Experimental Protein Molecular Dynamics: Broadband Dielectric Spectroscopy coupled with nanoconfinement

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Protein dynamics covers multiple spatiotemporal scale processes, among which slow motions, not much understood even though they are underlying protein folding and protein functions. Protein slow motions are associated with structural heterogeneity, short-lived and poorly populated conformations, hard to detect individually. In addition, they involve collective motions of many atoms, not easily tracked by simulation and experimental devices. Here we propose a biophysical approach, coupling geometrical nanoconfinement and broadband dielectric spectroscopy (BDS), which distinguishes protein conformations by their respective molecular dynamics. In particular, protein-unfolding intermediates, usually poorly populated in macroscopic solutions are detected. The protein dynamics is observed under unusual conditions (sample nanoconfinement and dehydration) highlighting the robustness of protein structure and protein dynamics to a variety of conditions consistent with protein sustainability. The protein dielectric signals evolve with the temperature of thermal treatments indicating sensitivity to atomic and molecular interaction changes triggered by the protein thermal unfolding. As dipole fluctuations depend on both collective large-scale motions and local motions, the approach offers a prospect to track in-depth unfolding events.

Proteins produce biological activities in living organisms thanks to their 3D-structure and the associated dynamics. The 3D structure relies on chemical interactions between atoms of the amino acids that compose a protein. The molecular dynamics is based on molecular fluctuations, which produce motions from a local scale (amino acid side chain fluctuations) to a larger scale (structural relaxation) to respond to the protein folding and the protein function1–3. Accordingly, proteins dynamics covers several orders of magnitude of spatiotemporal motions from femtosecond to second for molecular fluctuations from Ångströms to nanometers. Basically, fast motions (femtosecond to nanosecond) concern local atomic motions (vibration, side chain motions) while slow motions concern the collective motions of many atoms within larger size areas such as secondary and tertiary structural elements (microsecond to millisecond) up to domains (millisecond to second)4.

To track protein dynamics, several experimental and theoretical approaches are required. Ultra resolution X-ray crystallography and X-ray laser have been successfully applied on few cases to monitor time-resolution of conformational motions4–7. Ultra fast NMR, femtosecond simulated Raman spectroscopy and ultra fast transient Infra Red spectroscopy are measuring atomic motions from femtosecond to nanosecond4–10. Slower motions above nanosecond are monitored by low spatial resolution techniques such as AFM and fluorescence spectroscopy (FRET) where the molecular dynamics are inaccessible4–10.

Theoretical approaches such as molecular dynamics (MD) simulations have atomic resolution and cover motions up to millisecond now thanks to ANTON super computer1. But MD simulation at high resolution is limited in terms of size and it remains hard to assess motions above microsecond range7,11. Network models are also useful to study protein dynamics9,12.

Most successful approaches are integrative, combining experimental and multiple theoretical approaches in order to cover more length and time scale motions of protein structure dynamics12–23. They are particularly successful in monitoring the slow motions involved in protein assembly and pore-formation16–32.

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Measuring the spatiotemporal dynamics of slow collective motions in proteins is crucial because it is the range where protein folding and biological activity take place. It is also the range to assess the health state of a protein as pathological mutations are distinguished from robust mutations by different collective motions. Protein robustness to mutations or to external perturbations such as environmental changes relies on the diversity of structures adopted by a protein in order to function. But there are little measurements of such structural diversity of a protein or of its sequence variants on which to explore the set of atomic and molecular interactions that sustain robustness and from which to infer potential structural and dynamics fragility. This is because slow collective motions associate with short-lived poorly populated conformations or conformations globally close requiring high sensitivity and probing of atomic interaction fluctuations.

