Optical Tweezers: Unfolding the Unknown

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Abstract: Optical tweezers are a single-molecule characterization technique that can be applied in many fields. Though there are many biological applications for optical tweezers, one type of study that can be performed with optical tweezers is mechanical protein unfolding studies. This review paper focuses on the mechanical protein unfolding studies that have been performed. These studies can provide information on the folding pathway, intermediate states and misfolding of proteins. Specifically, slipknotted and knotted proteins can be investigated using optical tweezers. The function and folding pathway of these proteins are still unknown. Moreover, optical tweezers can be combined with surface-enhanced Raman spectroscopy to structurally characterize protein intermediates present at low concentrations.

Introduction

Optical tweezers were first introduced in 1970 by Arthur Ashkin when he showed that particles can be trapped using radiation pressure of light.[1] Optical tweezers can simultaneously exert forces and measure forces of dielectric particles, such as micro-sized beads or single molecules. Since its introduction, the single-molecule technique has been used in many fields, such as physics, chemistry and biology, to observe the behaviour of individual particles that is masked in bulk studies. Though optical tweezers are not the only instrument capable of performing single-molecule studies, the high spatial and temporal resolution distinguishes this technique from others. The biological applications of optical tweezers are wide-ranging. In particular, optical tweezers have been used to characterize the behaviour of molecular motors, such as kinesin and myosin.[2,3] Optical tweezers have also been applied to exert forces on living cells, cell organelles and large biomolecules.[4] Next to this, optical tweezers have been applied to mechanically unfold proteins, such as titin, since 1997.[5]

Using optical tweezers to perform mechanical unfolding studies, information about the refolding pathway, intermediates and misfolding of proteins can be obtained. Generally, mechanically unfolding proteins results in a linear polypeptide chain due to the low topological complexity of most proteins.[6,7] However, the mechanical unfolding of slipknotted and knotted proteins - proteins with high topological complexity - has gained much interest lately. Slipknotted proteins are proteins whose structure contains a threaded loop similar to that of a shoelace. Unfolding these proteins either results in tightening of the knot or complete unknotting. Although an exhaustive definition of knotted proteins is difficult to establish due to the incomplete mathematical definition of knots for open paths and the numerous possible knotted conformations,[8] these proteins are characterized by their high topological complexity.[6,9] The exact folding pathway and function of these proteins are yet unknown, however due to their complexity, they are speculated to have high folding barriers. Optical tweezers can be used to obtain specific details of the folding pathway of knotted and slipknotted proteins.[10–12]

Theory & Technical Overview

Optical tweezers are a characterization technique that can probe and investigate the mechanical characteristics of single molecules by optically trapping and exerting forces on those molecules. Simultaneously, optical tweezers can be used to

measure the forces of single molecules. The main benefit of performing single-molecule studies, such as mechanical protein unfolding studies, is that the properties of individual molecules, which are often masked in bulk or ensemble studies, can be observed.[13,14]

Optical tweezers are not the only available instruments for performing single-molecule studies. Another possible instrument is an atomic force microscope (AFM). The key difference between AFM and optical tweezers is the magnitude of the force that can be exerted on the molecules of interest. AFM can exert higher forces, exceeding 100 pN, whereas forces applied by optical tweezers are orders of magnitude lower, ranging between 0.1-100 pN.[13,14] Moreover, the spatial and temporal resolution that can be obtained with optical tweezers is higher than with AFM.[13]

One type of single-molecule study that can be performed with optical tweezers is mechanical protein unfolding, which yields information on the folding pathways of proteins. In these studies, optical tweezers are used to apply force on a protein molecule to study how the protein unfolds. Decreasing the applied force, the unfolded molecule refolds into a folded conformation. Specifically, these refolding studies yield information on the folding kinetics, thermodynamics and the structure of conformations. In particular, folding intermediates and their role in finding the native conformation of proteins can be directly studied.[15,16] Although experimental differences between thermal and mechanical unfolding studies have been reported,[17] the biological unfolding mechanism of misfolded proteins inside cells is based on mechanical denaturation. This shows the importance of performing mechanical unfolding studies. Moreover, the unfolding/refolding of proteins using force as opposed to heat or chemicals allows to selectively unfold and study individual domains of proteins.[14] These two factors make optical tweezers an interesting candidate for obtaining information on folding and unfolding pathways of proteins.

