PERSPECTIVE

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Pushing the limits of luminescence thermometry: probing the temperature of proteins in cells

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Abstract

Proteins are involved in numerous cellular activities such as transport and catalysis. Misfolding during biosynthesis and malfunctioning as a molecular machine may lead to physiological disorders and metabolic problems. Protein folding and mechanical work may be viewed as thermodynamic energetically favorable processes in which stochastic nonequilibrium intermediate states may be present with conditions such as thermal fluctuations. In my opinion, measuring those thermal fluctuations may be a way to access the energy exchange between the protein and the physiological environment and to better understand how those nonequilibrium states may influence the misfolding/folding process and the efficiency of the molecular engine cycle. Here, I discuss luminescence thermometry as a possible way to measure those temperature fluctuations from a single-molecule experimental perspective with its current technical limitations and challenges.

1 Introduction

Temperature is a fundamental physical parameter that can be defined thermodynamically as the degree of kinetic energy of the constituents of the matter. Macroscopically, temperature is generally associated with heat, and its measurement has been commonly used to monitor the proper operation of electrical, mechanical, chemical, and biological systems. Luminescence thermometers rely on the change of the luminescence intensity, bandwidth, peak wavelength, or lifetime, with the temperature. Luminescence thermometers are particularly useful for fast readout where adverse conditions are found, for example, temperature monitoring in electric power transformer networks, where quick rise of oil temperature and the presence of high magnetic fields limit the use of conventional thermocouples. As we moved towards miniaturization, nanoscale luminescence thermometers have been developed for use in, for example, studies of temperature gradients in electronic integrated circuitry, microfluidic devices, and intracellular structures [1]. Recently, studies of thermogenesis in organelles such as mitochondria, plasmatic reticulum, centrosome, and nucleus

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using luminescence-based thermometers have been reviewed [2]. Intracellular metabolic processes are diverse and very complex with the regulation of the biochemical reactions being carried out mainly by enzymatic activity. Understanding the mechanisms behind heat production in cells on a molecular level is a challenge. In this scenario, luminescence nano-thermometers may be a suitable tool to probe temperature gradients, especially in proteins.

2 Protein in a cell: a thermodynamic approach

Proteins participate in different cellular functions such as catalysis, structure, and transport [3]. Proteins are synthesized on ribosomes and the polypeptide linear chain folds spontaneously into a three-dimensional structure known as the native state. The amino acid sequence that is characteristic of each type of protein is responsible for the folding dynamics and the morphology of the three-dimensional folded state. Proteins may be unfolded by an external perturbation (physical, chemical, or mechanical agent), but they generally spontaneously return to the folded native state in physiological environments. Protein folding may be viewed as a thermodynamic process. The entire folding process is very complex with numerous possible stochastic thermodynamic pathways and meta-stable intermediate states that leads to a minimum Gibbs free energy equilibrium state, according to the folding energy funnel hypothesis. Figure 1 shows the energy landscape of a protein during folding. The width of the dominant funnel represents the configurational entropy, while the depth represents the free energy of an individual configuration averaged over the solvent alone. After the partially ordered molten globule is reached, another appropriate reaction coordinate to describe the folding process is Q, the fraction of native contacts. In the upper region of the funnel, helical order is established and the protein becomes compact [4].

While the difference in the Gibbs free energy, ΔG, between the initial and the final thermodynamic equilibrium states of the unfolding/refolding mechanisms has been