To overcome these difficulties, broadband dielectric spectroscopy (BDS) was used to monitor dipole fluctuations in the frequency range from 1 Hz to 10^6 Hz relevant to slow motions and combined with nanoconfinement for lowering the protein dynamics and enriching homogeneous conformations. The nanoconfinement was initially developed to analyze attograms (1 attogram = 10^-18 g) and zeptograms (1 zeptogram = 10^-21 g) of matter and investigate the dielectric behavior of matter on a length-scale becoming already comparable with molecular dimensions. The characteristic volume of 1 attogram (considering a density of ~1 g/cm^3) is ~10 × 10 × 10 nm, approaching thus the characteristic dimensions of one protein and offering the possibility to measure protein dynamics at the scale of the protein instead of the scale of a population of proteins.

Nanoconfined choler toxin B subunit pentamers (Ctxb) were incubated at different temperatures from 60 °C to 180 °C and the dielectric signal measured after each thermal treatment. The toxin dielectric loss ε'' varies with the thermal treatment showing the technic sensitivity to atomic and molecular interaction changes induced by the toxin thermal unfolding. To the best of our knowledge, investigating protein dynamics using nanoconfinement and BDS on the scale of attograms has never been performed before.

Material and Methods

Materials. Lyophilized cholera toxin B pentamer purchased from Sigma Aldrich (C9903) was diluted in phosphate buffered saline (PBS; 10 mM sodium phosphate, 150 mM sodium chloride, pH 7.4) at a final concentration of 2.6 mg/ml. This bulk toxin preparation was aliquoted and kept at −20 °C, thawed and frozen twice maximum, when used for the dielectric measurements. Ten-time concentrated solution of PBS solutions were purchased from BIOSOLVE and diluted 10-times in distilled water to prepare the PBS used to prepare the bulk toxin solution. In addition, the PBS solution was filtered in 22 μm filter to limit impurity contamination. The Aluminium Oxide (AAO) Films on Al are purchased from InRedox. The AAO membrane dimensions are 10 mm * 10 mm, and they contain nanopores of 40 nm-diameters and 10-μm lengths, with 12% porosity. The AAO membrane containing the nanopores is referred to as the nanomembrane. Before use, the nanomembranes were plasma treated for twenty minutes to remove organic impurities.

Methods. Protein sample preparation. For the dielectric measurement, a protein sample at 0.025 mg/ml was used. This protein sample was prepared by diluting 2 μl of the bulk toxin solution at 2.6 mg/ml in 204 μl of deionized water (Final pH 6.9). Since the bulk protein is prepared in PBS, the sample for the dielectric measurement contains protein, deionized water and PBS. A 200 μl drop of sample is deposited on the nanomembrane using the drop technique, then heated at 50 °C (323 K) for 15 min to evaporate the bulk water and allow proteins to enter the pores. The sample is cooled down to 30 °C (303 K) for 5 minutes with a speed of 2 K/min, placed between two Al electrodes of 7 mm and 40 mm diameter, respectively and in the cell holder for measurement. The procedure is the same for the control samples.

Dielectric measurements. The dielectric measurements were performed on a broadband dielectric spectrometer Novocontrol Alpha analyzer over a frequency range from 1 Hz to 10^6 Hz and over a temperature range from −80 °C (193 K) to 180 °C (453 K). The temperature ramps were carried-out with a rate of 2 K/min and a voltage of 0.2 V was applied. The dielectric loss ε'' varies with the temperature at different frequencies. For the temperature control, a flow of pure nitrogen gas was used in a closed cryostat, providing water-free and oxygen-free experimental conditions. The sample was first heated to 60 °C (333 K) for three hours, then cooled down to −80 °C (193 K) (Cooling, C) and maintained for 30 min before being heated back to 60 °C (Heating, H). A cycle is composed of the cooling (C) and the heating (H) temperature ramps. The sample was heated again for three hours at 60 °C and a second cycle of cooling/heating, repeated to measure the reproducibility of the signal. The curves of the cooling and heating signals of the cycle 1 and of the cycle 2 are almost superimposed for the protein sample (Supplementary Fig. S1A).