Optical tweezers consist of a very tightly focused laser beam, which acts as a trap for dielectric particles, such as small molecules or micron-sized beads made of silica or polystyrene to which molecules of interest can be attached. Various geometries for optical tweezers exist and can be distinguished by static and dynamic geometries. In static geometries, the optical trap remains stationary, whereas in dynamic geometries, the optical trap moves.[18] There are three frequently used static geometries in which optical tweezers using beads can operate: surface-based, in which one part of the molecule is adhered to a surface and the other to the bead. Suction-micropipette, in which one part of the molecule is adhered to a bead kept in place by the suction of the pipette and the other part is adhered to the bead in the trap. Finally, the dual trap geometry, in which the molecule is attached to two beads both in separate optical traps. These three geometries are shown in figure 1. Additionally, three dynamic geometries are: force clamp, where force is maintained constant by moving the trap, position clamp, in which force is measured by restricting movement of the tethered molecule, and lastly dynamic force spectroscopy, where rupture forces of bonds can be measured for various loading rates.[19] Generally, the used geometry is determined by the system of interest. If high spatial resolution and stability are required, dual trap geometry is preferred since it suffers less from stage drift. Additionally, drift between the separate traps can be reduced by creating dual traps from the same laser.[14,20]

Proteins are often indirectly attached to trapped beads using molecular handles, such as linear double-stranded DNA segments. One end of these DNA segments is attached to the protein and the other is labeled with biotin. The trapped beads are coated with streptavidin, which can bind to the biotin labeled end of the DNA segment. The bond between streptavidin and biotin is sufficiently strong that high enough forces can be applied without breaking the connection between the bead and the protein. Next to connecting proteins to the trapped beads, the DNA handles also limit the optical damage to the protein as well as limiting the interference of the mechanical properties of the beads with the protein. Since the handles do interfere in the measurement of the mechanical properties of the tethered molecules, the mechanical properties of the handles must be known before the study is performed.[13,15,21]

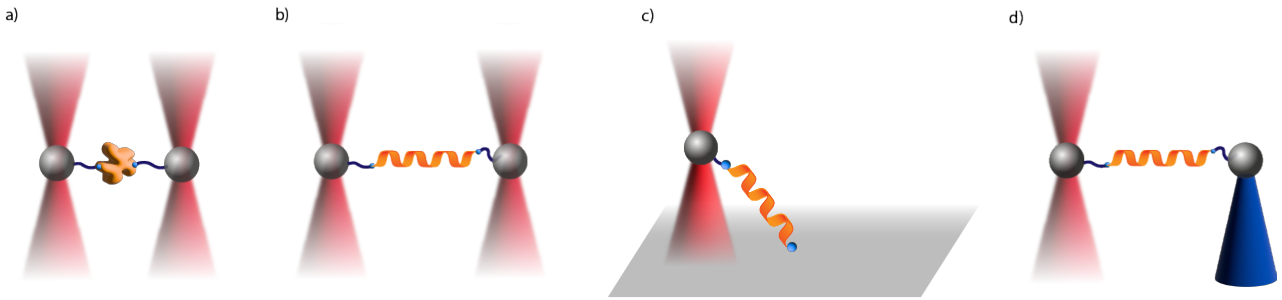

Figure 1: a) shows the dual trap geometry in which two beads are optically trapped. A protein is indirectly attached to two polystyrene-coated beads using DNA strands as molecular handles. In **b)**, the distance between the two trapped beads is increased, and the protein unfolds. **c)** shows the surface-based geometry in which a protein is tethered to one optically trapped bead and tethered to a surface. **d)** shows the suction-micropipette geometry in which a protein is tethered to two beads, one optically trapped and the other held in place through suction.

Gradient & Scattering Force

Photons can exert forces on objects through the transfer of momentum. For particles smaller than the wavelength of the laser, two important forces are relevant to the working principles behind optical trapping. Firstly, the electric field of the laser beam induces a fluctuating dipole moment in dielectric particles. If the electric field is inhomogeneous, the particles experience a gradient force directed towards the focus of the laser beam. This force depends on the polarizability of the particles and the intensity gradient of the light. In contrast, the scattering force, arising from scattering or absorption of photons impinging on the trapped particle, is directed away from the trapping center along the propagation direction of the light. This force depends on the absorption and the intensity of the light. If the gradient force dominates, the optical trap is stable. In order for the gradient force to exceed the scattering force, a large intensity gradient near the focus is required.[14,15]

Refraction & Reflection Forces

For biological samples, such as proteins, DNA or RNA strands, the wavelength of the laser used is often in the near infra-red region. Since proteins are often attached to micro-sized beads in mechanical unfolding studies, the particle size will be greater than the wavelength of the light. Particles larger than the wavelength of light can be considered as refractive objects, and the force experienced by the particles is explained by the transfer of linear momentum and Newton's second law of motion. As the momentum of the photons changes upon refraction or reflection through interaction with the particle, Newton's second law states that the change of momentum of the particle is equal but opposite to that of the photons. Refraction induces a force on the particle, which directs it towards the focus of the beam. Conversely, reflection induces a force which directs the particle along the propagation direction of the beam. If the refraction force is greater than the reflection force, a stable trap is obtained.[14,15,22] If a particle that is optically trapped is displaced, it will thus experience a restoring force directed towards the center of the trap. If the displacement of the particle is small, the restoring force is directly proportional to the displacement according to Hooke's law, $F = kx$. However, for larger displacements the force goes to zero and the particle is no longer trapped.[19] By measuring the displacement of an optically trapped bead, the force that is exerted by the tethered particle can be determined and a force-distance curve is obtained, from which information about the trapped object can be inferred.[23]