Fig. 1 The protein folding funnel for a fast-folding helical protein. Adapted from Ref. [4], with permission. Copyright Elsevier Cell Press 1996
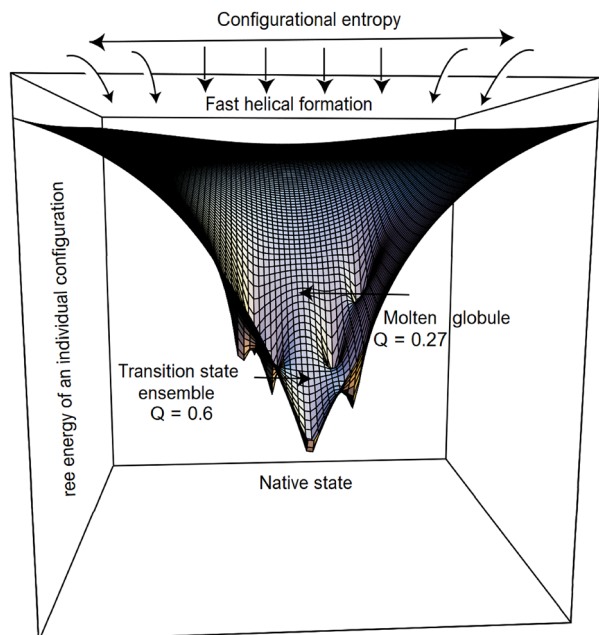
investigated from both a theoretical and experimental points of view [5], the study of inter-mediate nonequilibrium states is also important as these states may influence the folding process during biosynthesis and may help understand misfolding, which is the cause of some diseases [6]. Thermodynamically, the folding process might occur at constant temperature (the physiological environment is assumed to be a thermal bath), but stochastic nonequilibrium intermediate states may present thermal fluctuations that may be related to energy (heat) exchange between the protein and the physiological environment [7]. Therefore, the measurement of those temperature fluctuations/heat exchange is important to better understand the thermodynamics of those intermediate nonequilibrium states and its influence during the (mis)folding process. However, measuring those temperature fluctuations can be fairly challenging, as the folding mechanism can be quite fast. According to Kubelka et al. [8], both experimental and theoretical methods demonstrate that single-domain proteins can fold as fast as $\sim N/100$ μs, where N is the number of residues in the protein. However, a protein may also follow slow-pacing folding trajectories with trap states in the energy landscape that lead to much longer folding times (estimated to be as slow as ~ 1 s for $N = 100$) [9]. Besides the folding process, real-time temperature measurements would also be appealing for the study of fluctuations due to thermal noise in proteins that work as motors (rotary and linear engines) and shuttles [10]. These proteins generally use chemical reactions such as ATP hydrolysis as a fuel to generate mechanical work. Taking into account the first law of thermodynamics, part of the energy received by the protein is converted into useful work and the remaining energy is lost as heat. When proteins help transporting ions/molecules through cell membrane, for example, the work is considered thermodynamically favorable when the transport is performed from a region of high ion/molecule concentration to a region of low ion/molecule concentration. In this case, ΔG is associated to the chemical potential difference and the reversible work needed to perform the transport of ions/molecules across the membrane [11]. As a transport phenomenon, it may also be viewed as a nonequilibrium thermodynamical process in which heat conduction may lead to gradients of temperature and entropy production with time. Thus, the direct measurement of those temperature gradients may be relevant information to assess and perhaps optimize the efficiency of the energy cycle of the molecular motor.

3 Protein dynamics: theoretical and experimental analyses

Protein dynamics has been investigated by a number of theoretical methodologies and experimental techniques. From a theoretical point of view, computational and modeling have been used to analyze energy change in protein folding using statistical mechanics and nonequilibrium thermodynamics [12, 13]. From the experimental point of view, X-ray crystallography, optical spectroscopy, and nuclear magnetic resonance have been commonly used [14]. All these experimental techniques are employed in ensembles; i.e., the information retrieved is based on the collective behavior (average) of the objects of study, and therefore individual behavior associated to stochastic nonequilibrium fluctuations are not detectable. Single-molecule experiments have been focused on fluorescence correlation spectroscopy to study protein folding as a diffusive process on a free energy surface [15] and optical tweezer/atomic force microscopy to establish mechanical forces to stretch (unfold) the macromolecule and observe the dynamics of the refolding process [16]. Concerning proteins working as motors and shuttles, experimental techniques such as magnetic tweezers and electrorotation have been used to estimate the thermodynamic efficiency

