to shielded contacts. The liquid sample was loaded into the 5 x 5 mm opening of the polytetrafluorethen spacer. The thickness of the liquid layer was thus determined by the thickness of the polytetrafluorethen gasket, d, which was in the range of 50 micrometer. The $CaF_2$ plates were closed and sealed by parafilm and vacuum grease to prevent leakage of water. Since water loss, especially induced by the heating of the sample, would cause problems, in addition to these measures, the stability of water concentration was ensured by observing the intensity of the infrared water peak.

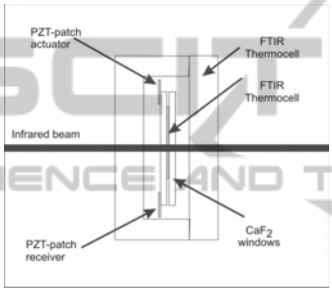


Figure 1: Ultrasonic cell for in-situ spectroscopic investigation of ultrasonic actuation. Scheme of operation mode, vibrations in the $CaF_2$ sample assembly in the transmission cell are generated by a PZT actuator and the response is monitored by the PZT receiver.

The $CaF_2$ plate assembly was mounted in a commercial SPECAC® temperature cell. In order to avoid the transfer of essential amounts of ultrasonic energy into the temperature cell, the ultrasonically active plates were firmly embedded within heat-resistant foam that isolated the plates due to the big mismatch of acoustic impedances. Placing the foam also reduced the thermal contact between the cell and the probed region in the sample, resulting in an offset between the registered temperature and the actual sample temperature.

For the ultrasonic excitation, the following approach was followed: a sinusoidal electrical signal consisting of a continuous wave (cw) with a frequency of about 50 kHz and an amplitude of 200 $mV_{pp}$ was generated by an Iwatsu SG-4511 pulse/function generator. The exact resonance frequency was fine-tuned using the output signal monitored at the oscilloscope. The electrical signal from the generator was connected to the input of a broadband amplifier (AR Worldwide 75A250). The PZT transducer finally transformed the amplified voltage signal into a mechanical oscillation that released plate waves into the $CaF_2$ plates. Due to the continuous operation mode, standing wave patterns were generated by multiple reflections that depend on the material, the frequency and the geometrical boundary conditions of the sample holder. The receiving PZT transducer was connected to a LeCroy 9310 AM oscilloscope in order to monitor the amplitude of the standing ultrasonic wave. For connecting the electronic devices, shielded standard BNC cables with an impedance of 50 Ohm were used.

For driving the PZT transducer, a resonance frequency was used that was fine-tuned by the output signal. Whereas the main resonance (thickness resonance) was in the range of 4 MHz, a resonance around 50 kHz was selected.

According to a number of simplifications, the sound intensity in the insulin solution can be estimated. The final intensity estimated is in the range of $I$ = 180 mW/cm$^2$ given an average displacement of $\xi$ = 110 nm. Note that the threshold of sound intensity in diagnostic medical applications is also in the range of $I$ = 100 mW/cm$^2$, such as mentioned above. On the other hand, the intensities are too low to enable the forming of acoustic cavitations. The present set-up thus meets the targets of the intended study.

## 2.2 FTIR Spectroscopy

The unfolding and aggregation of insulin was followed by FTIR-spectroscopy using a Bruker IFS-66 spectrometer equipped with a liquid nitrogen-cooled mercury cadmium telluride detector. 64 spectra with a resolution of 2 cm$^{-1}$ were co-added in order to achieve a good signal to noise ratio. The spectra were processed using the OPUS software of the spectrometer, and the positions of the peaks were determined using the peak seeking function of the software. The temperature was controlled by both a heating and cooling element, and it is measured by a thermocouple that was attached at the thermocell. The temperature was rising with a rate of $\alpha$ = 0.25 K/min. In this way, one obtains one spectrum for every Kelvin that represents an average of the spectroscopic signals in that temperature interval.

## 2.3 Preparation of Samples

Insulin from bovine pancreas [11070-73-8] was obtained from Sigma Aldrich and was dissolved in $D_2O$ [7789-20-0] obtained from Cambridge Isotope Laboratories, Inc. (99.9%). The solvent $D_2O$ was selected because the bending vibration of $H_2O$

interferes with Amide I band of proteins. The final concentration was 50 mg/mL. The pD of 1.2 was adjusted by adding DCl (20wt% in $D_2O$ 99+atom%D, [7698-05-7], Janssen Chimica). Between preparation and measurements, there was sufficient time for enabling deuterium water exchange (minimum one hour).

# 3 RESULTS AND DISCUSSION

## 3.1 Results on FTIR Study of Ultrasonic-assisted Fibrillogenesis

The unfolding and aggregation of insulin can be monitored by FTIR spectroscopy. This is possible using the amide I band that is sensitive to the transformation from the α helical into the β sheet structure. In Figure 2, the transition from the α-helix conformation which is located at around 1650 $cm^{-1}$ to the β-sheet conformation which is located at around 1628 $cm^{-1}$ is shown. The wavenumbers as well as the temperature agrees with literature values (Bouchard, Zurdo et al., 2000). At starting transition, a small shoulder at the lower wave number appears, which indicates the presence of β-sheet structure and as the temperature rises it slowly becomes a separate band. One should keep in mind that there are always remaining α–helical structures even after the complete formation of fibrils.