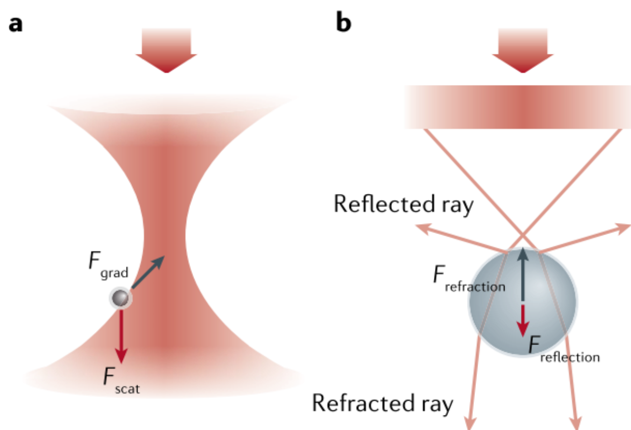

Figure 2: In **a**, the gradient and the scattering forces that the particle experiences are displayed. The gradient force is directed towards the focus of the laser beam, whereas the scattering force is directed along the direction of light propagation. In **b**, the reflection and refraction forces for particles larger than the wavelength are displayed. The refraction force is directed to the focus, and the reflection force along the light propagation direction. Reproduced from [14].

Spatial & Temporal Resolution

The high spatial resolution of optical tweezers allows for the measurement of the conformational changes and displacement of biological samples. The fundamental limit is set by the Brownian motion of trapped objects in a solution.[19] In reality, the limit of spatial resolution is determined by instrumental noise arising from electronic noise, mechanical oscillations, thermal contractions or expansions and pointing and power instability of the laser.[13,14,19] Various techniques are available to reduce these causes.[19]

The temporal resolution of optical tweezers is in the relevant time scale at which important biological interactions occur. The temporal resolution is limited by the relaxation time of molecules. In order to increase the spatial and temporal resolution, the back focal plane interferometry is often used to detect trapped objects. In this setup, the position of the trapped particle is determined using the inference pattern created by the transmitted light and light scattered by the particle.[14,24] In this way, the relative particle displacement can be measured.[25] With this interferometry, displacements of 1 Åwithin 0.1 ms can be measured.[14]

Experimental Setup

Originally, optical tweezers were often incorporated in optical microscopes. Nowadays, it is also possible to buy commercial optical tweezer setups. Due to the possibility of constructing optical tweezers from optical microscopes, the possible setups for optical tweezers range widely. However, certain aspects are common among all optical tweezer setups. Two essential parts for setups are: a high NA objective and the trapping laser.[13,14] The high NA objective is needed to focus the laser beam to a diffraction-limited spot and obtain a gradient intensity high enough to overcome the scattering force in order to create a stable trap. The minimum required value for the NA is 1.2, which means that most used objectives are either oil or water immersed. Oil immersed objectives are advantageous due to their higher NA. However, these objectives suffer from spherical aberrations, which affect trap performance for measurements that are performed deeper in the sample solution. For these experiments, it is better to use water immersed objectives. Moreover, the transmission of the objective is an important quality that must be taken into account. It is important that the transmission is high in the wavelength region of the used laser.[13,14,25,26] Various laser properties determine the correct laser choice. In order to obtain an inhomogeneous electric field, the laser beam intensity distribution should be a Gaussian distribution. Moreover, a high intensity beam is required for the gradient force to dominate over the scattering force, which means that optical tweezers require a high power output. The laser power also determines trap stiffness, and thus the maximum force that can be exerted. In practice, the emitted laser power is often 1 W.[14] However, there are certain factors that decrease the laser power as the beam reaches the sample, resulting in a lower laser power at the sample.[27] A drawback of the high laser intensity is that it causes heating of the local environment due to absorption and heating of the objective. The former can influence the behaviour of the system of study, whereas the latter can increase laser drift.[13,14] Both can be negated by incorporating feedback control of the sample stage and temperature control.[14] Additionally, thermal

drift and noise can be reduced by selecting a laser with high pointing and power stability.[25] Next to practical considerations, the sample also influences which laser can be used for the study. For biological samples, a laser with a wavelength in the NIR is often used to minimize optical damage to the sample. A possible laser that has been used in various studies on biological samples is, for example: diode pumped neodymium yttrium aluminium garnet with an excitation wavelength of 1064 nm.[13,25] This type of laser also has a high pointing and power stability as well as a high power output.[13]

