Rapid Characterization of Biomolecules’ Thermal Stability in a Segmented Flow-Through Optofluidic Microsystem

Zdenka Fohlerová1,2, Hanliang Zhu3, Jaromír Hubalek1,2, Sheng Ni4, Levent Yobas4, Pavel Podesva1, Alexandr Otahal2 & Pavel Neuzil1,2,3 ✉

Optofluidic devices combining optics and microfluidics have recently attracted attention for biomolecular analysis due to their high detection sensitivity. Here, we show a silicon chip with tubular microchannels buried inside the substrate featuring temperature gradient (∇T) along the microchannel. We set up an optical fluorescence system consisting of a power-modulated laser light source of 470 nm coupled to the microchannel serving as a light guide via optical fiber. Fluorescence was detected on the other side of the microchannel using a photomultiplier tube connected to an optical fiber via a fluorescein isothiocyanate filter. The PMT output was connected to a lock-in amplifier for signal processing. We performed a melting curve analysis of a short dsDNA – SYBR Green I complex with a known melting temperature (Tm) in a flow-through configuration without gradient to verify the functionality of the proposed detection system. We then used the segmented flow configuration and measured the fluorescence amplitude of a droplet exposed to ∇T of ≈ 2.31 °C mm⁻¹, determining the heat transfer time as ≈ 554 ms. The proposed platform can be used as a fast and cost-effective system for performing either MCA of dsDNAs or for measuring protein unfolding for drug-screening applications.

Temperature can significantly affect the biological systems of living organisms in terms of cellular morphology, metabolism, growth, and cell death1,2. On a molecular level, temperature influences the structure and function of biomolecules such as proteins and nucleic acid3,4. The denaturation of biomolecules under relatively high temperatures is utilized in real-time polymerase reactions (qPCR), subsequent melting curve analyses (MCA) of double-stranded deoxyribonucleic acid (dsDNA), and differential scanning fluorimetry (DSF) of proteins.

The MCA analysis of dsDNA stained with an intercalator dye such as SYBR Green I is conducted by gradually ramping the temperature while monitoring the intensity of the emitted fluorescence (F). The dsDNA melting temperature (Tm) is defined as the temperature at which 50% of presented dsDNA molecules separate into single-stranded forms. This value depends on the number of base pairs and their composition. Typically, while the MCA is performed using the qPCR systems, they can be also used to determine the protein-unfolding curve. However, qPCR systems currently available are bulky, costly, and slow.

Advances in microfabrication technology have enabled the miniaturization of bioanalytical tools with precisely controlled temperatures, allowing for quick investigations into the thermal effects on biological molecules using samples with a small volume. Heating methods and the thermal conductivity of the device’s materials are essential to create thermally stable systems with either homogeneous distribution of temperature or temperature gradient (∇T).

Heat conduction-based microfluidic techniques, especially in a droplet configuration, have attracted attention due to their applicability in DNA amplification, protein analysis, single-cell assays, and chemical synthesis.

1Central European Institute of Technology, Brno University of Technology, Purkynova 123, 612 00, Brno, Czech Republic. 2Department of Microelectronics, Faculty of Electrical Engineering and Communication, Brno University of Technology, Technicka 3058/10, 61600, Brno, Czech Republic. 3Ministry of Education Key Laboratory of Micro and Nano Systems for Aerospace, School of Mechanical Engineering, Northwestern Polytechnical University, 127 West Youyi Road, Xi’an, Shaanxi, 710072, P.R. China. 4Hong Kong, University of Science and Technology, Clear Water Bay, Hong Kong, P.R. China. ✉ e-mail: pavel.neuzil@nwpu.edu.cn
The characteristics of the thermal behavior of water in oil droplets within microfluidic channels were studied per the generation and transport of liquid droplets within local heating at the breakup location of T-junctions and flow-focusing systems either at the downstream channel or with the heater placed at the end of the transport channel. However, the temperature dependency of droplet physical properties represented by density, viscosity, and interfacial tension between the continuous oil phase and water-based droplet may complicate high-performance droplet microfluidics. Further, the mathematical simulation of droplet behavior under heating is complicated due to the necessity of 3D models of a droplet-based system. In addition to the investigation of the temperature dependence of a droplet’s physical properties with localized heating on the microfluidic chip, the creation of on-chip \( \nabla T \) has been challenging as one can study microtubule polymerization along the \( \nabla T \) or MCA for single nucleotide polymorphisms, both performed in a flow-through configuration. Moreover, the MCA in microfluidics has been conducted using either solid phase multiple analysis, which requires DNA immobilization, or the immobilization of a free liquid phase restricted for a single analysis. However, micro-/nanoscale dimensions of channels and the surrounding environment and flow-through configuration may complicate the determination of the temperature distribution in thermally driven microdevices. Thus, heat transfer in such a system should be characterized in heat fluxes by thermal convection, thermal radiation, or the heat transfer time between the sidewall of a chip and liquid.

