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Whispering-gallery mode (WGM) sensors: review of established and WGM-based techniques to study protein conformational dynamics

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Monitoring the conformational dynamics of proteins is crucial for a better understanding of their biological functions. To observe the structural dynamics of proteins, it is often necessary to study each molecule individually. To this end, single-molecule techniques have been developed such as Förster resonance energy transfer and optical tweezers. However, although powerful, these techniques do have their limitations, for example, limited temporal resolution, or necessity for fluorescent labelling, and they can often only access a limited set of all protein motions. Here, within the context of established structural biology techniques, we review a new class of highly sensitive optical devices based on WGM, which characterise protein dynamics on previously inaccessible timescales, visualise motions throughout a protein, and track movements of single atoms.

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Introduction

Proteins are macromolecules that perform numerous essential tasks within organisms, which include metabolic catalysis, DNA transcription and replication, signal transduction, and molecular transport. Most proteins gain their functionality from a complex three-dimensional structure and the ability to change this structure, often in direct response to interactions with other molecules or in response to changes in environmental conditions such as temperature and pH. Proteins can adopt many different conformations over very short time intervals. This characteristic is often described with a multidimensional energy landscape that defines the relative probabilities of the conformational states and the energy barriers between them [1•]. In 1975 Austin *et al.* first hypothesised that the functionality of a protein (rebinding of carbon monoxide and oxygen to myoglobin) can be described by such an energy-landscape picture [2•]. Subsequent studies [2•,3–5] reinforced the energy landscape picture to describe protein function, and suggested the landscape is influenced by both the surrounding environment and the presence of ligands. The dynamic motion within a protein's structure is extremely complex and involves varied timescales, amplitudes of motion and different directionalities associated with the structural motions and fluctuations. The energy landscape is hence multidimensional and also tied to a particular set of temperature, pressure and solvent conditions.

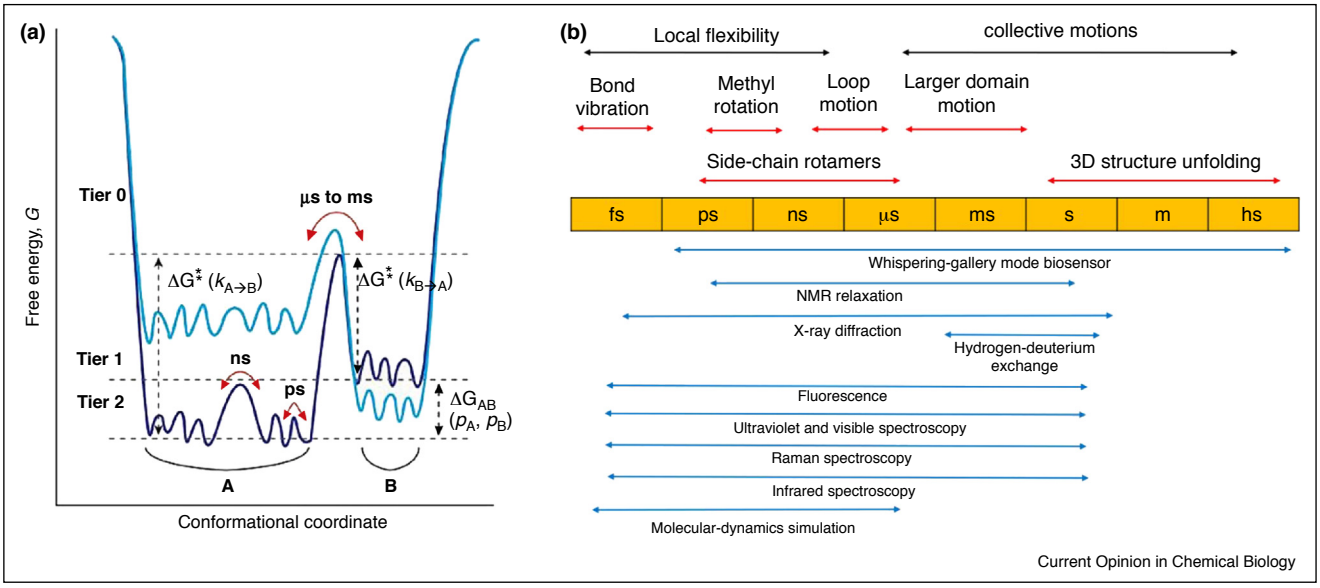
The classification of protein dynamics based on timescales of motion is a different and perhaps more straightforward approach. Henzler-Wildman and Kern [1•] classifies protein motions into different tiers (0–2) based on their timescales and free energy. Figure 1(a) shows a one-dimensional cross-section through the energy landscape of a protein, indicating the hierarchy of protein dynamics and energy barriers. Figure 1(b) shows the timescales of motions and experimental methods capable of detecting fluctuations on each timescale, including the emerging WGM biosensing technique. The changes in protein structure occur over varied timescales; some conformational changes occur in subpicosecond timeframes, and others take several hours.