Besides an objective with a high NA and an appropriate laser, optical tweezer setups also consist of a moveable sample stage, trapping chamber and a condenser lens. Additionally, most optical tweezers have methods, such as a camera, to observe the trapped objects, such as micro-sized beads.[25,28]

Mechanical Unfolding Studies

There are various ways in which mechanical unfolding studies can be performed. By moving one trap at constant velocity away from the other, a force ramp experiment can be conducted, in which the force on the protein increases as the traps are moved apart until it unfolds. By decreasing the distance between the beads, the force on the protein decreases and the protein refolds. These experiments can give information about unfolding steps of the protein, population of occupied states as the protein refolds and hysteresis between unfolding and refolding.[15]

In another experiment, a constant force is applied to the trapped proteins, and spontaneous unfolding/refolding of the proteins is studied. As the molecule folds or unfolds, the constant force is maintained by altering the distance between the beads. This type of experiment yields information on force-dependent lifetime of states.[15]

A similar experiment to constant force can be performed in which the beads first apply a certain force to the protein, but then remain stationary as the protein folds/unfolds. This method allows for mapping larger sections of the energy landscape of proteins and is able to measure more transitions in a shorter time than other methods.[15]

Finally, in cases where the previously mentioned methods are not suitable due to, for example, slow unfolding/refolding rates in the equilibrium force range, force jump experiments can be performed, in which the applied force is quickly increased or decreased and the time for a protein to unfold/refold is recorded. This experiment yields information on the lifetime of states and on intermediate states, provided the lifetime of intermediate states is long enough.[15]

Results & Discussion

One of the first mechanical unfolding studies in which a globular protein was unfolded and sequentially refolded using optical tweezers was performed in 2005 on ribonuclease H of *E. coli*.[15,18] The authors were able to map the folding energy landscape of the protein using the suction-micropipette geometry. Ensemble studies of the protein suggested the existence of an intermediate state between the folded and unfolded state of the protein, however, this intermediate had not yet been resolved. Using constant force experiments, this study was able to directly observe the intermediate state, and the results show that the intermediate state is not only thermodynamically stable, but essential to the folding pathway of the protein. Furthermore, the authors were able to characterize the mechanical properties, such as the elasticity, of this intermediate state, and from this alluded that the structure of the intermediate likely shares similarities to a molten globule structure, as was suggested in ensemble studies.[18]

Next to mapping energy landscapes and intermediate states, it is also possible to study the misfolding of proteins using optical tweezers. In particular, large proteins with complicated folding pathways can be studied to gain insight on the intermediate states generated during folding. The heat shock protein 90 of yeast has been studied, and information on the folding pathway was obtained. Specifically, the results indicate that there is a sequence in which the various protein domains refold. Additionally, the effect of force on misfolding was investigated in a force ramp experiment. The authors reported that applied force prevents formation of misfolded proteins, and can

guide the protein to its native folded state.[29]

A central question in the protein folding field is how unfolded proteins are able to find their stable folded conformation in a relatively short period of time. The number of possible conformations of a protein increases with its chain length, and if the folding process is random, it would take longer than the age of the universe before a protein would find its stable conformation. This problem is known as Levinthal's paradox. Currently, there are two proposed answers to this paradox: the defined pathway or the rugged energy landscape theory. By mapping the energy landscapes of various proteins using optical tweezers, the validity of these theories can be investigated.[14]

Many proteins with low topological complexity have been studied using optical tweezers, increasing the understanding of protein folding pathways and intermediates.[30] Recently, the mechanical unfolding/refolding of proteins with higher topological complexity has received more interest. Specifically, for proteins with conformations that were previously considered too complex to exist, mechanical unfolding studies can provide insight on the folding pathway.

Slipknotted & Knotted Proteins

The existence of knotted proteins was first proposed in 1994.[6] Due to the high topological complexity that knotted proteins have compared to unknotted proteins, knotted proteins were considered improbable. However, from the assumption that the native state of proteins is dictated by their potential energy landscape minimum, it was theorized that at least a small fraction of proteins should contains knots.[6] A schematic representation of a knotted protein is shown in figure 3a. The function and precise folding pathway of knotted proteins are unknown. Due to their high topological complexity, the folding process of these proteins is believed to be slow, and bulk studies indicate that the knotting is the rate limiting step of this process. Molecular dynamics (MD) simulations suggest that certain knotted proteins have on pathway slipknotted intermediates to reduce their high topological folding barrier.[10,11] Slipknotted proteins, as shown in figure 3b, differ from knotted proteins when it comes to the structure of the knot. A slipknotted protein contains a knot in a certain domain, but is unknotted if the entire protein is considered.[31] Unlike knotted proteins, the folding rate of slipknotted proteins is likely determined by the threaded and knotting loop. Investigating the refolding process of slipknotted and knotted proteins using single-molecule characterization techniques can provide useful insight on these matters.