This demonstrates that the proteins under study are stabilized in a state where no bulk water is present anymore and where the amount of adsorbed water is stabilized as well. It also indicates that three hours incubation at 60 °C is enough for the protein to reach a stable conformational state and this incubation time can be used for thermal treatments at higher temperatures. In contrast, the cooling signal of the PBS buffer control is higher than the heating signal showing that evaporation of the bulk water still occurs in the buffer sample after the first three hours at 60 °C (Supplementary Fig. S1B). Since this is not the case for the protein sample, it suggests more adsorbed water in the protein sample. At 1 Hz, the same is observed (Supplementary Fig. S1C and D).

From 60 °C to 180 °C, little hysteresis is observed indicating that the dielectric loss ε'' does not detect different cooling and heating processes (Supplementary Fig. S2A). The protein remains in a steady state across the measurement and undergoes thermal unfolding mainly during the three hours incubation time.

The thermal denaturation of the toxin is studied using temperature ramps with a series of 3-hours thermal treatments at 100 °C (373 K), 140 °C (413 K) and 180 °C (453 K) followed by cooling/heating cycles after each treatment.

It is worth noting that one full dielectric experiment uses as little as ~5 μg of toxin.
Dielectric data. The temperature dependence of the relaxation processes is modeled by an Arrhenius equation expressed as (Eq. 1)⁴³,⁴⁴.

\[ \tau = \tau_0 \exp\left(\frac{E_A}{k_B T}\right) \]  

where \(\tau\) is the relaxation time, \(\tau_0\) the relaxation time at high temperatures, \(T\) the temperature, \(E_A\) the activation energy and \(k_B\) the Boltzmann constant⁴⁵.

Control experiments. The dielectric response of the empty nanomembrane was measured for 10 min at 60 °C and from 60 °C to −80 °C to control that no dielectric dispersions are detected. The dielectric response of nanopores filled with a 200 μl drop of deionized water was measured for 10 min at 60 °C and from 60 °C to −80 °C to control that the deionized water used to prepare the sample show no dielectric dispersions too. Finally, the dielectric response of nanopores filled with a 200 μl drop of PBS buffer control, is measured following a complete temperature ramp experiment as for the protein sample. The PBS buffer control is prepared with 2 μl of PBS diluted in 204 μl of deionized water (no protein is added). This concentration is equivalent to the concentration used to prepare and measure the protein sample. This control experiment was carried out to investigate the dielectric response of PBS mixed with deionized water in the same conditions as the protein.

To make sure the toxin is in a native state when a dielectric experiment is performed, the toxin pentameric state is checked by Trp-fluorescence beforehand⁴⁶. The sample cannot be recovered from the nanopores after the BDS experiment so it is not possible to control the state of the toxin after.

Results

The goal of the study is to determine if combining nanoconfinement and broadband dielectric spectroscopy (BDS) allows distinguishing protein conformations by measuring different molecular dynamics. To test such a possibility, the cholera toxin B pentamer (CtxB₅) is chosen because of its high stability and functional resistance to freeze-drying, which shows that the dehydration of the toxin does not lead to irreversible conformational changes. This is important since the dielectric measurements are performed under dehydrated conditions where the toxin sample contains PBS and only adsorbed water (Methods and Supplementary Fig. S1).

Toxin and PBS buffer control samples are deposited in the nanomembrane and the dielectric loss \(\varepsilon''\) of the sample is measured by BDS, the whole setup is shown on Fig. 1. The samples are incubated for three hours at 60 °C and the dielectric loss \(\varepsilon''\) is measured as a function of temperature (Fig. 2) (Methods). The protein and the PBS buffer control spectra can be described by several relaxation processes, which give rise to peaks in the spectra of the dielectric loss (Fig. 2, *).

