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Coupling of a Microfluidic Mixer to a Fourier-transform Infrared Spectrometer for Protein-Conformation Studies

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Abstract: The biological properties of a protein critically depend on its conformation, which can vary as a result of changes in conditions such as pH or following the addition of various substances. Being able to reliably assess the quality of protein structures under various conditions is therefore of crucial importance. Infrared (IR) spectroscopy of the Amide I band of proteins is a powerful method for the determination of protein conformations and further allows the analysis of continuously flowing solutions of the target molecule. Here, a commercial Fourier-transform infrared spectrometer was coupled to a microfluidic mixer to allow the on-line monitoring of protein conformation under varying conditions. The validity of the concept was demonstrated by continuously recording the variations of the IR spectrum of poly-L-lysine resulting from repetitive, pH-induced conformational changes.

Keywords: FTIR · Microfluidic · Protein conformation · Protein folding

1. Introduction

The conformational state of a protein crucially affects its biological activity. For instance, several illnesses such as Alzheimer or Creutzfeldt-Jakob disease originate from the presence of misfolded proteins. It is therefore instrumental to have access to various techniques allowing the determination of the conformational state of proteins. Besides circular dichroism and nuclear magnetic resonance, infrared (IR) spectroscopy has been shown to be a powerful tool for the determination of the secondary structure of proteins and peptides.[1–3] In particular the Amide I band at ~1650 cm^{-1} exhibits features that are characteristic for the secondary structures present in a protein, such as α-helices or β-sheets, and is therefore an excellent indicator of its overall conformation.

The conformation of proteins depends on conditions such as pH, temperature or composition (*e.g.* presence of structure-altering solutes). Several microfluidic systems have been recently described in the literature,[4–8] which allow both the addition of various chemicals and the monitoring of their impact on the structure of proteins (usually in the context of protein folding or refolding investigations). They are combined with several types of IR spectrometers such as transmission-mode Fourier-transform IR spectrometers (TM-FTIR)[4–6,8] or attenuated-total-reflection-mode FTIR (ATR-FTIR).[7] These allow the monitoring of the kinetics of conformational changes. The use of microfluidic channels consumes low amounts of reagents, and permits a rapid and efficient mixing as well as the recording of continuous IR spectra. However, the various prototypes described involve dedicated and often very complex microstructures commercially not available and are therefore restricted to specialized applications.

Here we show that continuous monitoring of the conformational state of model proteins can be performed using a simple, commercial microfluidic mixer from Micronit© coupled to a FTIR spectrometer. This instrument makes use of TM-FTIR which was found to be superior to ATR-FTIR for this application. All the parts of this instrument are readily commercially available, thus allowing general application of this technology.

2. Experimental

2.1 *Reagents*

Hemoglobin (H2500), poly-L-lysine (P2636) and deuterium oxide (364312) were obtained from Sigma-Aldrich (St-Louis, USA).

2.2 *Description of the Instrument*

The layout and connection scheme of the equipment is shown in Fig. 1. Infrared spectra were recorded using a commercial Fourier-transform infrared spectrometer (FTIR) (Nicolet 5700, Thermo Fisher). This device was either equipped with a ZnSe ATR crystal or with a flow chamber consisting of two CaF_2 windows separated by a 50 µm Teflon spacer (both from Specac, Slough, UK). A flow of nitrogen (5.0) and silica gel were used to remove water vapor inside the FTIR. The FC TD26 microfluidic mixer and coupling tubes and nuts were from Micronit (PV Enschede, NL). Two VIT-FIT programmable syringe pumps from Lambda (Berno, CZ) were used to deliver the solutions.

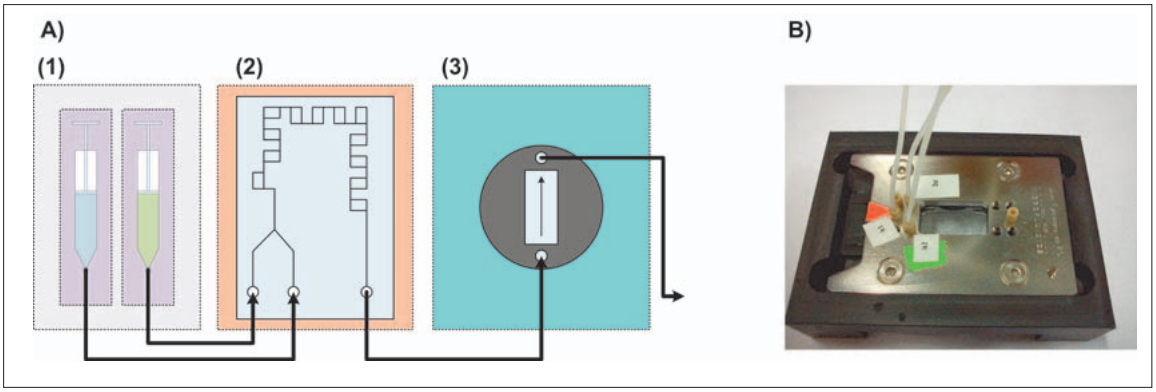

Fig. 1. A) Scheme of the instrument: (1) 'syringe' pumps, (2) microfluidic mixer with two inlets, (3) measurement cell for recording of TM-FTIR spectra. B) Photograph of the microfluidic mixer.

2.3 *Recording of IR Spectra*

Protein and NaOH solutions at 10 and 1.0 mg/mL respectively were freshly prepared using D_2O as a solvent and dispatched into two syringes connected to the pump devices. Slow flow rates (10 arb. units) were set for each pump. Background (spectrum of pure D_2O) and water-vapor spectra were subtracted to the recorded sample spectra.

