Protocol

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Protocol
A protocol to measure slow protein dynamics of the cholera toxin B pentamers using broadband dielectric spectroscopy

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SUMMARY
The present protocol describes how to measure experimentally the slow protein dynamics that take place upon the thermal unfolding of the B subunit cholera toxin pentamers using broadband dielectric spectroscopy (BDS) in weakly hydrated and nanoconfined conditions. Transient unfolding intermediates, rarely identified otherwise, are revealed thanks to the B subunit’s remarkable heat resistance up to 180 °C and distinct molecular dynamics. The frequencies detected experimentally are consistent with the spatiotemporal scales of motions of molecular dynamics simulation.

For complete details on the use and execution of this protocol, please refer to Bourgeat et al. (2021, 2019).

BEFORE YOU BEGIN
The whole experiment runs over two days, the measurement conditions and experimental set up are programmed on the broadband dielectric spectrometer (BDS) using the software WinDETA such that the experiment can be followed remotely. The user intervention is needed to load the samples on the nanomembrane. Here, the buffer and the protein samples are loaded the first day which hence requires an early start to: (i) prepare the samples, (ii) perform the measurement of the membrane and water controls, (iii) load the protein sample and (iv) start the protein measurement to check that everything is working fine. The BDS needs to be booked in advance, as the machine will be running over two days. Nitrogen gas is used for the temperature control so check there is enough to cover the two days experiment.

Preparation one: Protein sample

© Timing: 2 h 30 min

1. Protein stock solution.
   a. Solution preparation (60 min). Solubilize lyophilized cholera toxin B pentamer (CtxB5) purchased from Sigma-Aldrich (C9903) 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 (stock solution). The stock solution also contained 20 mM Tris buffer, 400 mM NaCl, 6 mM NaNO3 and 1 mM sodium EDTA, because it was prepared and packaged in such solution by Sigma-Aldrich.
b. Storage of the stock solution (30 min). Aliquot CtxB₅ stock solution and store at −20°C. Use immediately after preparation or freeze.

c. Freeze and thaw twice maximum for dielectric measurements.

2. Starting material.

a. Broadband dielectric spectroscopy (BDS) protein sample (15 min). Use a protein sample at 0.013 mg/mL for the dielectric experiment. Prepare it by diluting 2 μL of the stock solution at 2.6 mg/mL in 410 μL of deionized water reaching a final pH of 6.9. After the dilution, the protein sample for the dielectric measurement contains 0.1 mM Tris, 0.05 mM PBS, 50 mM salt, 0.01 mM EDTA and 0.03 mM Azide. The dilution in water reduces interferences with the protein dielectric signal. Troubleshooting 1. One full dielectric experiment uses as little as ~ 5 μg of toxin.

b. Fluorescence spectroscopy protein sample (45 min). Prepare a CtxB₅ sample at a final concentration of 0.1 mg/mL by diluting 15.4 μL of stock solution (same as for BDS) in 385 μL deionized water reaching a final pH of 6.9. This sample is used to check that CtxB₅ is in a native state before the BDS experiment (Lesieur et al., 2002). Because the sample cannot be recovered from the nanopores after a BDS experiment, it is not possible to also check the state of CtxB by fluorescence after the BDS experiment.

Preparation two: Buffer sample

@ Timing: 45 min

Buffer solutions. 10×PBS solutions were purchased from BIOSOLVE and diluted 10-times in deionized water to prepare the PBS used for the protein stock solution (PBSx1). The 10-times PBS solution is kept at 4°C and warm up for 30 min at room temperature before preparing the diluted solutions. All solutions are filtered in 0.22 μm filter before use for a BDS experiment, to limit impurity contamination.

⚠ CRITICAL: Check the structural state of the protein before the BDS experiment to make sure the protein is not already partially or fully unfolded before doing the dielectric measurement. For CtxB₅, the structural state is checked by Trp-fluorescence because the folded pentamer has a different spectrum than the unfolded monomer (Lesieur et al., 2002). A technique suitable to the protein under study must be chosen such as for example fluorescence spectroscopy, circular dichroism, SDS-PAGE for oligomeric state if appropriate. If the protein is partially or fully unfolded, take a new sample from the stock solution.