Established experimental methods for characterising protein dynamics

Established techniques that probe an ensemble of protein molecules

Various experimental and simulation techniques have been developed to describe the structure and dynamics of proteins in the last three decades. X-ray crystallography, cryo-electron microscopy (cryo-EM), small angle X-ray scattering (SAXS) and Nuclear Magnetic Resonance (NMR) spectroscopy have enabled the visualisation of a static protein structure, down to atomic resolution (X-ray, cryo-EM, NMR). X-ray crystallography requires preparation of homogenous crystals, trapping proteins in conformational substates [6]. However, it is possible to study conformational changes of small amplitudes in the

Figure 1



(a) One-dimensional cross-section through the high-dimensional energy landscape of a protein showing the hierarchy of protein dynamics and the energy barriers. An energy surface minimum is described as a 'state'. The transition state is the maximum energy difference between states. The populations of the tier-0 states A and B (p_A, p_B) are defined by Boltzmann distributions based on their difference in free energy (ΔG_{AB}). The barrier between these states ($\Delta G^{\ddagger}$) determines the rate of interconversion (k). Lower tiers describe faster fluctuations between a large number of closely related substates within each tier-0 state. A change in the system will alter the energy landscape (from dark blue to light blue, or vice versa). For example, ligand-binding, protein mutation and changes in external conditions shift the equilibrium between states. **(b)** Timescale of dynamic processes in proteins and the experimental methods that can detect motions and fluctuations on each timescale.
Figure adapted from Ref. [1•].

crystalline phase using kinetic X-ray crystallography techniques as function is still preserved after crystallisation [7]. Cryo-EM and SAXS on the other hand enable studies of the structural ensemble directly under experimental conditions [8,9]. However, it is difficult to apply these methods to characterise the timescales of state interconversion [1•]. NMR spectroscopy has been the most popular technique for studying protein dynamics over varied timescales in solution. By introducing spin 1/2 isotopes, ^{1}H, ^{13}C, ^{15}N and ^{31}P, site-specific local structure and dynamics can be monitored [10,11]. NMR also enables the characterisation of interconversion timescales. Different effects are employed depending on the interconversion timescale represented by rate k_{ex} which quantifies the average number of stochastic exchange events per unit time, relative to the chemical shift timescale represented by frequency Δv, the difference in chemical shift of two conformational substates. For slow timescales, $k_{ex} < \Delta v$, exchange rates vary between 0.1 s to tens of seconds can be measured using the Nuclear-Overhauser Effect (NOE) and EXchange SpectroscopY, EXSY (also known as zz-exchange spectroscopy) [12]. Physical processes in these timescales include protein folding, solvent hydrogen-exchange and slow conformational changes such as ligand binding and release [10]. For higher rates ($k_{ex} \geq \Delta v$), various relaxation dispersion experiments have been developed [13]. Dynamics in this time window include loop motion, secondary structure changes and side chain reorientation [10].