At 1 Hz (Fig. 2, top panel), the protein sample presents two dipole relaxation processes and hence two molecular dynamics (MDs). The main one, narrow, is the fastest with a maximum temperature position at −14 °C (MD₁). The second, broader and less distinct, is slower with a maximum temperature position at 30 °C (MD₂).
The broadness of MD2 suggests a relaxation process associated with a heterogeneous population of toxin conformations while the narrower MD1 peak suggests less heterogeneous toxin conformations. The buffer control also presents a relaxation process and a molecular dynamic (MDb) with a maximum dielectric loss $\varepsilon''$ at $-27^\circ C$, so a faster process than those observed for the toxin. At $10^3\text{Hz}$ (Fig. 2, middle panel), a relaxation process is still detectable for the protein with a maximum dielectric loss $\varepsilon''$ at $13^\circ C$ (MD1). For the PBS buffer control, a relaxation process is also observed but not well enough to have an accurate maximum temperature position (Fig. 2, middle panel). The maximum temperature positions shift to higher temperatures with increasing frequencies. At $10^6\text{Hz}$, neither the protein nor the PBS samples have a detectable signal in the experimental window of our investigations (Fig. 2, bottom panel).

To test whether the dielectric losses probe protein conformation and the relaxation processes two different toxin conformations, the toxin is submitted to thermal denaturation (Methods). Globular proteins go from well-packed structures with numerous atomic and molecular interactions that hold their native state at low temperatures to less compact unfolded structures at high temperatures, where conformations losing atomic and molecular interactions open to solvents and conserved only residual structures. Thermal stability significantly varies for proteins from mesophilic to thermophilic organisms. The CtxB thermal stability has been studied using differential scanning calorimetry and it exhibits a single transition centered at 74°C. The thermal treatments disturb the toxin native atomic and molecular interactions and are hence expected to alter dipole and/or dipole environments, and consequently modify the global dielectric losses.

Figure 2. Dielectric loss as a function of temperature, at different frequencies (as indicated) for Cholera toxin B pentamer (red) and PBS buffer control (black), after a thermal treatment of 3-hours at 60°C.
Three hours thermal treatments at 100 °C, 140 °C and 180 °C are performed since it is enough time for the protein to reach a stable state at 60 °C (Methods and Supplementary Fig. S1A). After the incubation step, the dielectric loss εʺ is measured from the incubation temperature (100 °C, 140 °C or 180 °C) to −80 °C (Cooling) and from −80 °C back to the incubation temperature (Heating) (Fig. 3). The dielectric loss εʺ is shown as a function of temperature for the cooling process but both the cooling and heating signals are similar over the four temperatures of the thermal treatments (Methods and Supplementary Fig. S2A).

The thermal treatments leads to a loss of the relaxation process of the PBS buffer control and only conductivity is observed after treatment at 100 °C and 1 Hz frequency (Supplementary Fig. S2B). In contrast, the toxin maintains multiple relaxation processes evolving across the thermal treatments up to 180 °C and over the frequencies from 1 Hz to 10⁶ Hz, confirming that the dielectric loss of the protein is conformation specific and sensitive to atomic and molecular interaction changes (Fig. 3).

At 1 Hz, the dielectric losses are similar after the thermal treatments at 60 °C and at 100 °C, suggesting that the detected molecular dynamics and protein conformations are stable up to 100 °C. The maximum temperature
positions of MD1 shift only from $-14^\circ C$ to $-7^\circ C$ and from $30^\circ C$ to $40^\circ C$ for the MD2. In the same time, the MD2 peak becomes even broader.

After incubation at $140^\circ C$ and at $180^\circ C$, the dielectric losses change significantly more. The intensity of the dielectric loss of MD1 drops at $140^\circ C$ and even more at $180^\circ C$ with, in addition to a broadening of the peak; a shift of the maximum temperature positions from $-7^\circ C$ to $10^\circ C$ at $180^\circ C$. The drop of the dielectric loss intensity of MD1 suggests a disappearance of the average conformations associated with MD1 as the temperature rises above $100^\circ C$.

The MD2 peak broadens and the maximum temperature position shifts from $40^\circ C$ to $80^\circ C$ after the $140^\circ C$ treatment (Fig. 3, top panel, blue curve). In contrast to MD1, the intensity of the dielectric loss $\varepsilon''$ of MD2 only slightly drops when the protein is heated up to $140^\circ C$ but the dielectric loss $\varepsilon''$ is undetectable after the treatment at $180^\circ C$. This suggests as for MD1 that the toxin conformations associated with MD2 disappear with increasing temperature above $140^\circ C$.