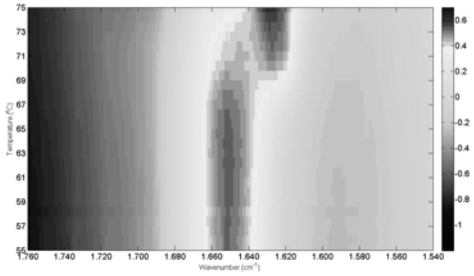

Figure 2: Contour plot of the infrared spectra. The amplitudes are represented by the grey-scale. The amide I band in the middle shows the unfolding at 71 °C.

The peak position of the amide I band as a function of temperature is shown in Figure 3. The temperature axis is shifted so that the average unfolding and aggregation temperatures of the respective sample series are set to zero. The spreading of the transition temperature does thus represent the variation around the average values. In the respective samples that were subjected to ultrasound, the temperature axis was also shifted by the corresponding unfolding and aggregation for the

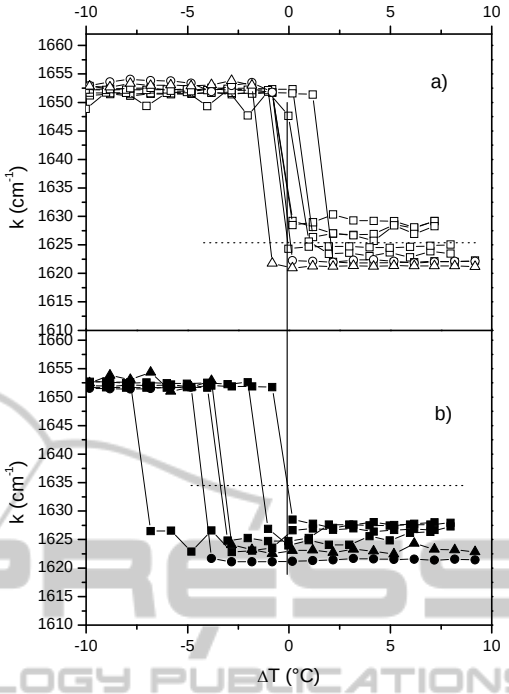


Figure 3: Thermotropic shift of the amide I band of insulin without ultrasound a) and under ultrasonic ultrasonic excitation b). The temperature axis is normalised according to the average transition temperature in the sample series without ultrasound.

non-ultrasonic case. According to Figure 3, b), the transition temperatures are always lower if ultrasound was applied. The variation of the ultrasound assisted shift (between 1 and 7 K) is essentially not due statistics but due to the spatial variation of the ultrasonic effects at the sample that arises from the standing wave pattern. The variations of transition temperatures should thus be attributed to the position of the infrared beam in different sound nodes and antinodes.

## 3.2 Discussion

There are different possibilities to explain the shift of the unfolding and aggregation temperature by ultrasonic excitation. A first and manifest reason is the heating due to ultrasound and the heat dissipation due to the operation of the ultrasonic transducer itself (transducer self-heating, (Wu and Nyborg, 2006)) that account for a difference of approximately 1 K according to thermography tests performed. But the highest difference in unfolding temperature observed is more than 7 K. Therefore it can be expected that other reasons must be taken into account. Electromagnetic effects can be excluded for this case, because the shift of

temperature would be much more constant due to the constant output control of the ultrasonic device, moreover that effect was checked to be negligible. Another possibility would be a local change of the conditions of the electrolytic buffer solution. It is known that ultrasonics is able to change the pH, a phenomenon that is called ultrasonic vibrational potential – for the case that the pH was lowered by ultrasonics a temperature decrease would be explainable. However, this effect was checked to be negligible.

The most probable explanation is the acceleration of the unfolding and aggregation process by ultrasonic induced acoustic micro-streaming (Wu and Nyborg, 2006). One distinguishes between two types of micro-streaming; the simplest type is acoustic streaming in a liquid bulk phase that is sometimes called “quartz wind”. The other type is related to interactions where several kinds of boundaries, surfaces or inhomogeneities are involved. Due to the small liquid layer thickness with respect to the wavelength, one should expect that the second type of acoustic micro-streaming applies to our experiment described above. Acoustic-micro-streaming leads finally to additional mobility inside the liquid that promotes mixing processes.

It is known, that unfolding and aggregation leading to fibrillogenesis is a kinetic process. Aggregation itself is determined by the frequency of mutual contacts between hydrophobic groups, and mixing of the solution would definitely enhance the probability of these contacts. Nonlinear ultrasonic effects will cause micro-streaming in liquids (Suslick, 1988), and they will have an effect similar to the direct mixing of components. In this way, a reduction of the transition temperature should anyway be expected. An interesting reference in this context are studies on the kinetics of protein aggregation on agitation (stirring). Stirring was clearly leading to an increase of the kinetics of insulin fibrillogenesis with respect to an unstirred reference sample (Grudzielanek, Smirnovas et al., 2006). This was accordingly explained by eliminating the diffusion control causing aggregation nuclei immediately start growing. Similar observations are reported for whey protein fibril formation (Bolder, Sagis et al., 2007), here, also the breaking up of immature fibrils is discussed, suggesting a similar behaviour for the shorter fibrils observed in the AFM images of our samples (not shown).

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