Established techniques that probe a single protein molecule

The above-mentioned experimental techniques are mostly bulk measurement techniques, requiring averaging across thousands of molecules at a time. More recently, another class of methods has emerged, capable of studying a single individual protein molecule. The main strength of single-molecule techniques is their ability to detect molecular heterogeneity, transient intermediates, rare events and the sequence of the structural states adopted by a protein over time [1•], all of which is often hidden in bulk measurements due to the averaging needed. Force spectroscopy using optical or magnetic tweezers attached to proteins and atomic force microscopy [14] has provided, for example, detailed measurements of elasticity of nucleic acids, measurement of bond energies, lifetime and entire energy landscapes. Single-molecule force spectroscopy generally measures the extension of a molecule attached to a force probe, when mechanical tension is applied across the molecule. The review from Woodside and Block [15] provides a detailed guide on the reconstruction of energy landscapes from various types single-molecule force spectra. The main concern with force spectroscopy is that the natural

dynamics of the protein might be hindered due the external forces applied and the probe-attachment protocols. Hence, alternative techniques to monitor proteins in the native state are sought, most popularly through observing single-molecule fluorescence [16,17]. The large range of fluorescent dyes commercially available, combined with optimised optics, efficient detectors and algorithms have made single-molecule fluorescence spectroscopy the method of choice for dynamic studies in the past decade. Additionally, single molecule Förster resonance energy transfer (smFRET), can accurately measure distances in Ångstroms, allowing characterisation of distance over time [18]. Single-molecule fluorescence experiments have finally reached a stage where critical molecular and biological questions are being answered. For example, in 2013 Chung and Eaton studied the dynamics of barrier-crossing in small, helical proteins using single-molecule fluorescence experiments [19•].

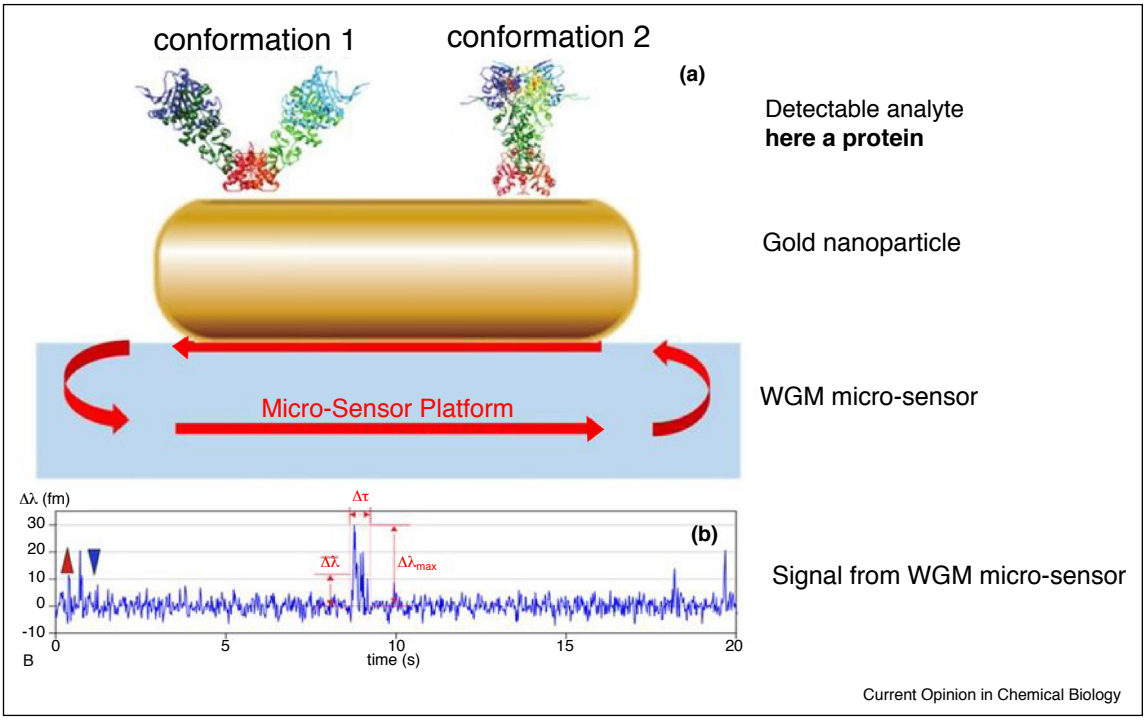
Complementary to experimental methods, computational techniques have been fundamental in understanding protein dynamics. Although experimental methods provide information on conformation substates, an atomic-resolution description of the change from one 'valley in the energy landscape to another' is currently out of experimental reach, due to extremely low probability and short lifetimes of the high energy conformers [1•]. Computational molecular dynamics techniques can describe the position of each atom in a protein over time starting from a known atomic resolution 3D structure. However, even with high computational resources, conducting long-timescale simulations (>1 μs) to include all atoms of even small proteins (~10 kDa or less) remains challenging. Although computational methods can provide critical insights into protein dynamics, experimental validation is always necessary.