Optical systems have been used as a detector for analyzing bio/chemical samples as well as their miniaturization utilized in lab-on-a-chip (LOC) applications such as portable qPCR for DNA or RNA diagnostics. Recently, the combination of optics and microfluidics has attracted great attention due to the combination of highly sensitive bio-detection with LOC technology. By fully integrating optical functions on a chip instead of using bulky external optics, these optofluidic systems lower the cost and downsize the system, making it promising for point-of-care diagnosis. The detection of fluorescence remains valuable in many bio-applications. Therefore, the development and characterization of a multifunctional optofluidic lab-on-a-chip was introduced for sample analysis by fluorescence and Raman spectroscopy or by the measurement of the absorbance and fluorescence of droplets in segmented flow. Further, the fluorescence detection and quantification of the Ebola virus using hybrid optofluidic integration have been reported, as have platforms for real-time visualization of viruses in complex media or cell phone-based imaging cytometers.

Here, we demonstrate the MCA of dsDNA with the sensitive detection of fluorescence inside a microchannel as part of an optical microsystem exposed to \( \nabla T \) value. We used a modulated laser as the light source and a photomultiplier tube (PMT) as the fluorescence detector with its output signal processed by a lock-in amplifier to suppress environmental noise. The proposed concept of a thermally regulated optofluidic platform was first characterized in flow-through configuration of dsDNA bulk. More importantly, we presented the MCA analysis in segmented flow configuration as a tool for rapid determination of a biomolecules’ stability.

**Materials and Methods**

**Chip fabrication.** We designed the chip layout with a size of \((6 \times 27) \text{mm}^2\) using the Nanolithography toolbox software with the aim of having all fluid/optics inputs/outputs at the chip sidewalls to provide a robust solution. The layout consisted of \(\approx 30 \mu \text{m}-\text{wide} \) lines subsequently forming buried microchannels by a process similar to earlier ones that used two parylene-\(C\) depositions. The first parylene-\(C\) was employed as a mask with conformal coating on the sidewalls and a second parylene-\(C\) layer to seal the created channels. We designed our device to insert both capillaries and the optical fibers from the sides (Fig. 1A), allowing a more robust configuration than the previous one, which had its capillaries inserted vertically.

We started the fabrication process using Si wafers with a diameter of \(\approx 100 \text{mm} \) and an unusual thickness: \(\approx 1 \text{mm}\), which is thicker than the ordinary wafer thickness of \(\approx 450 \mu \text{m} \) to \(550 \mu \text{m}\) since the targeted microchannel diameter was the same: between \(\approx 450 \mu \text{m} \) and \(\approx 550 \mu \text{m}\).

First, we coated the wafers with a \(\approx 10 \mu \text{m}-\text{thick} \) positive photoresist (PR), followed by a prebake at \(\approx 110 \text{°C} \) for \(\approx 165 \text{~s}\). The wafers were exposed to ultraviolet light with a dose of \(\approx 1600 \text{~mJ/cm}^2 \) for lithography and developed in a KOH-based developer for \(\approx 300 \text{~s}\), patterning the shape of the trenches at a designed width of \(30 \mu \text{m}\). The Si was etched with the Bosch process to a target depth of \(\approx 500 \mu \text{m}\), then, we stripped PR in N-methyl-2-pyrroldinone solution at \(\approx 80 \text{°C} \) for \(\approx 600 \text{~s}\) (Fig. 1B).