At $140^\circ C$, simultaneously to the drop of the MD1 signal, a new MD (MD3) appears with a narrow peak and a maximum temperature position at $10^\circ C$ (Fig. 3, upper panel). The MD3 peak broadens after treatment at $180^\circ C$ and the maximum temperature position shifts to $70^\circ C$.

MD3 signal drops with increasing temperatures but with no evident increase of the signals of MD2 and MD3 compensating for the loss of the MD3 signal. This indicates that the protein unfolds into conformations, others than MD1 and MD3, and not detected at frequencies between 1 Hz to $10^6$ Hz. This would suggest that unfolded states of proteins where most ‘native’ atomic and molecular interactions and initial dipoles are lost, might need to be investigated at lower frequencies to be detected.

To assess whether the protein initial conformation associated with MD1 could be recovered after the thermal treatment at $180^\circ C$ and the cooling down to $-80^\circ C$, the sample was heated back to $60^\circ C$ and kept at $60^\circ C$ for three hours (Fig. 3, purple curve). The dielectric losses measured after the treatment at $180^\circ C$ and after the subsequent three hours at $60^\circ C$ were similar, indicating that the thermal damages are irreversible and the protein does not refold into the initial conformation despite three hours at $60^\circ C$ (Fig. 3, green, purple and red curves, respectively). Finally, the sample was heated back to $180^\circ C$ and kept for three more hours and the dielectric loss measured again (Fig. 3, grey curve). The signal appears lower than after the first treatment at $180^\circ C$ and MD1 is almost undetectable indicating that the associated averaged conformations is almost gone. After the second treatment at $180^\circ C$, the MD3 and MD1 peaks seem merged.

At $10^3$ Hz, MD3 is barely detected while the MD1 temperature-dependent features change similarly to 1 Hz, and the MD1 can already be detected at $100^\circ C$ (Fig. 3, middle panel). At $10^3$ Hz, only MD1 is detectable, with a drop of the dielectric loss, a shift in the maximum temperature position with the increase of the temperature of the thermal treatments as observed for lower frequencies (Fig. 3, bottom panel). The signal is noisier at temperatures below $-20^\circ C$ but at $10^3$ Hz, it becomes noisier almost over the whole temperature range.

The frequency dependency of the three relaxation processes was investigated as function of temperature (Fig. 4). MD1 covers both low and high frequencies from 1 Hz to $10^6$ Hz over the thermal treatments from $60^\circ C$ to $180^\circ C$. The MD2 is detected only at low frequencies from 1 to $10^3$ Hz over the thermal treatments from $60^\circ C$ to $180^\circ C$. The MD3 is detected only at low frequencies from 1 to $10^3$ Hz over the thermal treatments from $60^\circ C$ to $180^\circ C$. Only few frequencies are shown in the insets for the sake of clarity.

**Figure 4.** Dielectric loss as a function of temperature for Cholera toxin B pentamer, at frequencies from 1 Hz to $10^6$ Hz after three hours thermal treatments at $60^\circ C$ (Top left), $100^\circ C$ (Top right), $140^\circ C$ (Bottom left) and $180^\circ C$ (bottom right). Only few frequencies are shown in the insets for the sake of clarity.
to 140 °C. MD3 covers high frequencies after the thermal treatment at 100 °C, both high and low frequencies after the thermal treatment at 140 °C, and only low frequencies after the thermal treatment at 180 °C.

The temperature dependencies of the relaxation times $\tau$ of the three relaxation processes are plotted on Fig. 5. The relaxation times linearly depend on the maximum temperature positions indicating that dipoles fluctuating at different frequencies are independent of one another and that is true for the three relaxation processes. Three signal features change with the temperature of the thermal treatments and with the relaxation processes.