Emerging WGM sensors for characterising protein dynamics

Whispering-gallery mode sensors are emerging micro-optical devices that exploit the precise measurement of an optical resonance frequency to detect proteins and other biomolecules with exceedingly high sensitivity [20••,21–27]. The optical resonance is excited by sending light into a small WGM micro-sensor, which circulates the light. An example of a commonly used WGM micro-sensors is a glass micro-bead, which is approximately 100 μm in diameter [28,29]. When light enters into the microsphere, it can remain trapped inside sphere due to the total internal reflection. The circulating light results in self-interference, producing an optical resonance phenomenon, the so-called whispering-gallery modes (WGMs). Light waves extend via a so-called evanescent field, somewhat beyond the surface of the micro-bead. If a protein adheres to the surface of the micro-bead, the protein interacts with the light. The effect is equivalent to increasing the circumference of the bead, the circular wall which traps the light. Consequently, proteins attached to a micro-bead alter the optical resonance wavelength of that bead. Although the change is minimal, a shift can be detected due to the sharpness, or high-quality factor (Q-factor), of the resonance. One of the most interesting properties of optical WGMs, such as microspheres made by melting of optical fibres, is their extremely high Q-factors for biosensing, which can reach 10^{7-8} [30•,21]. The Q-factor of a measurement is proportional to the decay-time of the waves, and depends on both the surface scattering loss and the absorption loss in the glass. WGMs can be further characterised by two polarisation numbers (TE and TM) and three mode numbers (radial n, angular l, and azimuthal m). 'High-Q' resonances enable WGM sensors to reach the high sensitivity that is required for detection of biomolecules in solution.

Dielectric WGM sensors, such as glass micro-beads, can detect multiple proteins bound to a sensor, even in complex media and in real-time [31]. From these ensemble measurements it is possible to determine the concentration of analyte molecules in various biosensing applications. It is difficult, however, to determine the behaviour of individual molecules from such ensemble measurements. To study the dynamics of a protein and its conformational changes it is necessary to observe the WGM signals from individual proteins. To achieve single-molecule detection with WGMs sensors, the sensitivity of the detection scheme has to be enhanced by several orders of magnitude [32]. One approach is to concentrate the light on a deep subwavelength length-scale in order to enhance interactions with a single protein. Such deep-subwavelength concentration of light beyond the scale of the diffraction limit is possible when utilising metallic nanostructures. The trick is to combine the optics of WGMs with a very different branch of optics: plasmonics [33–36,37••,38]. As it is conceptually shown in Figure 2, by attaching a plasmonic metal nanorod, of approximately 100 nm × 20 nm in size, to the glass microbead sensor it becomes possible to concentrate the light down to the dimensions of a protein, or about 10 nm. A detection signal (WGM resonance wavelength shift) occurs when the overlap of a biomolecule, such as DNA or protein, with this nanoscale light field changes [20••,39••], see Figure 3. Essentially, biomolecule intersecting with the light slightly changes the path-length for each roundtrip around the micro-bead sensor. This change in path-length shifts the optical resonance wavelength/frequency of a WGM. In this way, it has recently become possible to demonstrate detection of the conformational motions of an active polymerase enzyme immobilised on a WGM sensor [40••], see Figure 3. As described in this seminal publication, a WGM resonance wavelength-shift signal followed the opening and closing motions of the 'polymerase hand' [40••]. This work demonstrates an optical, label-free method capable of observing enzymatic

Figure 2



(a) A WGM micro-sensor platform recirculates the probing light (in red) and concentrates the probing light field with the use of a plasmonic gold nanoparticle to achieve detection sensitivity down to the observation of conformational changes from a single protein molecule. **(b)** The example signal trace (resonance wavelength change Δλ recorded over time) illustrate the signal shifts that occur due to transient conformational changes of a protein, see spike-like signals at time-point ~9 s. Positive wavelength shifts (red arrow) correspond to the protein adopting conformation 1, negative wavelength shifts (blue arrow) correspond to the protein adopting conformation 2.