**KEY RESOURCES TABLE**

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Chemicals, peptides, and recombinant proteins |  |  |
| Cholera toxin B subunit pentamer | Sigma-Aldrich | C9903 |
| Milllex-GS Syringe Filter Unit, 0.22 μm | Merck | SLG525SF |
| 10×PBS | Biosolve | 162323 |
| 10×PBS | InRedox | https://www.inredox.com/product/aao-al/ |
| Aluminum Oxide (AAO) Films on Al | InRedox |  |
| Other | Broadband dielectric spectrometer Novocontrol Alpha analyzer | Novocontrol | https://www.novocontrol.de/php/index.php |
| Deposited data | Cholera toxin B subunit pentamer | https://www.rcsb.org | PDB: 1EEI |
| Software and algorithms | Amino acid network (AAN) | Dorantes-Gilardi et al. (2018) | https://github.com/orpac/amino_acid_network |
| | Electrostatic dipole network (EN) | Bourgeat et al., (2021) | https://doi.org/10.5281/zenodo.6597454 |
MATERIALS AND EQUIPMENT

Proteins- The cholera Toxin B subunit (CtxB₅) used in this study was brought from Sigma-Aldrich (C9903) and according to SIGMA-ALDRICH CtxB₅ is produced from *Vibrio cholerae*.

Membranes- For the measurements discussed in this Protocol, the AAO membrane had dimensions of 10 mm × 10 mm, 12 ± 2% porosity and nanopores of 40-nm diameters and 10-µm lengths (Figure 1A). Before use, we treated the nanomembranes with plasma for twenty minutes, to remove organic impurities.

Note: The InRedox specifications indicate that the pore lengths and diameters are 10 ± 1 µm and 40 ± 4 nm.

Note: The AAO membrane containing the nanopores will be referred to as the “nanomembrane.”

Broadband dielectric spectrometer- For the measurements discussed in this Protocol, a broadband dielectric spectrometer Novocontrol Alpha analyzer (Figure 1B) was used over a frequency range from 1 Hz to 1 MHz (10⁶ Hz) and over a temperature range from −80°C (193K) to 180°C (453 K). The temperature ramps were carried-out with a rate of 2K/min and a voltage of 0.2 V was applied. The dielectric loss ε’’ which is the imaginary part of the complex dielectric permittivity, was measured as a function of temperature at seven frequencies: 1 Hz, 10 Hz, 100 Hz, 1 kHz, 10 kHz, 100 kHz and 1 MHz.

Note: The broadband dielectric spectrometer Novocontrol Alpha analyzer is equipped with a WinDETA software where the experimental conditions are programmed and which control the BDS.

Note: The WinDETA software can be installed on any computer to control the experiment remotely.
Note: For the temperature control, a flow of pure nitrogen gas was used in a closed cryostat, providing water-free and oxygen-free experimental conditions.

Program protein experiment on WinDETA. WinDETA is programmed for changing temperature and frequency, as well as for the temperature ramp. The details are indicated in the step-by-step method and it depends on the protein under investigation. The seven frequencies are measured almost simultaneously at each temperature point. The dielectric measurement is started from WinDETA and stop automatically once the program is finished.

Electrostatic dipole networks- The amino acid network (AAN) of CtxB5 is built using the atomic coordinates of CtxB5 X-ray structure (PDB: 1EEI) freely accessible on the RCSB website (https://www.rcsb.org/). The amino acids are the nodes of the networks linked if they have at least one atom each located within a threshold distance of 5 Å from one another. The AAN computes all atomic distances and hence models all atomic and amino acid interactions in the pentamer structure (Dorantes-Gilardi et al., 2018). The electrostatic dipole network (EN) is obtained from the AAN by keeping only opposite charged amino acids as nodes of the network linked if they have at least one atom each within 5 Å distance of one another (Bourgeat et al., 2021; Pacini et al., 2020). The EN models the Coulomb interactions between positive and negative charges in the structure. As the experiments are done at pH 6.9, the negative charges are the C-terminals, the glutamic and aspartic acids and the positive charges are the N-terminals, the lysine and the arginine residues. The orientation of the dipoles is not taken into account in the model although it could cancel out the participation of some of the dipoles to the dielectric signal when dipoles of equal strength have opposite orientation. Nevertheless, the amino acid chemical neighborhoods are anisotropic and no amino acids have same chemical environment so it appears unlikely to have dipoles with equal dielectric contribution but at opposite directions, making the approximation locally reasonable. The code to produce the electrostatic network is available on GitHub (see key resources table).