The wafer was then coated with a \(\approx 1.5 \mu \text{m} \) parylene-\(C\)-layer (Fig. 1C), and this layer was photo-blasted at the trench bottoms using a femtosecond laser working at a principal wavelength of \(\approx 515 \text{~nm} \) using pulses with a duration of \(\approx 300 \text{fs} \) and a maximum amplitude of pulse energy of \(\approx 200 \mu \text{J} \) (Fig. 1D). The wafer was exposed to \(\text{XeF}_2 \) vapor to isotropically etch Si through the opening in the trench bottom, forming a buried cylindrical microchannel with a diameter of \(\approx 500 \mu \text{m}\) (Fig. 1E). The parylene-\(C\) was removed using \(\text{O}_2 \) plasma with a set power of \(300 \text{~W} \) for a duration of \(1 \text{~h}\) (Fig. 1F). The wafer was diced into individual chips, and the second parylene-\(C\)-layer was deposited with a thickness of \(\approx 30 \mu \text{m} \) to cover the microchannel with hydrophilic materials and to seal the \(\approx 30 \mu \text{m}-\text{wide} \) trench (Fig. 1G). Both optical fibers and microcapillaries were inserted into the chip and sealed with epoxy resin. Details of one port are shown in Fig. 1H.

**Optofluidic.** The Si chip had a buried microchannel system consisting of a through channel and three inlets connected via a cross junction and a single outlet. The through channel also served as a light guide with an optofluidic path with a length of \(\approx 24 \text{~mm}\) (Fig. 2B). The diameter of the buried microchannel was chosen to be \(\approx 500 \mu \text{m}\) (Fig. 2A), sufficiently large for both the optical fiber and the capillary to be inserted inside the chip to form a stable, robust, and autonomous system. The chip was placed on two brass blocks with a distance of \(\approx 13 \text{~mm} \) (Fig. 2C). Each block had its own heater and sensor connected to a proportional integrative derivative temperature controller. The heaters’ temperature was set to \(\approx 50 \text{°C} \) and \(\approx 80 \text{°C} \), respectively, forming a \(\nabla T \) value of \(\approx 2.31 \text{~°C/mm} \). We inserted two multimode optical fibers with a core and cladding diameter of \((200 \pm 4) \mu \text{m} \) and \((220 \pm 2) \mu \text{m} \), respectively, both (mean \(\pm\) standard deviation) into the through microchannel of the chip. Each...
The high power of the laser interacting with the fluid inside the microchannel could affect the measurement as the power corresponds to the power density of $\approx 127$ mW·mm$^{-2}$. Nevertheless, the photothermal effect should...
not be very severe. First, the oil/water interacts with light at a rather low nominal wavelength of 471 nm, but there is, of course, an interaction with fluorescence-producing dyes, such as SYBR Green I in presence of dsDNA, which warms the water-based sample. The photothermal effect was determined earlier using 500 times more powerful laser per unit of area, which only warmed the water by \( \approx 6 \) K\(^3\). Finally, we performed a relative measurement, not absolute, and thus heating of the sample by photothermal effect is compensated.

The fluid was connected to the chip via silica capillaries with internal and external diameters of \( \approx 100 \mu m \) and 360 \( \mu m \), respectively, to inputs and output, which were sealed with epoxy resin (Fig. 2D). We used a pressure-controlled system to control the fluid flow rate (\( \nu \)). The chip was even designed to be able to generate segmented flow using a cross-junction; we formed it externally using a double T-junction as before\(^3\), since the externally generated segmented flow was more stable than the one formed internally.

**Chemicals.** Synthetically prepared dsDNA has a length of 17 bps with a sequence of 5‘-TCT GCT GTC ACA ACT AA-3‘. The \( T_m \) value of the dsDNA is \( \approx 60.6 \) °C as verified by a commercial qPCR system. We prepared the test solution by mixing this dsDNA at a concentration of \( \approx 20 \) nM with SYBR-Green I diluted 10,000 times in a Tris-EDTA buffer (1×). We also used this buffer with no dsDNA as a reference. An oil phase of hexadecane supplemented with 2% of SPAN-80 surfactant was used as an immiscible continual phase for segmented flow analysis.

**Results and Discussion**

### Mathematical calculation of heat distribution.

Devices operating at elevated air temperatures with the sample flowing through are subject to heat losses by convection, radiation, and heat flux due to sample flow, possibly affecting desired temperature distribution as well as its \( \nabla T \). We will only briefly describe the physical analysis, its numerical modeling, and experimental verification by infrared (IR) imaging. Details of those three methods were recently described\(^3\).