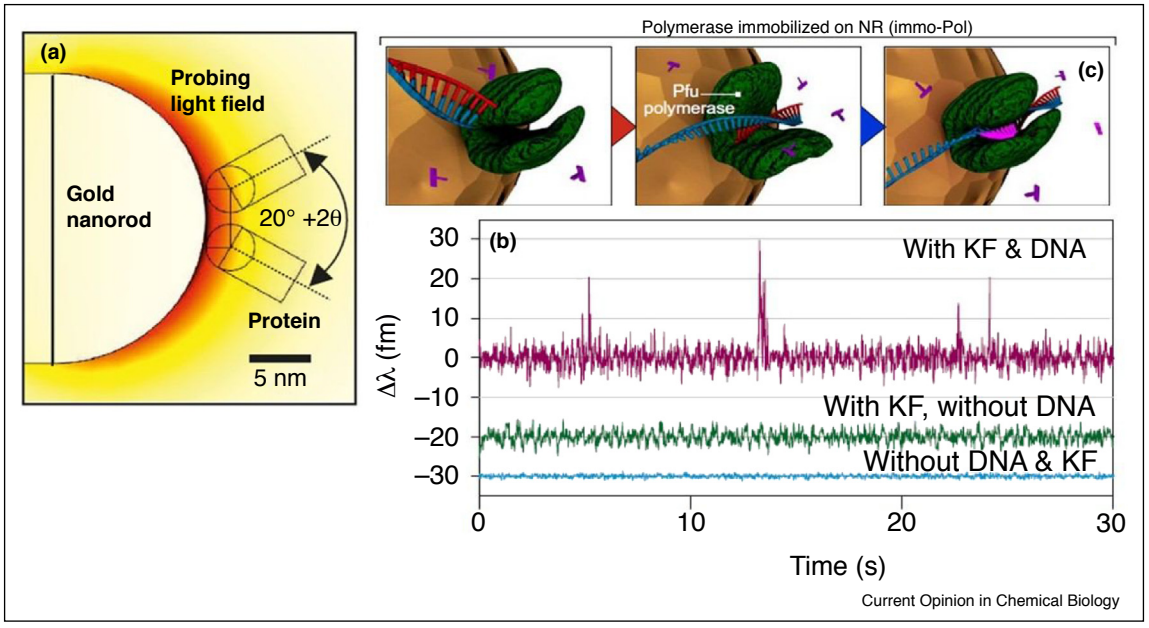
interactions and associated conformational changes on a single-molecule level. DNA polymerase from *Escherichia coli* (Klenow Fragment, KF) and *Pyrococcus furiosus* (Pfu) were immobilised in their active forms on plasmonic nanorods. DNA was mixed into the solution and then copied by the active enzymes. The active enzymes were inside the near-field focused by the plasmonic nanorod. In this way, it was possible to measure changes in the sensor signal as the KF and Pfu polymerase enzyme 'hand' opened and closed, a motion that is known to be part the conformational changes during polymerase enzyme activity [40••]. Figure 3b, top trace for KF and DNA, shows some of the sensor signals that were recorded for the opening and closing motions of the polymerase 'hand' which are illustrated in Figure 3c. The opening and closing motions reveal themselves as resonance wavelength shifts Δλ to longer and back to shorter wavelengths, respectively. A resonance red shift is observed when the overall overlap of the protein with the probing near-field increases, and a blue shift when this overlap decreases.

Using external cavity lasers to acquire the WGM spectrum often limits the acquisition speed to the piezo positioners of the cavity mirrors, which normally work up to 1 kHz. One technique to overcome this limitation is to use a frequency lock-in technique, where an error signal is obtained by demodulating the beat-note between the cavity field and a high-frequency side-band [41]. This error signal is used to lock the laser frequency to the cavity resonance instantaneously. This technique has been used for measurements of near-field WGM optomechanics [42] and for WGM single nano-particle detection [43]. Although, the frequency lock-in provides fast technique for detecting shifts in the cavity resonance, the complete cavity spectrum cannot be obtained. An alternative approach, cavity ring-up spectroscopy (CRUS), published by Rosenblum *et al.*, uses far-detuned pulses to acquire the complete WGM spectrum in the nanosecond time-scale [44••].