STEP-BY-STEP METHOD DETAILS

The broadband dielectric spectrometer Novocontrol Alpha analyzer is equipped with a WinDETA software where experimental conditions are programmed and from which the machine can be controlled remotely (materials and equipment). Below is described the detailed of the different steps that take place during the two days experimental course but once the sample is loaded, the experiment programmed on WinDETA and the dielectric measurement started using WinDETA, the user does not need to perform anything. The change of temperatures, frequencies, incubation times is programmed and controlled by the WinDETA software.

Part 1: Controls

© Timing: 1 day

The first day, after preparing the samples, perform dielectric measurements to control the membrane, the water and set up appropriate experimental conditions for the protein thermal unfolding.

1. Control of empty membrane (1h 30 min).
   a. Intercalate the nanomembrane between the 40 mm and the 7 mm diameter brass electrodes (Figure 1B).
   b. Put the whole on the cell holder (Figure 1B).
   c. Program the WinDETA to measure for 10 min at 60°C and from 60°C to −80°C (80 min).
   d. And from −80°C to 30°C (55 min).
   e. Start the dielectric measurement of the empty nanomembrane.
2. Control of water sample (3h 30 min).
   a. Stop the measurement using WinDETA.
b. Remove top brass electrode (Figure 1B) and deposit a 200 μL drop of deionized water onto the nanomembrane (do not put back the top brass electrode).

c. Program WinDETA to increase temperature to 50°C (10 min) and keep at 50°C for 15 min. The bulk water evaporates and the sample enters the nanopores and to.

d. Cool down to 30°C (10 min), keep for 5 min.

e. Put the top brass electrode on the nanomembrane for measurement.

f. Program WinDETA to increase temperature from 30°C to 60°C (15 min) and to.

g. Start measuring the dielectric response of water for 10 min at 60°C and from 60°C to −80°C (80 min), and to.

3. Control of the protein state after thermal treatments and during the measurement (13h 30 min).

a. Increase temperature from −80°C to 30°C (55 min).

b. Stop the measurement using WinDETA.

c. Remove top brass electrode and deposit a 200 μL drop of CtxB5 sample at 0.013 mg/mL (starting material).

d. Program WinDETA to increase temperature to 50°C (10 min) and keep at 50°C for 15 min. The bulk water evaporates and the sample enters the nanopores and to.

e. Cool down to 30°C (10 min), keep for 5 min.

f. Put the top brass electrode on the nanomembrane for measurement.

g. Program WinDETA for the whole 48 h protein experiment (from 3.h. to 4.d.), and start the measurement.

h. Cycle 1 (≈ 6 h): WinDETA is programmed to increase temperature from 30°C to 60°C (15 min), incubate for three hours at 60°C, cool down to −80°C (Cooling, C, 70 min), maintain at −80°C for 30 min and heat back to 60°C (Heating, H, 70 min). A cycle is composed of the three hours incubation time, the cooling (C) and the heating (H) temperature measurements, and to.

i. Cycle 2 (≈ 6 h): incubate again sample for three hours at 60°C, cool down to −80°C (Cooling, C, 70 min), maintain at −80°C for 30 min and heat back to 60°C (Heating, H, 70 min).

Note: The empty membrane and the water sample should exhibit a linear dielectric signal and no relaxation peaks (Figure 2A, inset).