First, the relaxation times of dipoles with similar maximum temperature positions become significantly slower in MD3 and in MD2 as the temperatures of the thermal treatment increase (Fig. 5, circles). In contrast, they remain similar up to 140 °C in MD1 and become slower only after the treatment at 180 °C (Fig. 5, circles). The relaxation times of dipoles with similar maximum temperature positions also become slower across the MDs (Fig. 5, vertical arrows). This means thermal unfolding leads to motions at larger spatial scale with no impact on the dipole local environment (no change in the maximum temperature positions). It could be interface motions or beta sheet motion for example. Moreover, regardless the maximum temperature positions, the relaxation times of MD3 are ten times slower at 140 °C than at 100 °C (Fig. 5, circles). Likewise, the relaxation times in MD2 are ten times slower than in MD1 at 100 °C and hundred times slower at 140 °C, regardless the maximum temperature positions. The relaxation times of dipoles in MD2 are thousand times slower than in MD1, regardless the maximum temperature positions. This indicates that dipoles fluctuating at different frequencies are independent of one another even as toxin thermal unfolding occurs. Dipole fluctuation influences as the toxin unfolds might happen during the incubation time and thus not be detected on steady state conformations.

Second, the maximum temperature positions of dipoles with similar relaxation times increase (1000/K decrease) with the increase of the temperature of the thermal treatments (Fig. 5, horizontal arrows). This is also
observed across relaxation processes as the maximum temperature positions of dipoles with similar relaxation times increase from MD$_1$ to MD$_2$ to MD$_3$. This means thermal unfolding also leads to changes in the vicinity of the dipoles (changes in the maximum temperature positions) with no impact on the dipole relaxation time (no change in tau). This could be a dipole going from a buried position to a surface position but keeping same fluctuation.

Third, dipoles change in terms of both relaxation times and maximum temperature positions. For example, there are no dipoles with slow relaxation times in MD$_3$ at 100°C but there are some at 140°C (Fig. 5). This means the MD$_3$ dipoles with fast relaxation times at 100°C have slowed down and changed their maximum temperature positions at 140°C. Here the thermal unfolding leads to local changes and larger scale collective motions.

The relaxation time temperature dependence of the three relaxation processes fits well with an Arrhenius equation (Methods) over the four thermal treatments at 60°C, 100°C, 140°C and 180°C (Fig. 5). They present similar activation energies of approximately 27–30 kcal mol$^{-1}$ across the three MD and for the different temperatures of the thermal treatments, except for the MD$_3$ at 140°C whose activation energy is approximately 41 kcal mol$^{-1}$ (Fig. 5, slopes). Such similar activation energies across the MD and the temperatures of the thermal treatments are consistent with having similar probes monitored in all processes but in different molecular environment as the toxin unfolds locally (around the dipole) or at larger scale.

**Discussion**

The main narrow peak MD$_1$ observed at 60°C suggests that the nanopores work as a conformational funnel, and reduce the structural heterogeneity of the toxin in solution by constraining the 3D space where the toxin can move (nanopore diameter). We are currently testing other nanopore dimensions to investigate this hypothesis. In addition to detecting MD$_1$, the nanoconfinement combined with BDS also distinguishes, via their respective molecular dynamics (MD$_3$ and MD$_4$) two other average toxin conformations induced by the thermal treatments.