During a typical experiment a poly-L-lysine solution was pumped through the micromixer. At specific moments the flow of NaOH was switched on and off to monitor the changes induced by this drastic pH modification on the recorded spectra. The time required to perform a measurement was about 2 minutes.

3. Results and Discussion

The system developed here for the continuous monitoring of protein conformational states is described in Fig. 1. It consists of a commercial microfluidic system of Micronit© comprising two inlets connected to a series of mixing chambers. The outlet is connected to a measurement cell made of two water-resistant and IR-transparent CaF_2 windows and a 50 µm thick Teflon spacer. The recording of IR spectra in transmission mode using this measurement chamber yielded spectra exhibiting an excellent signal to noise ratio (see Fig. 2A). Measurements were performed in D_2O in order to avoid spectral overlapping of the bending-vibration band of H_2O with the Amide I band.[2,6]

Several designs in the literature give preference to the recording of spectra using ATR-IR instead of TM-FTIR.[7,9] In our configuration however, the quality of ATR-FTIR was much poorer (Fig. 2B). The cause for this seems to be the smaller volume probed in the ATR mode. In addition, the use of ATR-FTIR resulted in a poor reproducibility of results and a kind of 'memory' effect that is believed to originate from the deposition of protein onto the surface. Since ATR-FTIR only probes a thin slice of a few µm above the surface, the recorded spectra can be strongly affected by the deposition of protein.

Correctly folded proteins could be distinguished from denatured, unfolded ones using their mid-IR spectra as shown in Fig. 2A for hemoglobin. Denaturation of hemoglobin resulted in a shift of the Amide I band from 1652 cm^{-1} to 1641 cm^{-1} and of the Amide II band from 1548 cm^{-1} to below 1500 cm^{-1}. The shift of the Amide I band is characteristic for a transition from α-helices to a disordered state.[3] The strong shift of the Amide II band might be due to an increase in the isotopic $^1H/^2H$ exchange of the amide N-H hydrogen atom, which is known to induce such dramatic changes.[1,3,10] Denaturation of the protein exposes hydrogen atoms buried in the α-helices allowing their faster replacement by deuterium.

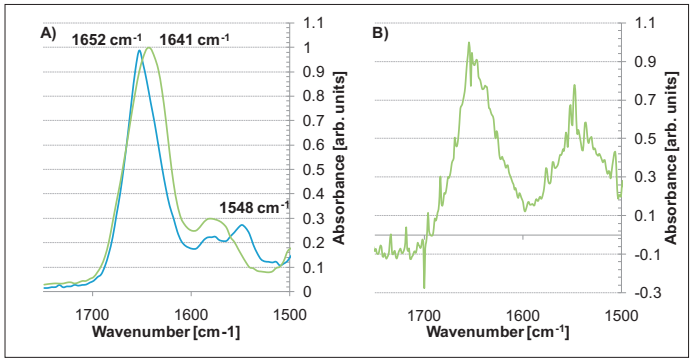

Fig. 2. Infrared spectra of the Amide bands of proteins A) Spectra recorded in the TM-FTIR mode: correctly folded hemoglobin at pH = 7 (blue curve) and unfolded hemoglobin at pH ~ 12 (green curve). B) Spectrum recorded in the ATR-FTIR mode: hemoglobin at pH = 7.

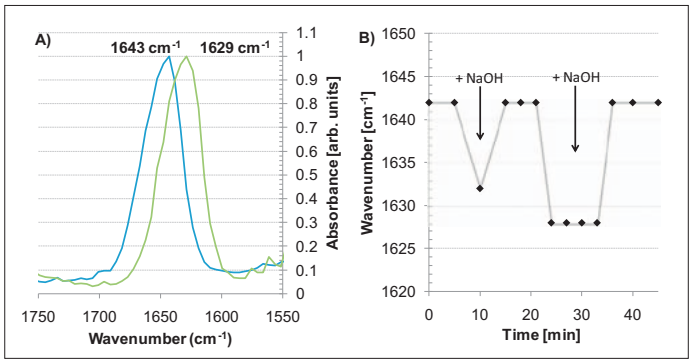

Fig. 3. A) Infrared spectra of the Amide band of poly-L-lysine at pH = 7 (blue curve) and pH = 12 (green curve). (B) Variation of the position of the maximum of the Amide I band of poly-L-lysine at the outlet of the microfluidic mixer as function of time. While poly-L-lysine at the inlet of the mixer is disordered at pH 7, it undergoes a transition to an α-helical conformation upon the repetitive addition (indicated by arrows) of a denaturing solution of NaOH 50 mM at pH ~12. The variations of the spectrum resulting from the change in conditions were continuously monitored.

Infrared spectra of proteins could be continuously monitored at the outlet of the microfluidic mixer. High-quality spectra were recorded in less than a minute. The second channel of the microfluidic mixer allowed the addition of various conformation-altering substances to the protein solution. Fig. 3A shows for instance the evolution of the infrared spectrum of poly-L-lysine following abrupt pH changes. Increase of pH above 10 resulted in a shift of the maximum for the Amide I band from 1643 cm^{-1} to 1629 cm^{-1}, an effect which has been attributed to a transition from an unordered to an α-helical conformation.[10,11] Equilibration to new conditions occurred in less than five minutes in accordance with the slow flow rate and could be continuously monitored. The IR spectrum reproducibly and repetitively varied following the pH jumps (Fig. 3B). As an alternative to the continuous-flow mode, slow kinetics of folding/unfolding could probably be monitored in a stopped-flow mode.

In conclusion, this instrument combining a microfluidic mixer and a FTIR-spectrometer can be assembled from commercially-available parts, is versatile and easy to use, and enables the monitoring of a variety of chemically-induced conformational changes.

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