Compare cycle 1 and cycle 2 dielectric signals (Figure 2A) to determine if the incubation time is enough to reach a stable unfolded state troubleshooting 2. Compare heating and cooling dielectric signals (Figure 2B) to determine if there is some hysteresis suggesting unfolding/refolding during the measurement troubleshooting 3.
△ CRITICAL: The state of the protein sample must be stable during the measurement otherwise the dielectric signal cannot be unambiguously interpreted as dipole fluctuations and molecular dynamics of a given protein state because it can also result from changes in the dipole environments due to protein unfolding/refolding taking place during the measurement.

**Part 2: Thermal unfolding of CtxB**

**Timing:** 1 day

The measurements at 60°C are protein controls but are also measurements of the early thermal unfolding stage of the protein (low temperature). The step 2 is the measurement of the dielectric signals of the protein for thermal treatments at higher temperatures and up to 180°C. WinDETA is programmed to run the whole experiment and measure the dielectric response for thermal treatments from 60°C to 180°C and at the seven frequencies (materials and equipment) such that the measurement is not stopped between steps 1 and 2, it stops itself at the end of the program.

4. Dielectric signals of the protein sample after thermal treatments at temperatures above 60°C.
   a. Total 380 min, 80°C: After cycle 2 at 60°C, WinDETA is programmed to continue measuring, increase temperature from 60°C to 80°C (10 min), incubate sample for three hours at 80°C, cool down to –80°C (Cooling, C, 80 min), maintain at –80°C for 30 min and heat back to 80°C (Heating, H, 80 min), and to.
   b. Total 400 min, 100°C: Increase temperature from 80°C to 100°C (10 min), incubate sample for three hours at 100°C, cool down to –80°C (Cooling, C, 90 min), maintain at –80°C for 30 min and heat back to 100°C (Heating, H, 90 min), and to.
   c. Total 450 min, 140°C: Increase temperature from 100°C to 140°C (20 min), incubate sample for three hours at 140°C, cool down to –80°C (Cooling, C, 110 min), maintain at –80°C for 30 min and heat back to 140°C (Heating, H, 110 min) and to.
   d. Total 360 min, 180°C: Increase temperature from 140°C to 180°C (20 min), incubate sample for three hours at 180°C, cool down to –80°C (Cooling, C, 130 min), maintain at –80°C for 30 min.

   **Note:** As for the thermal treatment at 60°C, the cooling and heating dielectric signals should be similar.

5. Data reproducibility.
   Repeat Major steps 1 and 2 for investigating experimental reproducibility. Figure 3 shows two experiments comparing the results for two thermal treatments (Figure 3A) and for three frequencies (Figure 3B).

   a. Compare thermal treatments at one frequency (Figure 3A):
      The dielectric signals are reasonably reproduced (similar dielectric loss intensity, peak position and peak number) with the peaks P1 overlapping across the two experiments and for both temperatures of the thermal treatments and the peaks P2 and P3 showing a shift of position of around 6°C and different dielectric loss intensities for P2.

      **Note:** P1 is likely to be associated with the least unfolded state as it has the lower temperature position meaning faster and smaller collective dipole fluctuations, hence less dipoles destabilized. This is also supported by the fact that P1 is detected from early (60°C thermal treatment) to late (180°C thermal treatment) stages of unfolding and is therefore the most stable state detected, hence likely to be the closest to the native state.

      **Note:** P2 and P3 are further unfolded states, which might explain lower reproducibility, as intermediate folding/assembly states are difficult to trap due to instability and a poor population.
Part 3: Data analysis and interpretation

Timing: 1 week

6. Background.
   a. Rational. A series of thermal treatments of three hours incubation at increasing temperatures from 60°C to 180°C triggers the thermal unfolding of CtxB5. Dielectric signal arises from dipole fluctuations, which depend on the local environment of the dipoles as well as on the morphological organization of matter. The dielectric signal monitors the protein thermal unfolding because when the protein unfolds with temperature, the local environment of the dipoles and the global conformation of the protein change as atomic interactions change.
   
   b. Dielectric signals.
      i. The linear response of the dielectric loss $\varepsilon''$ with increasing temperature is the conductivity contribution of the sample while the non-linear (i.e., peaks) increase is due to molecular relaxations (Kremer and Schönhals, 2002).
      ii. The relaxation peaks correspond to the molecular dynamics of B-subunits detectable in the temperature and frequency windows of the measurements.
      iii. One relaxation peak represents a set of dipoles collectively fluctuating; two relaxation peaks involve two different sets of dipoles.