under some constraint conditions such as quasi-static limit [17]. All the single-molecule experiments mentioned here were based on an external stimulus to induce some mechanical motion (folding, rotation) in order to study the dynamics of the system. However, biological systems under external stimulation and without external stimulation may undergo different metabolisms. Nothing has been tried concerning real-time temperature measurements on individual proteins spontaneously folding/performing a mechanical work. As mentioned earlier in the text, valuable information about misfolding during biosynthesis and poor engine efficiency, both possibly associated to stochastic thermodynamical nonequilibrium states, may be hypothetically retrieved by measurements of thermal fluctuations and, consequently, estimates of energy-related thermodynamic variables. Actually, the study of temperature fluctuations in nonequilibrium thermodynamical phenomena can be more widespread, not restricted only to proteins, but it can be extended to other types of biomacromolecules, such as, for example, DNA. Thus, the potential for using nanothermometers for probing nonequilibrium phenomena in biochemical reactions involving macromolecules is substantial.

4 Luminescence thermometry in a single protein: the experimental limitations and challenges

Optical temperature sensing using a single luminescent object linked to a biomacromolecule presents many challenges. For accurate real-time temperature monitoring of biomacromolecules, the luminescence nanothermometer has to be in contact with the biomacromolecule, while the temperature is estimated through some remote detection system that records certain change of the luminescence profile with the temperature on the object of study. Imaging and sensing of biological ensemble systems have been accomplished using different classes of luminophores, such as semiconductor quantum dots, lanthanide-doped nanocrystals, and fluorescent proteins [18]. Inorganic compounds have better photostability; i.e., they do not undergo photodegradation, a characteristic of organic compounds, and therefore they are more convenient for single-molecule studies. Due to its lower toxicity level, lanthanide-doped nanocrystals are generally more appropriate than semiconductor quantum dots, which contain elements such as Cd, Se, and S. However, due to its larger size and constitution, inorganic nanoparticles-biomacromolecules conjugates are much more complicated to fabricate than fluorescent proteins-biomacromolecules conjugates [18]. Thermometry using a single fluorescent protein is complicated by the fact that besides photodegradation due to long exposure to light excitation, organic molecules also show blinking, a characteristic of electronic population of dark states during relaxation. Lanthanide-doped nanoparticles, on the other hand, do not present blinking because a single nanoparticle has many luminophores, the lanthanide ions. Recent imaging and spectral analysis of the luminescence profile of individual lanthanide-doped nanoparticles are available in literature [19, 20]. Figure 2 shows the luminescence spectral profile of $NaYF_4$ nanoparticle-doped lanthanides (Yb^{3+} and Er^{3+}). The signal was recorded with a scanning confocal microscopy set-up [19]. Observe that the relative intensity of the two emission bands at 520–535 nm and 540–560 nm, which are radiative relaxations from thermally coupled electronic states $^4S_{3/2}$ and $^2H_{11/2}$ of Er^{3+}, changes with the temperature in a way that optical temperature sensing is feasible.

Single-molecule spectroscopy on nanoparticle-protein conjugate has not been reported so far. However, conjugation of proteins with nanoparticles has been proposed. For
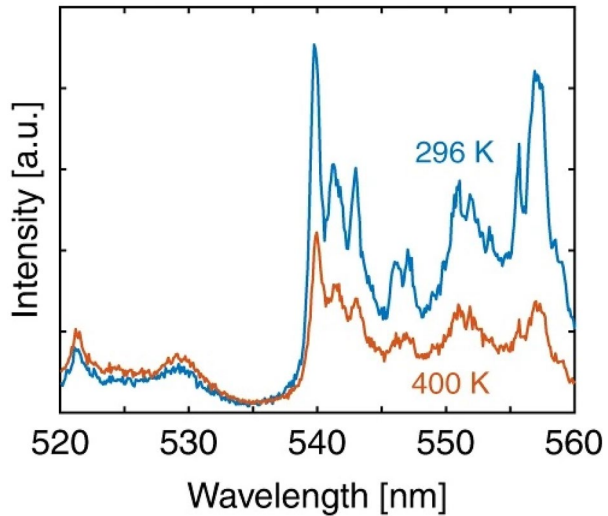
example, Fig. 3 shows an image of a space-filling modeling approach to study interactions between a maltose-binding protein and a semiconductor quantum dot [21], with surface sulfur atoms in teal and zinc atoms in pink. The red shell shows the estimated outer radius of the dihydrolipoic acid ligand. The side chain of Lys-370 is shown in blue.