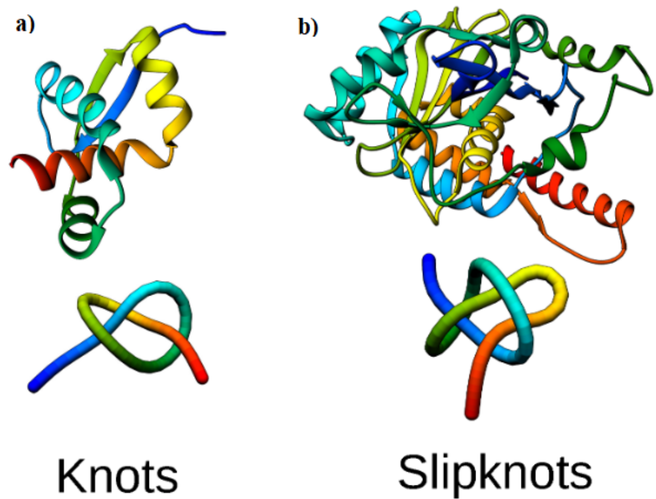

Figure 3: **a)** and **b)** show schematic representations of a knotted protein and a slipknotted protein. Adapted from [32].

Slipknotted and knotted proteins have previously been studied using AFM.[7,8] However, the force resolution of AFM is too low in order to directly observe the refolding of these proteins. In a study performed by He *et al.* the unfolding and refolding of slipknotted protein AFV3-109 was directly observed and studied using optical tweezers.[10] The protein was attached to beads with DNA handles, and the suction-micropipette geometry was used to fold/unfold the protein. The obtained force-distance curves are shown in figure 4. The authors determined that the GB1 DNA handles unfolded at forces higher than 30 pN, whereas the protein unfolded at forces around 10 pN.

Interestingly, the authors found inconsistency between studying the unfolding process using AFM or optical tweezers. In AFM, both two and three-state unfolding pathways were recorded, whereas with optical tweezers only two-state

pathways were recorded. This inconsistency is possibly caused by the difference in stiffness of the probe. The stiffness of AFM cantilevers is generally higher than optical tweezers. This indicates that the unfolding pathway depends on the probe stiffness. This was further supported when the authors performed the experiment using AFM probes with varying stiffness, and observed increasing two-state unfolding pathways as probe stiffness decreased. Additionally, the authors proposed that the use of DNA handles in optical tweezers could influence the results,[10] but since the authors report similar forces for unfolding and refolding using AFM and optical tweezers and have information about the unfolding/refolding characteristics of the handles used, this influence is likely small.

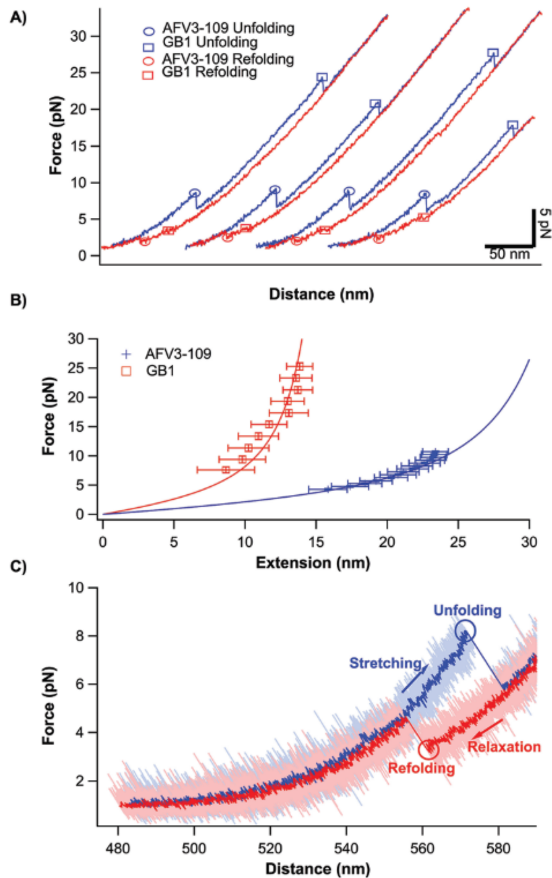

Figure 4: **A)** shows the force-distance curves obtained with optical tweezers for AFV3-109 attached with GB1 DNA handles. The protein is unfolded as the distance between the beads increases(blue curve), and refolds as the distance decreases(red curve). In **B)**, the extension of the protein and DNA handles are plotted as a function of the applied force. **C)** shows the force-distance curve of AFV3-109. Generally, the protein unfolded at 10 pN. Reproduced from [10].