   △ CRITICAL: A relaxation peak is associated with a set of conformations and not a unique conformation so the peak features result from conformational averages. Now, for the sake of clarity, we refer to an unfolded state associated with a peak and not averaged unfolded states associated with a peak.

   c. Peak features.
      i. A peak position is characterized by the molecular dynamics of the set of dipoles contributing to the signal. The lower the temperature of the peak position the faster the dipoles fluctuate and the smaller the collective motions is (less dipoles fluctuating together).
      ii. The intensity of a peak depends on multiple parameters (concentration of the conformations, contribution of the dipoles) so it cannot be interpreted.
      iii. The shape (broadness, symmetry) of the peak reveals the conformational heterogeneity.

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Figure 3. Reproducibility
(A) The 1 Hz dielectric loss $\varepsilon''$ is plotted against temperature (cooling) for two experiments and after thermal treatments at two different temperatures.
(B) The dielectric loss $\varepsilon''$ is plotted against temperature after the thermal treatment at 100°C and for three frequencies (1 Hz, 1 kHz and 1 MHz).

b. Compare frequency dependencies of one thermal treatment (Figure 3B):
The dielectric signals are reasonably reproduced (similar dielectric loss intensity, peak position and peak number) after the thermal treatments at 100°C. The peaks P1 are detected for frequencies from 1 Hz to 1 MHz while P2 is detected from 1 Hz to 1 KHz and P3 only at 1 KHz.


**CRITICAL:** Cautious that the dielectric loss $\varepsilon''$ monitors the molecular dynamics of conformational averages and hence do not provide direct structural information on conformations but a peak indicates the dynamics of a set of dipoles that associates with a conformation (averaged conformations).

d. Protein dynamics. According to the knowledge on protein dynamics, the frequencies from 1 Hz to 1 MHz correspond to slow collective motions (Henzler-Wildman and Kern, 2007; Munoz and Cerminara, 2016). From microsecond (1 MHz) to millisecond (1 kHz) there are motions of 2D- and 3D- structures. From millisecond to second, there are motions of large domain, interface and chain.

**Note:** Side chain motions are faster and not detected in the frequency window of our experiments.

**Note:** Functional slow dynamics is also not detected simply because the function of the protein is not triggered in our experiments. Slow and collective motions that take place during the thermal unfolding are monitored.

7. Peak and unfolded state identification.
   a. Export the data from WinDETA to Excel (or else).
   b. Sort the data in Excel by temperature of the thermal treatments and frequencies.
   c. Plot the dielectric loss $\varepsilon''$ measured after each thermal treatment as a function of temperature and at a given frequency (Figure 4A). Here the cooling measurement is plotted at 1 Hz as an example.
   d. Count the number of peaks to determine the number of different CtxB unfolded states observed during the experiment. On Figure 4A, two peaks P1 and P2 are detected at 60°C while three peaks are detected at 140°C.
   e. Determine the conformational heterogeneity of the peaks by comparing the peak broadness. P2 is broader than P1 and P3 suggesting a relaxation peak associated with larger conformational heterogeneities than the two others. This can suggest assembly intermediates (P2) versus unfolding intermediates (P1 and P3).

**CRITICAL:** This is assuming that in the assembly intermediates, the fluctuating dipoles have the same local environment but have different spatiotemporal dynamics when they are in dimers, trimers or tetramers, hence a response covering a larger range of temperature but still appearing under the same peak because same dipoles are contributing.

8. Thermal resistance of unfolded states.
   a. Characterize the thermal resistance of the unfolded states by looking over which thermal treatments the peaks remained detectable.
   b. Example 1: The peak P1 is detected over all thermal treatments from 60°C up to 180°C with a shift of the temperature peak position from $-14°C$ to 10°C with the increase of the temperature of the thermal treatments.
   c. Example 2: The peak 2, P2 is detected after the thermal treatments from 60°C to 140°C with a shift of the temperature peak position from 30°C to 80°C with the increase of the temperature of the thermal treatments.
   d. Example 3: The peak 3, P3 is detected after the thermal treatments from 140°C to 180°C with a shift of the temperature peak position from 10°C to 60°C with the increase of the temperature of the thermal treatments.

