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Communication

A novel microfluidic system for the rapid analysis of protein thermal stability

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We describe a simple microfluidic device for the rapid analysis of protein thermal stability using a novel imaging method. The change in UV absorption upon thermal denaturation or aggregation of proteins is used to get a spatial image of proteins' folding or aggregation state along a linear temperature gradient.

Introduction

Proteins can self-assemble in the cellular environment under specific conditions such as pH, temperature, ionic strength etc. Protein folding is the physical process by which a polypeptide folds into its characteristic and functional three-dimensional structure from a random coil[1-4].

When the protein folding process goes wrong, (commonly referred to as misfolding), it can induce many diseases including cancer[3, 5-7]. Hence it is very important to study the protein folding process. Through research, the true cause of many diseases might be found and may enable the design of more effective drugs.

Moreover, therapeutic functional proteins must have the correct sequence of amino acids and also the correct structure, i.e. tertiary structure. However, during production, formulation, storage, transportation etc, proteins tends aggregate due to protein folding intermediates and misfolding[8]. Understanding, monitoring and controlling such processes are crucial for the production and ultimate safety and efficacy of such therapeutic proteins.

The physical and chemical processes of protein folding have generated tremendous interest in the multidisciplinary field of protein science. Protein folding research paves the way for crucial research directions such as structure-function relationship of proteins, interactions between proteins, and between ligands and proteins[4, 9-11]. The mainstream thinking is that the answer to this question can be attained only by looking at individual protein folding processes. However, due to the small scales of protein conformations and their constantly changing nature, observation of protein folding is faced with severe challenges[12].One of the main parameters to monitor protein folding and unfolding are their thermal stabilities. Thermal stability is a general means to maintain tertiary structure in proteins. Hence the rapid determination of the thermal stability of proteins has many useful applications in protein engineering, drug discovery and productions[8, 13].

The protein folding field has advanced greatly over the past 50 years due to the experience acquired from a multitude of experimental, theoretical and computational approaches[3, 14, 15]. To date there have been many methods for studying the process of protein folding. Experimental strategies have included ensemble methodologies such as NMR, fluorescence and CD spectroscopy, and protein engineering, among many others[16, 17]. Over the last decade, single-molecule FRET (smFRET) has emerged as a prevalent and powerful tool to probe the complex distributions, dynamics, pathways and landscapes in protein folding and binding reactions, leveraging its ability to avoid averaging over an ensemble of molecules[17]. UV absorption is also known to be sensitive to the conformational changes in proteins[18].

Absorbance at 280nm by aromatic side chains, especially tryptophan, is frequently used to monitor conformational changes in proteins as well as to determine protein concentrations[19]. Ultraviolet absorption spectroscopy can be used to provide information concerning protein tertiary structure. Ultraviolet spectroscopy can also be used to monitor the aggregation behavior of a protein by recording the optical density in a non-absorbing region of a protein's UV spectrum. The main advantages of using ultraviolet absorption to detect protein conformational changes are simplicity, speed and the lack of need to introduce additional reagents, although there are disadvantages such as the sensitivity of the measurement[18, 20].

In this paper, we describe a simple microfluidic device for the rapid analysis of protein thermal stability using a novel UV absorption imaging method. The core innovation is that a spatial rather than temporal method is used. I.e. the temperature of the sample does not have to be ramped up over time, as an imposed temperature gradient allows simultaneous measurement of the sample over a wide range of temperatures. This method allows for the rapid determination of the temperature of denaturation and we have applied it to investigate the thermal stability of proteins under differing pH buffer conditions and differing protein concentrations[21, 22]. Changes in pH of buffer solution and protein concentrations often induce changes in the protein's thermal stability which manifest as changes in the midpoint denaturation temperature. By imposing a linear temperature gradient across the microfluidic channel and imaging the UV absorption spatially along the channel, we are able to obtain a rapid determination of the protein's denaturation characteristics. The use of a microfluidic device enables rapid analysis, low sample volumes and low-cost of analysis per sample. This microfluidic approach would also enable more advanced strategies in the future; for

example, microfluidic mixing can provide enhanced opportunities for studying protein folding in systems involving multiple components or using chemical denaturation agents (e.g. applying chemical rather than thermal gradients).

Furthermore fluorescence based techniques can be implemented in a similar system to provide additional information and sensitivity and with greater structural complexity.

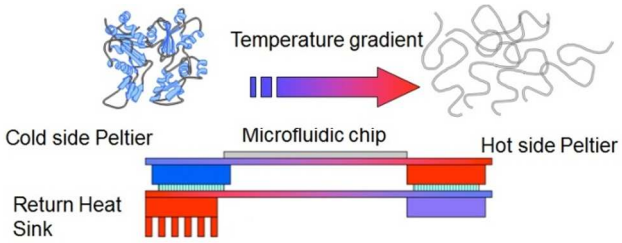

Fig.1 Schematic of thermal setup.