It has been discussed that the structure and function of the protein can be influenced by many factors such as the size of the nanoparticle, the nanoparticle ligand, the material of the nanoparticle, the stoichiometry of the conjugate, the labeling site of the protein, and the nature of the linkage [22]. Folded proteins have an average size of a few nanometers (typically a diameter of 5–10 nm). Thus, ideally, the nanoparticle should be much smaller than that to avoid physical and chemical interference over the morphological dynamics of the protein. Recently, Kopp et al. surveyed the information available in literature regarding experimental investigations on the effects of the nanoparticles' size on their interaction with the proteins [23]. While proteins can be adsorbed and denatured on the surfaces of large nanoparticles (> 20 nm), ultrasmall nanoparticles (~ 2 nm) can leave the protein in its native structure. The reason being that the high surface curvature of the small nanoparticle significantly decreases the intensity of the van der Waals forces acting on the protein
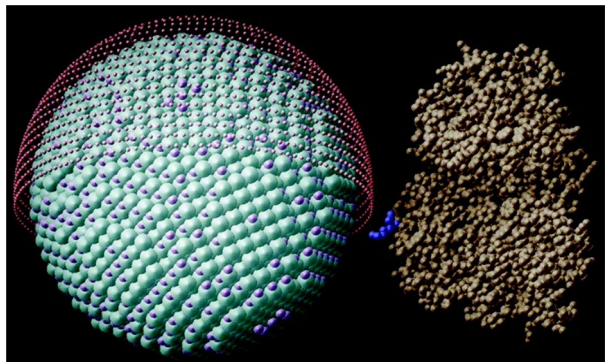
structure. Another characteristic that should be avoided is fluorescence resonance energy transfer (FRET), in which energy migrates from the excited luminescent temperature probe to the conjugated protein. FRET must be avoided as (a) it may decrease the intensity of the luminescence from the nanoparticle, which is used for thermometry, and (b) it may induce change of the protein energy state. Additionally, direct photon excitation of the protein must be avoided. As the nanoparticle-protein conjugates are much smaller than the spatial resolution of the optical system, typically larger than a few hundred nanometers (the Rayleigh diffraction limit), photons of the excitation source strike both the nanoparticle and protein. Therefore, excitation sources have to operate at wavelengths outside the absorption bands of electronic transitions of the organic structure, the so-called biological windows. Another important point that should be taken into consideration is the excitation power density. If the excitation power density is too high, the reading of temperature may be compromised by optically induced heating caused by strong absorption of light by the nanoparticle. A possible solution is the use of very small excitation powder densities, which besides avoiding optical heating also prevents nonlinear optical processes such as multi-photon absorption in both the nanoparticle and the protein. Interestingly, analysis on the luminescence from single sub-10 nm size lanthanide-based nanoparticle showed that self-heating is apparently absent at power densities as low as 3.8×10^4 W cm^{-2} [19]. As the luminescence intensity is proportional to the excitation power density and the amount of luminophores, the intensity of the luminescence is expected to be very low for a single nanoparticle. A mechanism called photon avalanche has been proposed as a way to improve imaging resolution and brightness at a single nanoparticle level [20]. For thermometry application, fundamental parameters are the absolute temperature sensitivity S, defined as the ratio in which the thermometric parameter changes with temperature, and the uncertainty in temperature δT [24]. To detect small thermal fluctuations, as should be the case found at a molecular level, S has to be very high, while δT has to be extremally small. Quintanilla and Liz-Marzán summarized the results available in literature for thermal resolution of various types of luminescent nanothermometers [25]. Nanodiamonds containing color centers (nitrogen-vacancy defects) showed the best performance with a reported thermal resolution of ~ 0.05 K. Engineering nanomaterials with tailored structural and bonding characteristics help improve S. For example, luminescence thermometry has been studied using the luminescence intensity ratio (LIR) technique in lanthanide-doped dielectric nanoparticles [24]. Assuming a Boltzmann population distribution among two thermally coupled electronic states (TCES) of lanthanide ions in solids, it is observed that S is a function of the barycenter of the energy bandgap between the TCES. In this case, the choice of the host material is a key factor because the crystal field influences the spectral peak position of emission bands. The parameter δT, on the other hand, is related to different factors such as, for example, the signal-to-noise ratio and integration time of the reading of the thermometric parameter. Thermal fluctuations related to nonequilibrium thermodynamic states are expected to be fast compared with other time scales of events happening during the protein activity and dynamics (microseconds to seconds). As a result, integration time of the reading of the thermometric parameter during the thermal fluctuation event has to be relatively short. Thus, the need of advanced instrument technology with superior spectral and temporal resolutions associated with high electronic noise reduction is fundamental to minimize δT.