9. Frequency characteristics of the peaks and CtxB unfolded state dynamics.
   a. Plot the dielectric loss measured as a function of temperature but for different frequencies and for a given thermal treatment (Figures 4B and 4C).
b. Count the number of peaks and determine their frequency range (i.e., the frequency at which a peak is observed).

c. Examples: P1 is detected from 1 Hz to 10^5 Hz (Figure 4B) and from 1 Hz to 10^6 Hz (Figure 4C) so this unfolded state involves microsecond to second motions while P2 is detected from 1 Hz to 10^3 Hz (Figures 4B and 4C) and its unfolded state involves only motions between millisecond and second. P3 is detected from 1 Hz to 10^5 Hz and its unfolded state involves microsecond to second motions as P1.

*Note:* According to protein dynamics (see background 6d), P1 and P3 would be unfolded states involving 2D, 3D and 4D structural motions while P2 will be involving only slower motions such as 4D chain motions, consistently with its larger broadness and the detection of assembly intermediates.

10. Dipole contribution to the dielectric signal (only if PDB of protein is available).

Under ionized buffer conditions, electrostatic dipoles are the main contributors to the dielectric signal and the contribution of non-ionized dipoles is negligible in comparison.

a. If proficient in protein structure program: determine electrostatic dipoles from the protein PDB using PDB viewer (or else).

b. Download PDB: 1EEI from [https://www.rcsb.org/](https://www.rcsb.org/).

c. Select opposite charged residues within 5 Å of one another as electrostatic dipoles.

d. Determine the structural level of the electrostatic dipoles to infer the frequency range they are expected to contribute to the dielectric signals (see background 6d).
e. If proficient in python, determine electrostatic dipoles from the electrostatic network model (materials and equipment).

f. Download the code from GitHub (see key resources table) and run it on the PDB of the protein of interest (1EEI for CtxB).

g. Determine the structural level of these electrostatic dipoles to infer the frequency range there are expected to contribute to the dielectric signals (Figure 4D).

For CtxB with PDB: 1EEI, the electrostatic model (or the structure analysis) reveals 5 electrostatic dipoles involved in 1D structure (first neighbors along the protein sequence), 11 in 2D structures, 7 in 3D-structures and 11 in 4D-structures per monomer. This is consistent with the relaxation peaks observed over the frequencies from 1 Hz to 1 MHz and the detection of slow collective motions of 2D, 3D and 4D structures.

EXPECTED OUTCOMES

This protocol aims at detecting protein dynamics experimentally, precisely the frequency window is such that the slow motions, which are involved in folding/unfolding and functional protein dynamics can be studied. From this point of view this is a unique technique. Moreover, thanks to the nanoconfinement which reduces conformational heterogeneity compared to macroscopic measurement, several unfolding and assembly intermediates are populated enough to be identified and characterized through thermal resistance and frequency ranges. More classical biophysical techniques (e.g., circular dichroism, fluorescence spectroscopy) will have a macroscopic global response making difficult the identification of structural intermediates and providing no information on the dynamics of the intermediates or of the conformations detected. This approach has limitations due to the present lack of measurement on many protein samples but because it gives access to information not easily accessible otherwise it is worth using it to gather more and more data. We have used it to compare variants and discovered that the method can monitor dynamics differences due to mutations, again an information not easily accessible experimentally by alternative methods (Bourgeat et al., 2021). Functional dynamics could also be investigated using large nanopores already used for enzymatic tests (de la Escosura-Muniz et al., 2013, 2018; Soubias and Gawrisch, 2013). InRedox sales nanopores of different geometries from 2.5 nm to 300 nm diameters and from 0.1 to 300 μm length and it is also possible to make your own nanopores (Houachtia et al., 2015; Serghei et al., 2013).