The toxin conformations and dynamics measured by BDS combined with nanoconfinement are clearly different from the toxin dynamics measured under macroscopic and hydrated conditions as illustrated by the toxin resistance to thermal treatments up to 180°C instead of 80°C$^{31}$. Such impact on the toxin stability is consistent with the detection of disassembled and/or unfolding species, rarely observed otherwise, made more stable under the conditions. The nanoconfinement, the dehydration or a combination of both could be responsible for the high temperature-resistance. Dehydration conditions are known to impact protein dynamics$^{52–54}$. Hydrogen bonding might be reduced under dehydration while the presence of the PBS buffer could favor salt bridges and lead altogether to a more stable toxin. This would be consistent with the role of stabilizing salt bridges on temperature resistance observed in thermophilic proteins$^{55–57}$. BDS experiments on myoglobin and other proteins under macroscopic hydrated conditions report one fast relaxation process corresponding to free water dynamics which disappears upon dehydration, and slower relaxation processes associated with protein-water and protein dynamics still detected upon dehydration$^{61–63}$. Schiro and co-authors have also shown using BDS that myoglobin confined in silica gels presents only slow relaxation processes as observed in our experiments, and little dynamic changes with the dehydration level of the sample$^{34}$. This would tend to suggest that with the nanoconfinement, the dehydration impacts less the protein dynamics. The nanoconfinement could increase the packing of the toxin structure and thus stabilize the protein as observed for thermophilic proteins$^{57}$. Repeating the experiments using different nanoconfineds and different dehydration conditions will be one way to test the role of both parameters in the temperature-resistance of the toxin.

Assessing nanoconfined protein dynamics is of interest as in cells geometrical nanoconfinement of proteins, for example in lipid microdomains (e.g. RAFT), regulate functional protein dynamics such as pore-formation, protein sorting and protein function$^{31,59,60}$. Protein dynamical structures detected under unusual experimental conditions (nanoconfinement and dehydration) show the robustness of protein structure and protein dynamics to a large range of conditions, going from crystal to dehydrated state in agreement with the sustainability of proteins in time and environmental conditions$^{61–63}$. This is thanks to structural and dynamics diversity which also fits with the function of a protein provided by an ensemble of structures and dynamics rather than unique solutions$^{37,64}$. Importantly, the dielectric signal is protein conformation specific, evolves with the temperature of the thermal-treatments and is therefore sensitive to the perturbation of local atomic and molecular interactions. Thus the technique allows characterizing a protein in terms of conformation, stability (to temperature) and dynamics with multiple measures such as frequencies and maximum temperature positions. The technique could therefore be used to detect mutations by comparing the dielectric signals of a protein and its sequence-variants and diagnose the impact of mutations. Such experimental measurements would be useful to classify mutations and develop customized therapies accordingly.

Nanoconfinement allows us to investigate the dynamics of proteins at length scales comparable to the protein geometrical dimensions, which is expected to enhance the sensitivity of dielectric measurements and help detecting the impact of protein mutation. At the macroscopic scale, this ability is strongly limited by disorder-effects arising from a very broad distribution of possible protein conformations.

Presently, only the global dielectric signal can be used to distinguish molecular dynamics and conformations. It is impossible to determine the local dipoles responsible for the global signal or the scale of the collective fluctuations probed. In other words, whether the entire protein is probed or only sub-domains, and if so which one cannot be inferred using only the BDS data. This is because the sample is a mixture of three compounds, the protein, the PBS and protein bound-water. Each compound has many dipoles and the individual participation of each compound or how the individual signals combined to give the global signal is intractable.

What can be done instead is to challenge the data against known experimental and theoretical data on protein dynamics to draw hypotheses on the scale of the collective fluctuations detected by BDS. Putting the results into such perspective, we propose a model of the mechanism of the toxin thermal unfolding with the supplementary
constraint that the model agrees with previously established mechanisms of the toxin unfolding and refolding46,51,65,66 (Fig. 6).

Amino acid side-chain motions determined by other experimental approaches and molecular dynamic simulations are several decades (≤ nanoseconds) faster than the range of frequencies measured by BDS so it is reasonable to presume that such motions are not detected here.

According to molecular dynamics simulations and integrative approaches, microsecond to millisecond motions are attributed to loop, 2D and 3D structural elements whereas millisecond to second motions are folding and interdomain motions1,3. Microsecond to second dipole fluctuations on other proteins have been observed previously by BDS and attributed to protein backbone, large-scale domain or buried side chain motions44,58.