Experimental limitations must be confronted with estimates obtained through theoretical modeling and computer simulations involving the interactions among proteins and functionalized nanoparticles. However, these interactions are quite complex, especially from an atomistic molecular dynamics approach. The situation is even more complicated if

the interactions of both the protein and the functionalized nanoparticle with the surrounding fluid are also taken into account, which is definitely the case from a thermodynamic point of view. A coarse-graining (CG) methodology has been recently applied to study the interactions among proteins and functionalized nanoparticles. In CG, the resolution of the system is decreased, and as a consequence, the number of degrees of freedom (number of interacting atoms) is reduced while maintaining a good balance between accuracy and resolution of elements. As such, a multiscale approach may be applied, in which the protein and functional groups mediating the protein-nanoparticle interactions are represented at the minimalist residue-based level, and the nanoparticle core is usually represented at the mesoscale level, for example, as a hard sphere [26]. This approach has been used in studies of adsorption, conformation change, and corona formation of proteins on the nanoparticles' surfaces, especially for drug delivery applications [27]. For temperature sensing and imaging applications, minimum perturbative interaction among the protein and the nanoparticle is desired because the nanoparticle shall not disturb the native structure and original physio-chemical performance of the biomacromolecule in its environment. In a recent paper published this year [28], the CG approach was used to estimate the influence of attaching a tracker (a gold nanoparticle) in the stepping trajectories a molecular motor (kinesin). The authors concluded that careful placement of the probe is necessary in order to minimize the bias induced by the presence of the nanoparticle on the stepping trajectory of the protein. The theoretical study of nanoparticles-proteins-bath interacting systems from a temperature sensing perspective, i.e., heat exchange, is a new and yet to be explored field. In the literature, analysis of heat transfer has been considered among proteins and their surrounding media. For example, Lervik et al. [29] used transient nonequilibrium molecular dynamics simulations to probe the heat transfer process in protein-water interfaces. Simulations were performed considering the temperature relaxation of proteins towards the temperature of the surrounding bath, a water droplet. They found that the temperature relaxation occurs within tens of picoseconds. The thermal conductivities of the studied proteins (myoglobin, GFP and Ca^{2+}-ATPase) were lower than that of water (0.1–0.2 W K^{-1} m^{-1} compared with 0.6 W K^{-1} m^{-1}), and the authors concluded that proteins should be able to sustain large thermal gradients across their structures. They also found that the heat capacities of the two Ca^{2+} ATPase conformations, 1SU4 and 1KJU, are the same (180 kJ m^{-1} K^{-1}), within statistical computation accuracy, indicating that the heat capacity is not sensitive to the structural detail of the proteins. In a recent review on luminescence nanothermometry, Suzuki and Plakhotnik [30] made an estimate of the temperature fluctuations due to mechanical fluctuations for a system of 10 nm size in thermodynamic equilibrium with a thermal bath. The authors obtained a temperature fluctuation of the order of 1 K with a characteristic relaxation time of energy exchange among the system and the bath of around 0.1 ns. From an experimental point of view, the authors believe that more accurate and less prone to artifacts methods of temperature measurement at the nanoscale are yet to be developed.