This study revealed that the protein unfolded by untying the slipknot before complete unfolding occurred. Furthermore, after decreasing the force in order to refold the protein, it was observed that the protein refolded rapidly into its native state, with no observation of misfolded or intermediate states. This rapid refolding indicates that this slipknotted protein does not have a high topological barrier, which supports slipknotted intermediates as on pathway intermediates for knotted proteins. However, the authors indicate that the AFV3-109 protein might not be representable due to its relatively short slipknot, and therefore suggest that more complicated slipknotted proteins should be investigated.

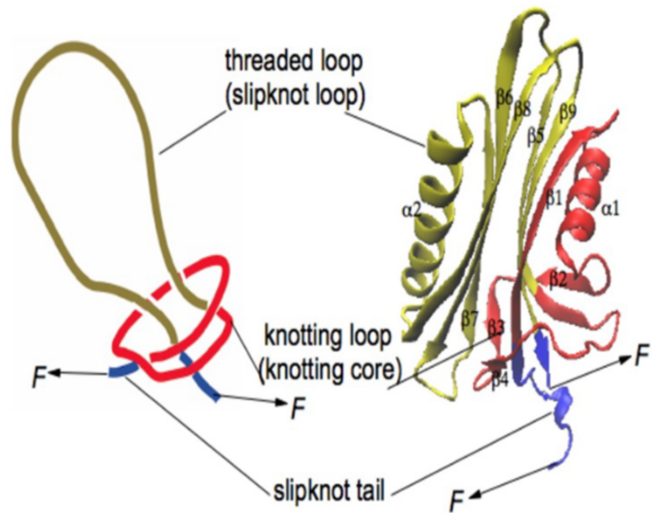

Figure 5: Schematic illustration of a slipknotted protein. During mechanical unfolding, the threaded loop and knotting loop compete, resulting in either untying the threaded loop or tightening the knotting loop. Reproduced from [11].

Wang *et al.* studied the refolding process of pyruvoyl-dependent arginine decarboxylase (PADC), a protein with a longer slipknot.[11] Using the suction-micropipette geometry and NuG DNA handles, the protein refolding process was studied. Complete unfolding of the protein as it was stretched was observed. The majority unfolded in a two-state manner, however a minority was observed that unfolded in a multiple-state pathway through a not well-defined intermediate. Interestingly, the refolding often resulted in misfolded states as opposed to the native state. Using steered MD simulations, the authors proposed that the observed two-state unfolding manner was caused by the

energy barrier associated with breaking certain β strands.

Comparing the results to the one obtained on AFV3-109, the authors determined that the unfolding of slipknotted proteins depends on two competing processes: untying the treaded loop and tightening of the knotting loop, shown in figure 5. Instead of refolding to the native conformation after relaxation, the majority of the proteins were observed in a misfolded state. The authors indicated that coarse grained MD simulations are needed to shed more light on the folding mechanism of this protein. Moreover, they also highlighted the reported differences between in vivo and in vitro protein unfolding studies, and indicated that studies of the protein as it is synthesized by a ribosome should be performed. Specifically, to yield information on whether this allows the threaded loop to form faster as predicted by simulations.[11]

In a study published in 2020, the refolding process of a knotted TrmD protein was studied using suction-micropipette geometry.[12] Unfolding of the knotted protein occurred in two or three-state manner at approximately 15 pN, and subsequent refolding occurred fast to the native state. The unfolded length obtained from the force-distance curve was smaller than the one calculated. The authors indicated that this is likely caused by the α-helix present in the structure, which is known to unfold at forces too low to be detected by optical tweezers. This hypothesis is supported by deleting the α-helix and measuring the length of the truncated protein, which is similar to that of the original unfolded protein. Moreover, the authors determined that the unfolded structure contains a tightened knot.

During refolding, the authors observed the reverse process of unfolding, which indicated that loosening of the tightened knot may be part of the original folding process of the protein. After investigating the unfolding/refolding of the knotted protein, the authors aimed to unfold the knot of the protein using a truncated protein. They determined that the protein was unfolded and untied at a force of 8 pN, and refolded at 4 pN with the majority of the proteins in an untied, misfolded conformation. By comparing the kinetics of folding for the untied truncated protein and the original protein, the authors determined that knot formation is the rate limiting step in the folding process of this protein.[12]

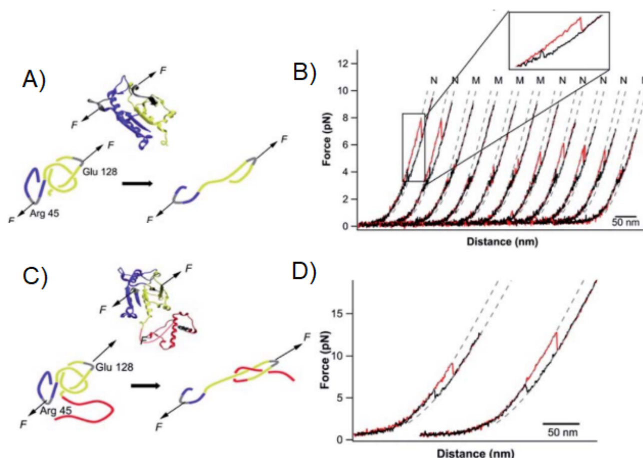

Figure 6: **A)** displays the unfolding of the truncated protein, and **B)** shows the corresponding force-distance curves. Stretching(red) results in unfolding at approximately 8 pN to an untied state, and relaxation(black) resulting in refolding at 4 pN. Adapted from [12].