However, even in its current form, we believe this method will provide fast, cheap and convenient analysis of the stability of proteins and could find useful applications such as for QC/QA[23, 24].

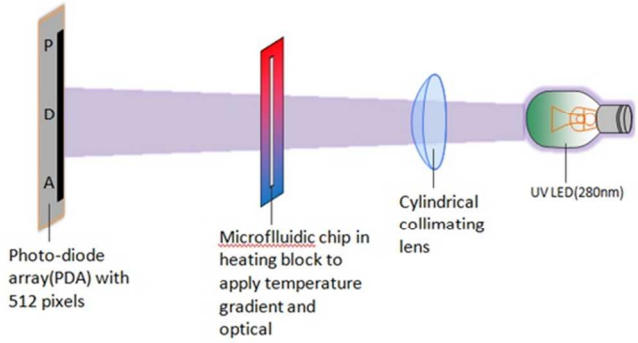

Fig.2 Schematic of optical setup.

PMMA microfluidic chips were used to rapidly determine melting temperature of proteins. To fabricate the microfluidic chips, three 500 micron wide parallel channels were machined in 500 micron thick PMMA. Fig.S1 shows a photograph of the actual chip. Two 200 micron thick PMMA sheets (one with access holes) were then bonded on top and bottom to enclose the channels (using thermal bonding).To ensure good optical transmission at 280nm, a UV transparent grade of PMMA was used for all three layers (Clarex UV transmission filter).

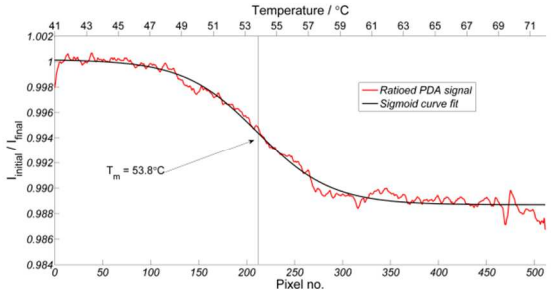

Fig.3. Ratio of final state versus initial state PDA intensities showing sigmoid curve. The midpoint of the curve corresponding to the temperature of unfolding, Tm, was calculated to be 53.8°C.

Fluidic connections were bonded to the inlet and outlet channels. A one-way check valve was used to ensure fluid did not travel backwards after injection. The sample was loaded into a 1ml plastic syringe and approximately 50µL injected rapidly (within ~1s) into the chip for each run. Although this injection volume is smaller than most common cuvettes, we can reduce the sample volume to an even smaller size in the later stage work. A manual valve on the outlet was closed immediately after injection to prevent flow during data acquisition. This system also allowed some pressure to be maintained in the channel after injection which served to minimize bubble formation. Fig.S2 shows a schematic of the fluidic system. Two Peltier elements were used with aluminium blocks to apply a thermal gradient across the chip. One Peltier element was used to apply heat at one and the second, (reversed), was used to remove heat from the cold end (Fig.1). The aluminium blocks were used to distribute an even temperature gradient across the chip and were clamped either side of the chip and also connected at the ends to complete a thermal "circuit".

A copper sheet with a 500 micron slit was used both to assist heat transfer and also to provide an optical slit such that light only passed through the microfluidic channel. The chip was clamped into the heating blocks using screws. Three thermocouples were bonded to the copper sheet to provide temperature measurements across the chip surface and thermocouples were embedded into the hot end and cold end of the heating blocks. The five thermocouple readings were recorded using a data acquisition card and Labview program. Two PID controllers were used to control the temperature of the hot and cold end by regulating the current applied to each Peltier element. Fig.S3 shows a photograph of the experimental setup.

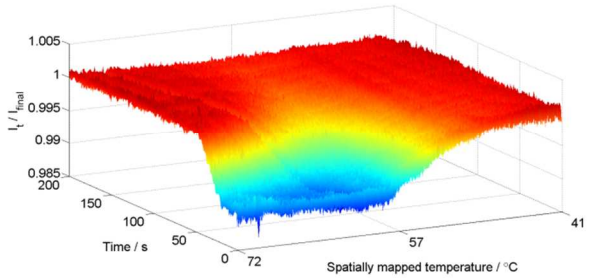

Fig.4 3D plot of ratioed PDA data (intensity, I at time t / final state intensity) of 2mg/ml HEWL showing change in absorbance over both time and space (pixel number) which has been mapped to the temperature gradient across the chip. (Note: axes have been reversed to provide better perspective view).