The system comprised a silicon chip with a thicknesses \( t \) and widths \( w \) supported by a pair of heaters (Fig. 2B), resulting in a heat flux \( P_1 \) between the heaters through the microfluidic chip as:

\[
P_1 = \frac{\lambda_{Si} \cdot w \cdot \Delta T}{L},
\]

where \( \lambda_{Si} \) is the thermal conductance of Si, \( L \) is the chip length, and \( \Delta T \) is the \( T \) difference between the heaters. The resulting value of \( P_1 \) was calculated to be \( \approx 2.42 \) W.

The convection (\( P_2 \)) in air was:

\[
P_2 = D \cdot w \cdot h \cdot (T - T_i),
\]

where \( D \) is the distance between the heaters, \( h \) is the convection coefficient and \( T_i \) ambient temperature, giving an amplitude of \( P_2 \) as \( \approx 259 \) mW, which is 10.8% resulting in minor non-uniformity of the \( \nabla T \) along the microchannel.

The power dissipated (\( P_3 \)) due to a water-based sample (sample) \( \nu \) influence can be calculated by:

\[
P_3 = \nu \cdot \rho \cdot c \cdot \Delta T_w,
\]

where \( \rho \) is the specific mass of the sample and \( c \) is the sample heat capacitance.

Finally, there is also a radiation power (\( P_4 \)) emitted influencing the \( \nabla T \) value. The \( P_4 \) amplitude is defined by the Stefan-Boltzmann law:

\[
P_4 = D \cdot w \cdot \varepsilon \cdot \sigma \cdot (T^4 - T_1^4),
\]

where \( \varepsilon \) is surface emissivity, and \( \sigma \) is the Stefan-Boltzmann constant. We calculated the values of \( P_1, P_2, P_3, \) and \( P_4 \) as before\(^3\) and determined the total values of \( (P_2 + P_3 + P_4) \) are only 9% of the \( P_1 \); thus, their influence on the \( \nabla T \) can be neglected.

We neglected different temperatures along the chip to make the analytical estimation simpler. It should be done more complexly, using the integral of the function along the gradient. Nevertheless, the finite element modelling (FEM) does take the local temperature into account.

**FEM and IR imaging.** We modeled the microfluidic chip as well as simplified heaters in CAD software and transferred them to the finite element modeling software COMSOL Multiphysics. Then we modeled temperature distribution along the microchannel due to convection, radiation, and sample \( \nu \) using Heat Transfer in Solids and Fluids and Creeping Flow modules in a fashion similar to before\(^3\). Here, we only show the model mesh (Fig. 3A) and calculated temperature distribution within the chip and the heaters (Fig. 3B). The simplified detail of the chip edge showing the buried microchannel is shown in Fig. 3C, where the red line shows the location of temperature data extraction for subsequent analysis. We performed FEM of the system with no external influence, with convection, radiation, and both to extract the temperature values inside the channel (Fig. 3D). We also showed the effect of convection and radiation in the inset. Finally, we checked the influence of the sample \( \nu \) (Fig. 3E), shown in detail in the inset.

Then we assembled the system and imaged the surface temperature using an IR camera after attaching the carbon tape on the chip surface to achieve a uniform emissivity close to unity. The chip was mounted on two heaters with the temperature set at \( \approx 50 \) °C and \( \approx 80 \) °C in order to create the \( \nabla T \) along the microchannel inside the chip. The first set of IR images was captured without \( \nu \) of the water sample (Fig. 4A), and the second set of images was taken with the \( \nu \) set to 0.5 \( \mu L \cdot s^{-1} \) (Fig. 4B), all in a monochromatic fashion. Here, we used false colors for contrast.
enhancement. The temperature profile alongside the center microchannel—indicated by the black line in Figs. 4A and 4B—was extracted from the IR images and plotted in Fig. 4C. We found that the value of $\nabla T$ between the two heaters was constant, confirming the negligible influence of convection and radiation on heat losses as simulated by FEM. The $\nu$ values of up to $0.5 \, \mu$L·s$^{-1}$ had only a marginal difference on the setup system shifting the $T$ amplitude by $(-2.8 \pm 4.5) \times 10^{-4}$·A.U. (mean ± measurement error) (Fig. 4C inset). This temperature shift does show the induced influence, albeit only of a marginal value with a relatively large fitting error.

