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Single-molecule force spectroscopy on polyproteins and receptor–ligand complexes: The current toolbox

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ABSTRACT

Single-molecule force spectroscopy sheds light onto the free energy landscapes governing protein folding and molecular recognition. Since only a single molecule or single molecular complex is probed at any given point in time, the technique is capable of identifying low-probability conformations within a large ensemble of possibilities. It furthermore allows choosing certain unbinding pathways through careful selection of the points at which the force acts on the protein or molecular complex. This review focuses on recent innovations in construct design, site-specific bioconjugation, measurement techniques, instrumental advances, and data analysis methods for improving workflow, throughput, and data yield of AFM-based single-molecule force spectroscopy experiments. Current trends that we highlight include customized fingerprint domains, peptide tags for site-specific covalent surface attachment, and polyproteins that are formed through mechanostable receptor–ligand interactions. Recent methods to improve measurement stability, signal-to-noise ratio, and force precision are presented, and theoretical considerations, analysis methods, and algorithms for analyzing large numbers of force–extension curves are further discussed. The various innovations identified here will serve as a starting point to researchers in the field looking for opportunities to push the limits of the technique further.

1. Introduction

The field began in earnest with the introduction of fluid cells for the (at that time) newly developed atomic force microscope (AFM) (Drake et al., 1989). The early 1990s then saw an explosion of the bio-AFM field, which opened the door to high-resolution imaging of proteins and cell surfaces under near-native conditions (Müller et al., 1995; Radmacher et al., 1996, 1992). Shortly thereafter came the realization that individual proteins and DNA molecules, or single receptor–ligand complexes, could be probed with the help of nano- to microscale force transducers (e.g., cantilevers, optically trapped beads, magnetically trapped beads) (Block et al., 1990; Florin et al., 1995; Lee et al., 1994a,b; Smith et al., 1992; Svoboda et al., 1993). It was furthermore discovered that natural polyproteins (e.g., Titin) with repetitive multi-domain structures provided regularly repeating saw-tooth like features in force extension data (Rief et al., 1997a). Artificial (i.e., recombinant) polyproteins quickly came into fashion as internal molecular controls for investigating mechanical properties of protein domains of interest. Since then, engineering of polyproteins has provided a wealth of information about mechanostable motifs in protein folds (Carrion-Vazquez et al., 1999; Oberhauser et al., 1998; Oesterhelt et al., 2000), directional dependence of protein mechanostability (Brockwell et al., 2003; Carrion-Vazquez et al., 2003; Dietz et al., 2006; Kim et al., 2011), and modulation of mechanostability by molecular recognition (Hu and Li, 2014).

Today, force spectroscopy and bio-AFM in general are well established as standard tools in the nanobiosciences, and are regularly used for investigating cell adhesion and cell surface properties (Helenius et al., 2008; Müller et al., 2009; Preiner et al., 2014; Tsukasaki et al., 2007; Wildling et al., 2012), interrogating membrane proteins (Beedle et al., 2015b; Janovjak et al., 2004; Müller, 2008; Müller and Engel, 2007), and measuring mechanical properties of proteins (Beedle et al., 2015a; Bu et al., 2012; Cao et al., 2011; del Rio et al., 2009; Geisler et al., 2010), polysaccharides (Kocun et al., 2011; Rief et al., 1997b) and DNA (Albrecht et al., 2003). Recent studies have already begun to characterize membrane proteins *in vivo* by probing their response to external forces on native living cells (Alsteens et al., 2010; Pfreundschuh et al.,

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2015). There are a number of review articles that thoroughly cover the field from the early years (Carvalho et al., 2013; Casuso et al., 2011; Hoffmann and Dougan, 2012; Lee et al., 2007; Li and Cao, 2010; Marszalek and Dufrêne, 2012; Müller and Dufrêne, 2008; Neuman and Nagy, 2008; Noy, 2011; Rief and Grubmüller, 2002; Sirbuly et al., 2015; Woodside and Block, 2014).

Despite the high level of interest and well-developed method of AFM-SMFS (Single Molecule Force Spectroscopy), there have remained several limitations to the technique that prevent researchers from fully taking advantage of mechano-phenotyping of molecules and cell surfaces. Specifically, low experimental throughput and low yield of useable single-molecule interaction curves have both hampered the widespread adoption of the method, and its application for studying a large number of proteins. The purpose of this review is to highlight recent developments in bioconjugate chemistry, instrumentation, and data processing/algorithms which aim at improving the design process, yield, measurement quality and throughput of AFM-SMFS experiments.

2. Unfolding fingerprints

In typical AFM-SMFS experiments, many thousand force–extension curves are recorded, but only a fraction of these curves contain useable data that describe the behavior of a single molecule. Typically, the majority of curves (~80–99%) contain no interaction, a multiplicity of interactions that are difficult to interpret, or unspecific adhesion events as measurement artifacts. The experimenter is left searching for a needle in a haystack, looking for single-molecule interactions among a vast excess of unusable force–extension curves. In order to filter the data efficiently, the SMFS community has identified a broad range of proteins that can be used as specific identifiers in unfolding traces. We refer to these domains as 'fingerprints' because they provide a unique unfolding step or 'contour-length increment' of defined length that can be used as a filter during data processing. These fingerprint domains are typically globular protein domains with individual unfolding forces and length increments varying across a large range. This ability to choose the length increments and unfolding forces of the fingerprint domains has enabled the design of custom fusion proteins with well-controlled unfolding behaviors. Recent surveys of mechanical properties of different protein domains are provided by Sułkowska and Cieplak (2007), Hoffmann and Dougan (2012).