Outlook for WGM sensing of single-protein structural dynamics

Work is underway to develop a WGM sensor platform with several sensing channels, which probe the protein molecular dynamics with different laser light, thereby gaining information on the dynamics in different parts of a protein. In principle this is possible, due to the overlap of the probing near-field with a protein being dependent on its wavelength. By varying the probing wavelength, it

Figure 3



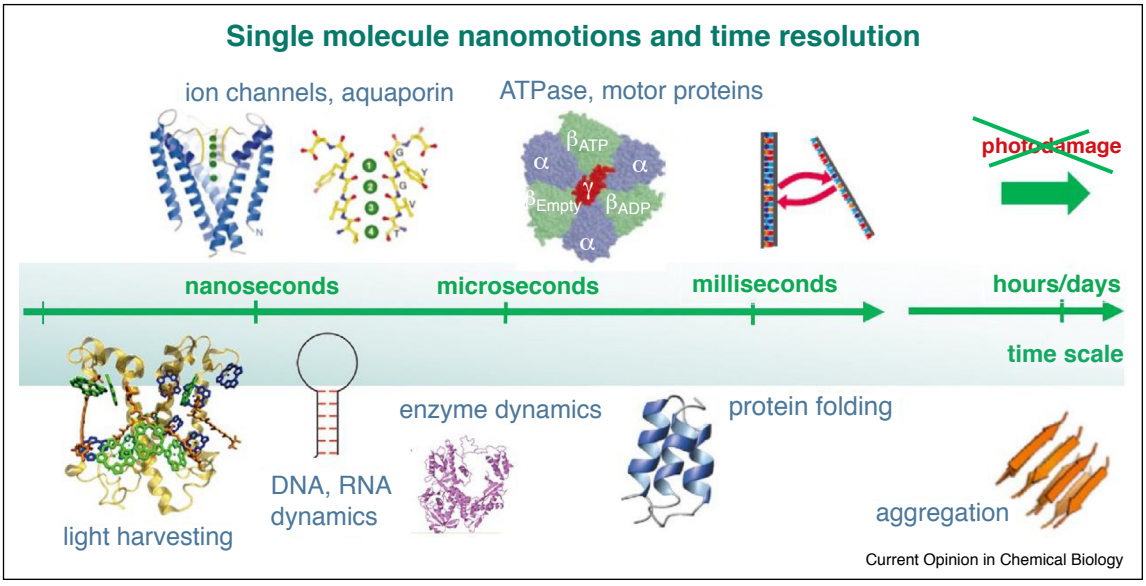
(a) Signal transduction mechanism of a single-molecule WGM sensor. WGM resonance wavelength shifts occur when the overlap of a protein with the nanoscale WGM light field (in red) changes. This is shown conceptually for the opening motion of a model protein. **(b)** WGM wavelength-shift signals $\Delta\lambda$ (in femtometer) measured for structural changes of an active polymerase enzyme (Klenow Fragment, KF). The spike-like signals correspond to the opening and closing motions of an active polymerase enzyme in presence of DNA template. **(c)** The three images illustrate the opening and closing motions of a Pfu (*Pyrococcus furiosus*) polymerase. The polymerase enzyme remains immobilised on a gold nanorod (NR) during the WGM sensing experiments.
Adapted from Ref. [40••].

should be possible to probe single-protein dynamics in different volume sections of a protein. In order to achieve this one could envisage several probing lasers, with different nominal wavelengths, to obtain a more complete characterisation of protein dynamics. Such a multi-modal WGM sensing may even enable the reconstruction of a protein's 3D dynamics to atomic resolution. The WGM signal analysis could thereby take advantage of external input from high-end molecular dynamics simulations, and from structural data already available for a protein from X-ray crystallography as well as also from cryoEM, NMR, and SAXS experiments.