$T_M$ determination by a flow-through system. Previously, we showed a method of precise $T_M$ determination, temperature calibration, or heat transfer rate measurement using dsDNA in the presence of a fluorescent intercalator such as SYBR Green I or Eva Green for stationary droplets, as well as a flow-through system. For the latter, we used a microscope to capture and analyze fluorescence images from the microchannel having $\nabla T$. Here, we replaced a bulky microscope with a thermally regulated optofluidic platform to characterize the proposed detection system in a bulk sample without $\nabla T$. First, we filled the microchannel with a dsDNA sample. We set $T_1 = T_2$ and gradually increased their temperature values, starting from $\approx 30^\circ$C to $\approx 85^\circ$C with $\approx 5$ K increments while monitoring the $V_L$ amplitude (Fig. 5A). The measurement at a determined temperature was followed by washing the solution that interacted with laser light with a fresh solution; then, we waited for $\approx 10$ s for the $V_L$ signal to stabilize the photobleaching effect. The $V_L$ value for each temperature was read when the temperature changed on both heaters, and the signal dropped and stabilized as indicated by the arrows in Fig. 5A. Then we plotted the $V_L$ values as a function of temperature and performed a nonlinear curve fitting using the sigmoidal Boltzmann function (Fig. 5B black line). Figure 5B also shows negative numerical derivative of $V_L$ with respect to temperature (Fig. 5B blue line). The maximum of this curve is the value of $T_M$ as $(59.9 \pm 0.2)^\circ$C (mean ± fitting error), which was close to the $T_M$ value measured earlier using the commercial qPCR system.

$T_M$ and heat transfer determination using segmented flow. Further, we conducted an experiment using segmented flow to demonstrate a rapid MCA of the dsDNA at $\nabla T$. The segmented flow was generated...
Figure 4. Infrared image of $\nabla T$ on a chip (A) without liquid flow and (B) with the $\nu$ of $\approx 0.5 \mu L/s$. (C) The extracted temperature as a function of $L$ with the slope representing the $\nabla T$. Black line and red line represent $\nu$ of $0 \mu L/s$ and $\approx 0.5 \mu L/s$, respectively. Inset: the plot of difference in extracted values with and without $\nu$.

Figure 5. (A) $V_L$ amplitude as a function of time with the temperature of both heaters set to values in the range from $\approx 30^\circ C$ to $\approx 85^\circ C$ using dsDNA with $T_{M}$ value of $\approx 60.6^\circ C$ with stagnant sample. Each arrow indicates the instance when the $V_L$ value was read for the stated temperature. (B) Nonlinear curve fitting of $V_L$ values as a function of temperature extracted from (A) (black squares) using the Boltzmann (sigmoid) curve (black line with squares) and its derivative (blue line) to determine the value of $T_M$. The error bars correspond to standard deviations (SD) from three measurements.
off-chip using a double T-junction, as it was found that the uniformity of produced segments was more stable than one generated within a heated chip.

We controlled the droplet generation as well as the ratio between oil and water phases using external p values set from \( \approx 25 \text{kPa} \) to \( \approx 75 \text{kPa} \), respectively. We recorded the \( V_L \) signal for \( \approx 250 \text{s} \), getting its value to alternate between \( \approx 300 \text{mV} \) and \( \approx 3 \text{V} \) for reference droplets and the sample, respectively (Fig. 6A), with the length of the droplet estimated to be \( \approx 200 \mu \text{m} \) (Fig. 6A inset).

The \( V_L \) amplitude increases above the baseline corresponding to the droplet entering the optofluidic path, reaching the maximum when the whole droplet is inside the optofluidic microchannel (Fig. 6B). The droplet there was exposed to \( \nabla T \) inside the microchannel; thus, the value of \( V_L \) dropped. This decrease of the \( V_L \) value is nonlinear due to the nature of dsDNA's melting characteristic. We extracted the part of the curve corresponding to \( \nabla T \) exposure (red arrowed line) and performed a non-linear curve fitting using the Boltzmann (sigmoid) function with respect to time, subsequently converting time to location and temperature39. The fitted curve was processed by derivation \(-dV/dT\) (Fig. 6C) with flow speed \((r)\) values (Table 1) as a parameter. The values of \( r \) were determined by the time \( t \) required for a droplet to pass the whole length of \( \approx 24 \text{mm} \) of microfluidic microchannel and then \( r = t/24 \). Subsequently, we expressed the \( T_M \) as a function of \( r \) values (Table 1 and Fig. 6D). Figure 6D shows how the \( T_M \) values increase with increasing \( r \) corresponding to the heat transfer rate. From this curve, the