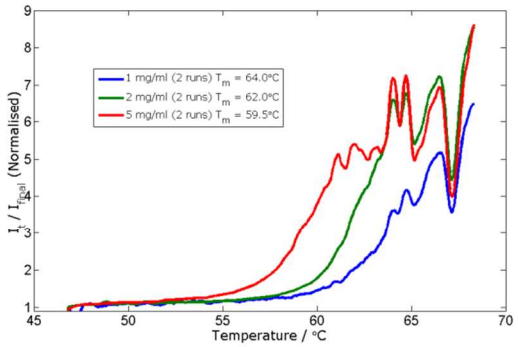

Fig 5. Carbonic Anhydrase unfolding at different concentrations (all at pH 5.3): At higher concentrations, 1 mg/ml, 2 mg/ml, and 5 mg/ml, Tm is reduced.

A 280nm UVTOP LED was used as the light source for detection (Sensor Electronic Technology, Inc). Fig.2 shows a schematic of the optical path. The LED provided a stable and intense monochromatic UV source with approximately 10nm bandwidth. The LED was current pulsed using a drive circuit at 1kHz with a 1% duty cycle and synchronized with the acquisition rate of the PDA detector. Pulsing is necessary to ensure maximum lifespan of the UV LED and synchronization is required to avoid aliasing errors. A quartz cylindrical lens was used to collimate the UV light before passing through the optical slit and microfluidic chip. A Hamamatsu linear 512 pixel photodiode detector array (PDA) was used to image the absorption signal passing through the microfluidic channel. The data acquisition rate of the detector was set to 5Hz. A USB data acquisition module (Data Translation, Inc.) and Labview software were used to control and acquire data during experimental runs.Further detailed experimental setup and procedure are described in the Supporting Information.

The system was first tested with Hen Egg White Lysozyme. Approximately 50 µL of 2 mg/ml HEWL in sodium acetate buffer at pH 5.3 was injected into the microfluidic chip as described in the experimental procedure. Data were acquired and analyzed with typical results shown in Fig.3. More experimental data on HEWL can be found in Fig S4. After unfolding, aromatic side chains such as tryptophan can yield a drop in absorbance at 280 nm upon unfolding which exposes these side chains to the buffer environment. In order to better visualize the data, 3D plots of the unfolding in both time and space can obtained. Figure 4 shows such a plot which clearly shows how the unfolding progresses over time at over the length of the channel (corresponding to the temperature gradient imposed across it).

This results in a sigmoid curve which is shown in Fig.3. From this curve we can obtain the temperature of unfolding which we calculated to be 53.8°C ± 0.5°C. To confirm this response was due to the protein rather than optical/density changes across the chip, we verified that no response could be observed when using only buffer solution.

We also performed protein stability studies using Carbonic Anhydrase. In contrast to the HEWL data, we observed an increase in UV absorption at the hot end rather than a decrease. In addition, the change in absorption signal is much greater. We attribute these results to protein aggregation rather than the exposure of aromatic side chains upon unfolding. Despite this, it is still possible to obtain a relative measure of the unfolding temperature in a similar manner as before.

Both protein concentration and the pH of buffer solution can affect the stability of the proteins. In this study, typical UV absorption data of CA at different protein concentrations (1, 2 and 5 mg/ml all at pH 5.3) and at different buffer pHs (4.4, 5.3, and 7.0) where measured using our system and are displayed in Fig. 5 and S5, respectively.

Fig.5 shows CA concentrations of 1 mg/ml, 2 mg/ml, and 5 mg/ml, melting temperatures of 64.0°C, 62.0°C and 59.5°C were observed respectively, indicating at higher concentrations T_m is reduced. It is known that there should also be a destabilizing effect at lower pH on the melting temperature value. This is what we have validated in our system as shown in Fig. S5. We further validated our results against data obtained with a conventional UV-Vis spectrophotometer with broad agreement (Figs S6-S7).

In summary, we used a simple microfluidic device for the rapid analysis of protein folding and thermal stability using a novel label-free imaging method. Changes in UV absorption at 280 nm along the length of the microfluidic channel were correlated to a temperature gradient imposed across it. By imposing a temperature gradient, spatially along a microfluidic channel, we avoid the need to ramp up and down the temperature of the system over time. Since the microfluidic system exhibits a low thermal mass, rapid analysis can be achieved when combined with our injection system. This allows determination of thermal protein denaturation in less than three minutes. In addition, the total sample volume consumed is typically less than 100µL, including waste due to filling of the chip and flushing the channel prior to injection. In future work, we are going to monitor a thermal shift i.e. ΔT_m. Thermal shift is very useful for research into protein-ligand interactions. Further work will be done to set up such ΔT_m verifying systems.

Whilst further work is required to reduce systematic noise and more fully characterize the system, these results indicate that rapid analysis of the thermal stability of proteins can be achieved using our system. Critical solution parameters (e.g., pH, concentration, and temperature) that perturb the stability of proteins can be rapidly evaluated. The new approach has the potential to significantly shrink the sample volume, cost, and assay time, providing an alternative tool for rigorously investigating protein biology. We believe this system could be particularly useful for QC/QA applications in which the rapidanalysis of thermal stability parameters is required with minimal sample loss.

Notes

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