Because MD1 is the main peak detected from 60 °C to 140 °C, we will assume it corresponds to the most stable state of the toxin and its most folded average conformation, a ‘native-like’ pentamer. Because MD3 as MD1 has a narrow peak, and is similar in terms of frequency ranges but with slower relaxation times, we will assume it is a partially folded pentamer where the dipole have less compact environment allowing larger scale motions. Because MD2 is the slowest relaxation process and a broad peak, we will assume it to be the least folded CtxB species detected and a mixture of CtxB assembly intermediates (CtxB tetramers, trimers and dimers). Alternatively, MD1 could be a folded CtxB monomer, MD3 a slightly less folded monomer and MD2 multiple unfolded states of CtxB monomers. But because the three relaxation processes disappear at high temperatures, it is difficult to picture what would be the undetected species if unfolded monomers are detected. The first scheme is also consistent with the CtxB (dis)assembly and (un)folding intermediates previously identified macroscopically46,65. The activation energy of Proline trans-cis isomerization is 30 kcalmol⁻¹, and maybe the toxin relaxation processes and dipole fluctuations are under the influence of such reaction. As the toxin assembly is inhibited by the isomerization of Pro93 which slows down the formation of the main β-interface, it is possible although speculative46.

Under the first scheme, it is reasonable to assume that the toxin subunit interfaces are monitored during an experiment since CtxB assembly intermediates are detected (MD2). It follows that folded and unfolded CtxB monomers would be among the undetected species. The native toxin pentamer has three distinct interface areas I₁, I₂ and I₃ as shown in Fig. 6A, but native interfaces are unlikely to be detected because they involve local atomic motions with fluctuations over the nanosecond scale³. In MD₁, fast and slow relaxation times are detected and as the toxin unfolds with high temperature treatments only slow relaxation times are detected for MD₂ and MD₃. The toxin interfaces I₁, I₂ and I₃ would exist as native (nanosecond scale not detected), destabilized (microsecond to millisecond fluctuations: 2D/3D segment mobility) and impaired with larger and slower motions (millisecond fluctuations).
to second: domain motion) (Fig. 6B). These assumptions are consistent with the microsecond to millisecond time-scale motions, measured by hydrogen exchange, involved in protein thermal unfolding87. The model assumes that CtxB intermediates are detected through the slow motion interfaces, the interfaces with no adjacent chains are assumed undetected. Having I1, I2 and I3 interfaces with slow motions in a monomer, is assumed to trigger the monomer dissociation extremely fast.

Now, in MD, even at 180 °C fast relaxation times (microsecond to millisecond) are observed but for different maximum temperature positions than at 140 °C. This suggests that some undetected native interfaces present in MD1 at 140 °C, become destabilized and detected at 180 °C but at different maximum positions than the one detected at 140 °C because they are different interfaces (Fig. 6). Alternatively, all the interfaces in MD1 could be destabilized and detected with fast motions from 60 °C, and the change in maximum temperature positions with the thermal treatements could indicate local environmental changes around the dipoles with fast relaxation times due to local unfolding with no consequences at larger scales. This would have to be further investigated.

The model highlights the large combinatory of interface dynamic states within a single monomer, the CtxB assembly intermediates and the pentamers. The dielectric loss measurement is performed on hundreds of nanopores, each containing some toxin molecules so it is capturing a large statistics of structural states, referred to as average conformations and appearing within each molecular dynamics.

Conclusion

The molecular dynamics of distinct protein conformations, among which conformations rarely detected macroscopically, become accessible experimentally by combining nanoconfinement with broadband dielectric spectroscopy. This novel biophysical approach captures the dipole fluctuations underlying the molecular dynamics of average protein conformations whose slow motions associate with protein unfolding. The level of description provided by the BDS offers a large experimental potential to study protein dynamics and is an experimental equivalent to molecular dynamics simulation over the frequency range from 10^3 Hz to 10^6 Hz.

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L.B. performed research and analyzed data, A.S. designed research, analyzed data and wrote the manuscript, C.L. designed research, analyzed data and wrote the manuscript.

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The authors declare no competing interests.

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