Optical Tweezers & SERS

It can be challenging to characterize intermediates of proteins present in low concentrations. In bulk studies, these intermediates are masked by other conformations present in higher concentrations. Another method for characterization is surface-enhanced Raman spectroscopy(SERS). However, locating the nanoparticles to which the proteins are tethered can be challenging. Furthermore, this method is limited by its low reproducibility. Recently, a study was performed combining optical tweezers and SERS in an effort to resolve the intermediates present at low concentration in their native state.[33] Though optical tweezers and SERS were already combined in previous studies,[34–36] the resolution and mechanical control of the technique remained challenging. In this study, micro-sized silica beads coated with Ag nanoparticles were optically trapped and used as a controllable probe to perform in situ characterization of four proteins. In particular, the structural characterization of alpha-synuclein, an intrinsically disordered protein linked to Parkinson's disease, is reported.

The obtained data is shown in figure 7. Whereas

the recorded CD spectrum indicated random coil conformation, the SERS spectra recorded using optical tweezers revealed the additional presence of α-helix and β-sheet conformations. The authors created a controllable SERS probe using optical tweezers, which was then employed to characterize the conformations of alpha-synuclein present in low concentrations. Specifically, the observation of β-sheets is linked to the fibrillation of the protein at later stages. The authors also indicate that this setup could possibly be adapted in order to perform mechanical unfolding studies.

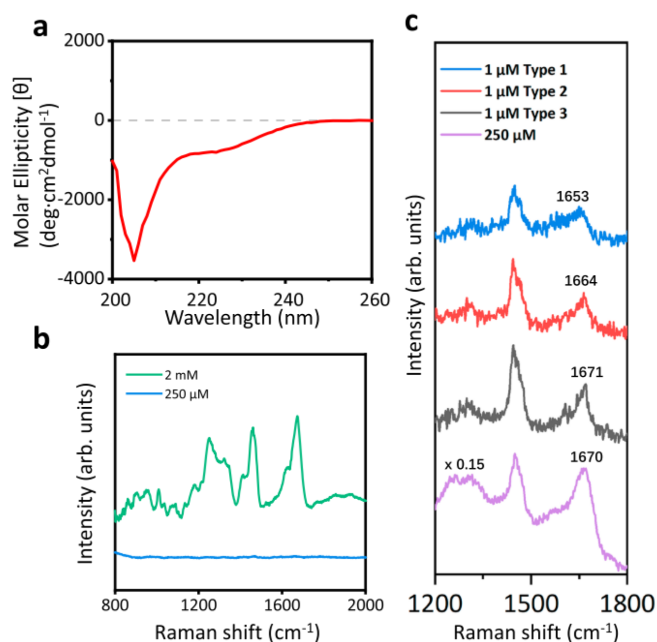

Figure 7: a) shows the CD spectrum of alpha-synuclein suggesting random coil formation. **b)** shows the Raman spectra of alpha-synuclein at two different concentrations. **c)** shows three SERS spectra of alpha-synuclein at 1 μM suggesting α-helix(blue), β-sheet(red) and random coil(grey) conformations present at physiological concentration. Reproduced from [33].

Limitations & Challenges

One main limitation regarding all single-molecule studies, including optical tweezers, is the low amount of molecules that are studied at a specific amount of time. The fact that single proteins - as opposed to ensembles - are studied is simultaneously an advantage as well as a limitation of these studies. In order to report results that are statistically representative for the system, a large amount of proteins have to be investigated, which can be time consuming. Since this limitation stems from the main advantage of the technique, it is difficult to overcome. However, approaches to make the process less time consuming have been proposed. For example, some propose to automize certain steps, such as optically trapping the beads, using artificial intelligence.[14] These suggestions have yet to be incorporated into existing optical tweezer setups. Therefore, it is not possible to completely determine how effective this will be. However, though the automation of the process might make the process less intensive, the amount of time that could be saved by automation is not immediately clear. Furthermore, the inconsistency that exists between different single-molecule techniques, such as AFM and optical tweezers, makes interpretation and comparison of data difficult. Since the stiffness of the probe influences the obtained unfolding/refolding force-distance curves, appropriate choice of stiffness is essential.