5 Conclusion

Proteins have a fundamental role in biological processes. The dynamics of such bio-chemical activities are complex, and a complete understanding of nonequilibrium states is currently lacking. In this scenario, there must be a tremendous effort to overcome all the current limitations that restrains measurements of thermal fluctuations involved

in nonequilibrium thermodynamic states present on the complex dynamics of proteins. However, the insight to be gained with those measurements will help better understand the mechanisms involved in the energy balance of important biochemical reactions and processes involved in the correct/incorrect operation of those key biomolecules. Luminescence thermometry is an attractive option to probe protein activity. Despite our current experimental limitations, it is a matter of time for the progress in technological tools and methods to achieve a level in which it will be feasible to unveil the details of the fascinating world of proteins at work, especially the thermodynamics of nonequilibrium states.

Declarations

Conflict of interest The author declares no competing interest.

References

1. Carlos, L.D., Palacio, F.: Thermometry at the Nanoscale: Techniques and Selected Applications. The Royal Society of Chemistry, Cambridge (2016)
2. Okabe, K., Sakaguchi, R., Beini, S., Kiyonaka, S.: Intracellular thermometry with fluorescent sensors for thermal biology. Pflügers Arch – Eur. J. Physiol. **470**, 717–731 (2018)
3. Buxbaum, E.: Fundamentals of Protein Structure and Function. Springer, Cham (2015)
4. Onuchic, J.N., Socci, N.D., Luthey-Schulten, Z., Wolynes, P.G.: Protein folding funnels: the nature of the transition state ensemble. Fold. Des. **1**, 441–450 (1996)
5. Fang, Y.: Gibbs free energy formula for protein folding. In: Morales-Rodriguez, R. (ed.) Thermodynamics - Fundamentals and its Application in Science, pp. 47–82. IntechOpen, London (2012)
6. Chiti, F., Dobson, C.M.: Protein misfolding, functional amyloid, and human disease. Annu. Rev. Biochem. **75**, 333–366 (2006)
7. Toyabe, S., Sano, M.: Nonequilibrium fluctuations in biological strands, machines, and cells. J. Phys. Soc. Jpn. **84**, 102001 (2015)
8. Kubelka, J., Hofrichter, J., Eaton, W.A.: The protein folding 'speed limit.' Curr. Opin. Struct. Biol. **14**, 76–88 (2004)
9. Thirumalai, D., O'Brien, E.P., Morrison, G., Hyeon, C.B.: Theoretical perspectives on protein folding. Annu. Rev. Biophys. **39**, 159–183 (2010)
10. van den Heuvel, M.G.L., Dekker, C.: Motor Proteins at work for nanotechnology. Science **317**, 333–336 (2007)
11. Gura, M., Golcuka, M., Yilmaza, S.Z., Taka, E.: Thermodynamic first law efficiency of membrane proteins. J. Biomol. Struct. Dyn. **38**, 439–449 (2020)
12. Li, Z., Scheraga, H.A.: Monte Carlo-minimization approach to the multiple-minima problem in protein folding. Proc. Natl. Acad. Sci. USA **84**, 6611–6615 (1987)
13. West, D.K., Olmsted, P.D., Paci, E.: Free energy for protein folding from nonequilibrium simulations using the Jarzynski equality. J. Chem. Phys. **125**, 204910 (2006)
14. Bartlett, A.I., Radford, S.E.: An expanding arsenal of experimental methods yields an explosion of insights into protein folding mechanisms. Nat. Struct. Mol. Biol. **16**, 582–588 (2009)
15. Nettels, D., Hoffmann, A., Schuler, B.: Unfolded protein and peptide dynamics investigated with single-molecule FRET and correlation spectroscopy from picoseconds to seconds. J. Phys. Chem. B **112**, 6137–6146 (2008)
16. Ritort, F.: Single-molecule experiments in biological physics: methods and applications. J. Phys.: Condens. Matter **18**, R531–R583 (2006)
17. Toyabe, S., Muneyuki, E.: Experimental thermodynamics of single molecular motor. Biophysics **9**, 91–98 (2013)