LIMITATIONS

BDS combined with nanoconfined and weakly hydrated conditions allows detecting structural intermediates difficult to capture by other means and provides experimental information on protein dynamics. These are two good reasons to use it for investigating proteins.

At this stage, few proteins have been studied using BDS limiting the data analysis, in particular to establish structural-dynamics relationships (Costa et al., 2021; Frauenfelder et al., 2009; Jansson et al., 2005; Khodadadi et al., 2008; Olsson et al., 2020; Schiro et al., 2009; Tsukahara et al., 2021).

Nevertheless, the data can be analyzed in terms of peak features, similarly to what is done for fluorescence spectroscopy and literature on protein dynamics can be used to analyze the frequency dependencies so proteins can be characterized by their dielectric properties.

Again because of the lack of studies, there are no typical experimental conditions and each protein needs to be investigated to find appropriate experimental conditions. The ideal is to use buffers, which do not contribute to the dielectric signal because they evaporate, hence water or volatile buffer as those used in mass spectrometry. However, because protein structural integrity depends on buffer and pH of solutions, if one wants to investigate native conditions, a compromise needs to be adopted as in the present protocol.
The number of electrostatic dipoles in the protein, their spatial organization and interrelation may also be limiting the type of proteins that can be studied by BDS. If the electrostatic dipoles are scattered and fluctuate independently they might not be any detectable signals within the frequency and temperature windows of the experiments appropriate for collective slow motions.

**TROUBLESHOOTING**

**Problem 1**
Ideally the sample should be prepared in pure water or volatile buffer so during the evaporation phase only the sample of interest remains. But maintaining the pH and have salt is necessary to preserve the structural integrity of proteins. Moreover ionized conditions are preferable to have electrostatic dipoles as main contributors to the dielectric signal and non-ionized dipoles negligible in comparison.

**Potential solution**
The dilution of a protein solution in water is a compromise.

**Problem 2**
The dielectric signals of cycles 1 and 2 should be similar if three hours incubation at 60°C is enough time for the protein to reach steady unfolded states (Figure 2A). If the dielectric signal decreases for the cycle 2 compared to the cycle 1 that may mean a longer incubation time is necessary to reach steady unfolding states. If the dielectric signal is lower over the entire temperature range, it could mean the bulk water evaporation was not complete after the first three hours of incubation.

**Potential solution**
Try different incubation times, the shortest time that exhibits no signal difference with longer incubation time is the incubation time for which a steady unfolding state is reached. Increase the evaporation time (see 3.c).

**Problem 3**
The cooling and heating dielectric signals should be similar (no hysteresis) (Figure 2B) if no unfolding or refolding takes place during the measurement.

**Potential solution**
If cooling and heating are different, it suggests that some unfolding/refolding takes place during the measurement. Longer incubation time can be attempted to reach unfolded states that will be steady during the cooling and heating measurement. The temperature rate can be increased to shorten the measurement time and make it faster than the time for conformational changes. The dielectric signal could be monitored in time to follow kinetics of conformational changes instead of spectral properties of steady states.

**RESOURCE AVAILABILITY**

**Lead contact**
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Claire Lesieur (claire.lesieur@ens-lyon.fr).

**Materials availability**
This study did not produce new unique reagents.

**Data and code availability**
Two codes are used during this study to produce two different networks: the amino acid network (AAN) and the electrostatic dipole network (EN). The codes to produce the AAN and EN are already published.
The AAN is produced using the code available at: https://github.com/lorpac/amino_acid_network.
To create the full AAN, the option “dim: ‘all’” should be used in the configuration file (analysis_config.json).

The EN is produced using the code available at: https://github.com/lorpac/amino_acid_network/blob/master/electrostatic_network.ipynb. To create the full EN, the option “dim: ‘all’” should be used in the configuration file (electrostatic_network_config.json).

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AUTHOR CONTRIBUTIONS
L.B. performed research, analyzed data, and contributed to writing the manuscript; L.P. performed research and analyzed data; A.S. designed research and C.L. designed research, analyzed data, and wrote the manuscript.

DECLARATION OF INTERESTS
The authors declare no competing interests.

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