**Table 1.** Melting temperature as function of flow rates in droplet-based configuration, showing the duration for MCA as the value of time \((t)\) required for the droplet to pass though the microchannel.

| Set \( p \) (kPa) | \( \nu \) (µL·s\(^{-1}\)) | \( t \) (s) | \( r \) (mm·s\(^{-1}\)) | \( T_M \) (°C) (mean ± SD) |
|-----------------|------------------|---------|-----------------|---------------------|
| \( \approx 25 \) | 0.22             | \( \approx 96.0 \) | \( \approx 0.25 \) | 60.13 ± 0.03        |
| \( \approx 40 \) | 0.28             | \( \approx 40.2 \) | \( \approx 0.59 \) | 60.64 ± 0.08        |
| \( \approx 50 \) | 0.33             | \( \approx 20.9 \) | \( \approx 1.15 \) | 61.19 ± 0.08        |
| \( \approx 60 \) | 0.37             | \( \approx 17.0 \) | \( \approx 1.41 \) | 61.55 ± 0.04        |
| \( \approx 75 \) | 0.45             | \( \approx 14.5 \) | \( \approx 1.65 \) | 61.97 ± 0.05        |

**Figure 6.** (A) Fluorescent emission signal from dsDNA sample and reference droplets moving across \( \nabla T \) on the chip formed by setting the temperature of heaters at values of 50 °C and 80 °C with their distance of \( \approx 13 \text{mm} \). Inset shows the generated fluorescence droplet \( \approx 200 \mu \text{m} \) in the microchannel. (B) Interpretation of fluorescence signal from a droplet passing through the \( \nabla T \) zone in the optical microchannel with the \( r \) value of \( \approx 1.65 \text{mm·s}^{-1} \). (C) Derivation of fluorescence amplitude generated from a droplet as a function of \( r \). (D) Plot of \( T_M \) values as a function of \( r \).
slope can be extracted to calculate the time required for heat to reach the center of the microchannel from the channel sidewalls.

Thus, we performed linear fitting to obtain the slope ($S$) as $(1.28 \pm 0.04)$ K s$^{-1}$ mm$^{-1}$ (mean $\pm$ fitting error). The $S$ value divided by $\nabla T$ of $\approx 2.31$ K mm$^{-1}$ produces a heat transfer time of $\approx 554$ ms. Knowledge of this value can be used to correct the system results when they are collected at different values of $\rho$.

The MCAs are typically performed using commercial real-time PCR systems with a typical temperature ramp rate between $\approx 0.1$ and $\approx 0.5$ K s$^{-1}$, thus corresponding to 500 s to 100 s per test (50 K temperature ramping), excluding sample loading. An ultrafast method to perform the MCA in an astonishing $\approx 50$ ms has been reported. It was based on a droplet placed on a cold Cu substrate with the sample heated by laser irradiation that required a complex setup and careful sample preparation. Our optofluidic platform enables rapid MCA of the sample containing biomolecules such as DNA or proteins. The time needed for the analysis is less than $\approx 30$ s, which makes this platform faster than other systems. That can significantly speed up the MCA and make it suitable for high-throughput screening.

Conclusion
We proposed an optofluidic chip having $\nabla T$ along the microchannel to perform the MCA of dsDNA or protein unfolding. We first numerically analyzed the influence of convection, radiation, and a sample $v$ on the temperature distribution, as well as $\nabla T$ deviation from a constant value. The optofluidic chip was equipped with a buried microchannel with a diameter of $\approx 500 \mu$m. We inserted both optical fibers and microcapillaries inside the chip, forming an integrated and robust autonomous system. The microfluidic channel served as a light guide for sensitive fluorescence detection along the $\nabla T$ inside the chip. We demonstrated this concept by determining the dsDNA $T_m$ in a continuous-flow configuration. We also conducted the MCA in a droplet-based configuration using segmented flow. This concept of fluorescence monitoring in a microchannel exposed to $\nabla T$ represents a fast and cost-effective approach for the characterization of thermal properties of biomolecules. It could be crucial in many areas of biology and chemistry, including the stability of proteins, by exposing them to different environments such as pH, buffer composition and ionic strength, purity control, and protein–ligand interaction. Those applications could have a great impact on drug discoveries as well as the molecular diagnostics of infectious diseases or genotyping.

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