The advantages of WGM sensors become a single-molecule method able to resolve the protein dynamics can be manifold. No fluorescence labelling is required in order to measure protein conformational dynamics, and a nanosecond time resolution would allow WGM sensors to resolve dynamics that are currently accessible by most of the fluorescence-based single-molecule techniques, see Figures 1 and 4. The measurement signals contain information about conformational dynamics recorded from an entire volume section of the protein that is probed in the near-field. Given enough sensing channels that record dynamics from different volume sections, and given a WGM sensitivity down to movements of individual atoms, it could become possible to reconstruct 3-D atomic image from the sensor data with some degree of accuracy and at several million frames per second. Even without recording and reconstructing entire movies of biomolecular machines, the sensor data are a fingerprint specific for chemical interactions and reactions recorded at the single-molecule level. Fingerprinting single-molecule interactions and reactions in nanoscale detection volumes can allow us to realise single-molecule laboratories on chip, and every-molecule analysis of extremely small sample volumes approaching that of a single cell. Achieving these lofty goals can be aided by the possibility of using WGM sensors to record vibrational signatures of individual protein molecules in response to light, such as Raman spectra including the low-frequency vibrational modes which are difficult to access in conventional Raman spectroscopy and which can identify a protein and its fold [45].

What limits the application of the WGM sensing technique is the need to immobilise enzymes at a gold surface while maintaining their activity. In order to repeatedly measure enzymes in a similar orientation it is necessary to modify the protein to attach the protein to the surface at the desired orientation. This requires surface immobilisation techniques such as those based on his-tagged

Figure 4



Examples of dynamic biomolecular processes across a timescale of pico-seconds to hours/days. The high-sensitivity WGM sensor platforms envisaged in the near-future have the potential to be able to detect all of these.

enzymes which can be bound to gold nanoparticle sensors via Ni-NTA (nitrilotriacetic acid) linker molecules or directly via thiol–gold reaction of cysteine amino acids. Another limitation is the difficulty in controlling where the protein attaches and which of the sensing hotspots probe the protein dynamics if a more complex plasmonic nanostructure is in use. Another challenge lies in avoiding interactions of the protein with the gold surface to prolong activity when attached at the surface [46] in order to enable prolonged measurements up to hours. The challenge lies in avoiding interactions of the protein with the gold surface. This can be achieved by coating of the gold surface with (mono)layers of small molecules that prevent a direct gold–enzyme interaction and that favour active enzymes, examples for this can be found in the electrochemistry literature and include glutathione [46], and also citrate as demonstrated with the WGM sensing technique [40••].

The time-resolution of a WGM-based sensor is limited by the cavity lifetime τ, with $\tau = Q/\omega$ where ω is the angular frequency of the light, or about one nanosecond for a typical glass WGM sensor operated in water. However, given the envisaged extreme high-sensitivity capability of future WGM sensors, it may not be necessary to wait for the completion of all of the circular trips of the light in the micro-sensor. In this case, resolution down to a pico-second time-frame may become possible. The probing volume of the plasmonic near-field limits which size of proteins can be studied for their dynamics. On the one hand, the nanometre extension of typical plasmonic near-fields limits the WGM technique to the study of proteins smaller than 100 kDa and to the study of only parts of a larger protein such as a ribosome. On the other hand, it may be possible to position plasmonic nanoparticles strategically around a protein using for example DNA origami approaches or 3D plasmonic nanostructure which could lead to 'film studios' that probe also larger proteins with different nanoparticle sensors in parallel.

The quality of the structural analysis of protein dynamics will depend crucially on the sensitivity of WGM sensors. WGM sensitivity can further be enhanced by utilising more optimal plasmonic nanostructures, such as bow-tie antennas or nano-stars. Utilising narrow line-width (kHz), tuneable laser systems, and possibly quantum and squeezed light [47], are approaches that both can further enhance detection-sensitivity. By combining all these advances on a single WGM sensor platform, it may become possible to reconstruct the movement of each atom of a protein, and achieve the ultimate goal of reconstructing molecular dynamics movies from WGM sensor signals. Because of their high time-resolution, combined with a large dynamic range and an extreme ultimate detection sensitivity, WGM sensors can become an important tool for studies of molecular dynamics. Such sensors have the potential to become the first single-molecule method able to resolve the entire range of nanomotions and biomolecular transitions occurring in protein biochemistry as depicted in Figure 4.

Conflict of interest statement

Nothing declared.

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