A technique similar to optical tweezers is acoustic force spectroscopy. As opposed to light, sound waves are used to trap particles. Therefore, unlike optical tweezers, acoustic tweezers do not require a high NA objective and a high laser power. In particular, the absence of the high laser power is an advantage when performing experiments with biological samples, since high laser power can damage these samples. However, the spatial resolution that can be obtained with optical tweezers, 0.1-1 nm, is higher than that of acoustic tweezers, 1-10 μm.[37] Mechanical unfolding studies can be performed with acoustic tweezers in similar fashion as with optical tweezers. However, the force range of acoustic tweezers, 0.3 fN - 200 pN, is wider than that of optical tweezers, 0.1 pN - 100 pN. Moreover, with acoustic tweezers it is possible to unfold multiple proteins at once and thus have a higher throughput than optical tweezers.[37,38] These factors make acoustic tweezers another interesting technique for mechanical unfolding studies, provided the spatial resolution of the technique can be improved.

Finally, another challenge is the discrepancy between in vivo and in vitro studies reported in studies using optical tweezers. Ideally, experimental environments should be as similar as possible to biological conditions. However, recreating these environments in vitro is

challenging. To overcome this challenge, the formulation of protocols has been proposed in order to provide common ground between the different environments.[30] Using optical tweezers, the folding process is often inferred from intermediate states observed between the folded and unfolded state. This method, however, neglects the role that biological folding guiding mechanisms, such as chaperons, can play in protein folding.[12] To gain more insight on in vivo protein folding, these factors have to be taken into account.

Summary & Outlook

Due to the transfer of momentum of photons, dielectric particles can be optically trapped using optical tweezers. By attaching proteins to optically trapped micro-sized beads using molecular handles, the proteins can be unfolded/refolded by increasing/decreasing the distance between the beads. The resulting force-distance curves provide information about the folding pathway, possible intermediates or misfolded states of proteins. Although using force to study protein unfolding has been questioned, the cellular unfolding mechanism shows that understanding the mechanical denaturation of proteins remains relevant.

Next to using optical tweezers to unfold and refold proteins, there are other relevant biological fields in which the technique is used to gain insight on specific areas. For example, optical tweezers are currently also used to yield information on virus assembly.[39] Additionally, optical tweezers as a method to characterize the mechanical properties of coacervates are also being developed.[40]

Optical tweezers are not the only method used for performing single-molecule studies. Other techniques, such as AFM, are also often employed. However, there are cases where different folding pathways of proteins are reported depending on the characterization technique used, as illustrated by He *et al.* The authors report that this could be due to the difference in probe stiffness or the influence of molecular handles on the recorded force-distance curves. This inconsistency should be resolved in order to conceptually interpret the obtained force-distance curves. Next to AFM, acoustic tweezers can also be used for mechanical unfolding studies. Although, the spatial resolution of the technique is lower than that of optical tweezers, the throughput that can be obtained with acoustic tweezers is higher.

Mechanically unfolding proteins using optical tweezers occurs in vitro. Thus, the environment of the proteins is significantly different from their biological environment. Moreover, the unfolding/refolding of proteins is often studied in the absence of chaperones, and the observed protein refolding can therefore be from the actual folding pathway of the protein. In order to resolve these discrepancies, protein unfolding studies should be performed in conditions that reflect the natural environment as closely as possible and preferably in the presence of chaperones. Moreover, protein folding as the protein is synthesized by ribosomes should also be further investigated using optical tweezers.

Currently, optical tweezers are used to untie knotted or slipknotted proteins in an attempt to understand their folding pathways. Generally, it is observed that unfolding of slipknotted proteins depends on competition between knotting and threading loop, however, more proteins must be investigated to fully characterize the pathway of these proteins, since their refolding behaviour also depends on the size of the threaded loop. Moreover, the study of knotted proteins indicates that knotting is the rate limited step for the folding of proteins. MD simulations have played a key role in interpreting the results of these studies. Showing that MD simulations and mechanical unfolding studies are a powerful combination. Knotted proteins make up a small fraction of the characterized proteins, however, there are various different knotted proteins that have been observed.[32] Moreover, the precise function and folding pathways of these proteins are not yet completely understood.[41] Therefore, more studies combining optical tweezers and MD simulations to investigate various knotted proteins should be performed.

Additionally, optical tweezers can be combined with SERS to characterize protein intermediates linked to neurodegenerative diseases that are present in low concentrations. This method has been used for the in situ study of a protein linked

to Parkinson's disease. Performing more studies on various proteins might yield more information about early misfolding of proteins relevant to specific diseases. Moreover, the authors suggest adapting the setup to perform mechanical unfolding studies as well as SERS.[33] This combination could reveal not only information about the conformation of misfolded proteins in early stages, but also on the folding pathway of the misfolded proteins.

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