18. Himmelstoß, S.F., Hirsch, T.: A critical comparison of lanthanide based upconversion nanoparticles to fluorescent proteins, semiconductor quantum dots, and carbon dots for use in optical sensing and imaging. Methods Appl. Fluoresc. **7**, 022002 (2019)
19. Pickel, A.D., Teitelboim, A., Chan, E.M., Borys, N.J., Schuck, P.J., Dames, C.: Apparent self-heating of individual upconverting nanoparticle thermometers. Nat. Commun. **9**, 4907 (2018)
20. Lee, C., Xu, E.Z., Liu, Y., Teitelboim, A., Yao, K., Fernandez-Bravo, A., Kotulska, A.M., Nam, S.H., Suh, Y.D., Bednarkiewicz, A., Cohen, B.E., Chan, E.M., Schuck, P.J.: Giant nonlinear optical responses from photon-avalanching nanoparticles. Nature **589**, 230–235 (2021)
21. Medintz, I.L., Konnert, J.H., Clapp, A.R., Stanish, I., Twigg, M.E., Mattoussi, H., Mauro, J.M., Deschamps, J.R.: A fluorescence resonance energy transfer-derived structure of a quantum dot-protein bioconjugate nanoassembly. Proc. Natl. Acad. Sci. USA **101**, 9612–9617 (2004)
22. Aubin-Tam, M.E., Hamad-Schifferli, K.: Structure and function of nanoparticle–protein conjugates. Biomed. Mater. **3**, 034001 (2008)
23. Kopp, M., Kollenda, S., Epple, M.: Nanoparticle−protein interactions: therapeutic approaches and supramolecular chemistry. Acc. Chem. Res. **50**, 1383–1390 (2017)
24. Brites, C.D.S., Balabhadra, S., Carlos, L.D.: Lanthanide-based thermometers: at the cutting-edge of luminescence thermometry. Adv. Optical Mater. **7**, 1801239 (2019)
25. Quintanilla, M., Liz-Marzán, L.M.: Guiding rules for selecting a nanothermometer. Nano Today **19**, 126–145 (2018)
26. Brancolini, G., Tozzini, V.: Multiscale modeling of proteins interaction with functionalized nanoparticles. Curr. Opin. Colloid. Interface Sci. **41**, 66–73 (2019)
27. Sajib, S.J., Sarker, P., Wei, Y., Tao, X., Wei, T.: Protein corona on gold nanoparticles studied with coarse-grained simulations. Langmuir **36**, 13356–13363 (2020)
28. Hasnain, S., Mugnai, M.L., Thirumalai, D.: Effects of gold nanoparticles on the stepping trajectories of kinesin. J. Phys. Chem. B **125**, 10432–10444 (2021)
29. Lervik, A., Bresme, F., Kjelstrup, S., Bedeaux, D., Rubi, J.M.: Heat transfer in protein–water interfaces. Phys. Chem. Chem. Phys. **12**, 1610–1617 (2010)
30. Suzuki M., Plakhotnik T.: The challenge of intracellular temperature Biophys. Rev. **12**, 593–600 (2020)