3. Receptor–ligand SMFS

Protein–protein and protein-small molecule interactions have been widely analyzed with SMFS. Reports of receptor–ligand SMFS include measurements on biotin–avidin (Florin et al., 1994; Lee et al., 1994a,b; Moy et al., 1994; Rico and Moy, 2007; Yuan et al., 2000), antigen–antibody interactions (Hinterdorfer et al., 1996; Morfill et al., 2007; Schwesinger et al., 2000) along with several other protein–protein or small molecule interactions (Lee et al., 2007; Mitchell et al., 2007; Schmidt et al., 2012).

One limitation in the standard method of receptor–ligand SMFS is that the signal lacks single-molecule specificity. Depending on the proteins involved and the experimental conditions (i.e., blocking/passivation steps), and since typically no fingerprint molecules are used, it can be difficult to differentiate non-specific interactions from specific protein-protein recognition. A second limitation of many receptor–ligand SMFS experiments is that pulling geometry is not strictly controlled. While in a standard polyprotein experiment, the force is applied strictly between the N- and C-termini of each domain, coupling of receptors and ligands to AFM tips and substrates is often done through amide linkages formed between amine groups on the proteins and activated NHS-ester groups on the surface or cantilever. This implicates a diversity of pulling geometries which are not strictly controlled, resulting in rupture force distributions that are smeared out or otherwise distorted.

4. Receptor–ligand SMFS with fingerprints

Our group has worked on improving the technique for receptor–ligand SMFS out of sheer necessity (Fig. 1). We were interested in studying a family of receptor–ligand proteins (i.e., cohesin–dockerin, Coh–Doc) involved in carbohydrate recognition and degradation by anaerobic bacteria (Jobst et al., 2015, 2013; Otten et al., 2014; Schoeler et al., 2015, 2014; Stahl et al., 2012). These protein receptor–ligand complexes are responsible for building up large extracellular networks of structural scaffold proteins and enzymes. They are linked into these structural networks in well-defined and known orientations (e.g., N-terminal or C-terminal anchoring points). It is important to note that when pulling apart a receptor–ligand complex consisting of two proteins, there are four possible terminal pulling configurations (i.e., N-N′, N-C′, C-N′, C-C′) (Fig. 1B). Many of the Coh–Doc complexes we are interested in possess a clear 'physiological' pulling configuration found in nature, and 'non-physiological' or 'non-native' configurations. To understand their natural mechanical adaptations giving rise to their remarkable assembly strategy, we sought to characterize the mechanical stability of these receptor–ligand complexes in both their native and non-native loading configurations. We found a way to ensure specific interactions by basically combining two previously separate modes of AFM-SMFS (i.e., on polyproteins and receptor–ligand complexes). We fused the Coh and Doc domains separately to different fingerprint domains, and recombinantly produced each construct as a single fusion protein. The fingerprints serve two purposes: (1) they provide site-specific attachment sites through engineered cysteine residues or peptide ligation tags (see section 5) to strictly control loading geometry; (2) they provide predetermined increments in contour length which allows us to filter the datasets for specific single-molecule interactions (Jobst et al., 2015, 2013; Otten et al., 2014; Schoeler et al., 2015, 2014; Stahl et al., 2012).

This configuration yields several advantages: We now have the ability to study mechanical stability of receptor–ligand pairs and unfolding of individual domains (i.e., the fingerprints) in a single-experiment with high yield and specificity, eliminating measurement artifacts. We also have a systematic and straightforward way to probe effects of pulling geometry on receptor–ligand unbinding, and to compare native and non-native pulling configurations. The gene design (i.e., N- or C-terminal fingerprint domains) directly reflects the conformation to be investigated. Furthermore, a specific protein domain of interest can now easily be fused to a mechanostable Coh–Doc receptor–ligand pair for characterization. Depending on the expected domain unfolding forces, an appropriately fitting protein receptor–ligand pair can be chosen from a wide range of well-characterized molecules (Table 1). We note that this table does not include every receptor–ligand probed by AFM. For an extensive list of receptor–ligands that were explored with AFM, see Lee et al. (2007). Currently, the mechanically most stable receptor–ligand pair is a Coh–Doc type III complex derived from *R. flavefaciens*, with loading-rate dependent rupture forces between 600 and 800 pN (Schoeler et al., 2015, 2014). Another interaction in a similar force range is the trimeric titin–telethonin complex described by Bertz et al. (2009).

5. Site-specific bioconjugation

Many polyprotein experiments rely on non-specific adsorption of polyproteins onto surfaces (e.g., mica, gold). Receptor–ligand

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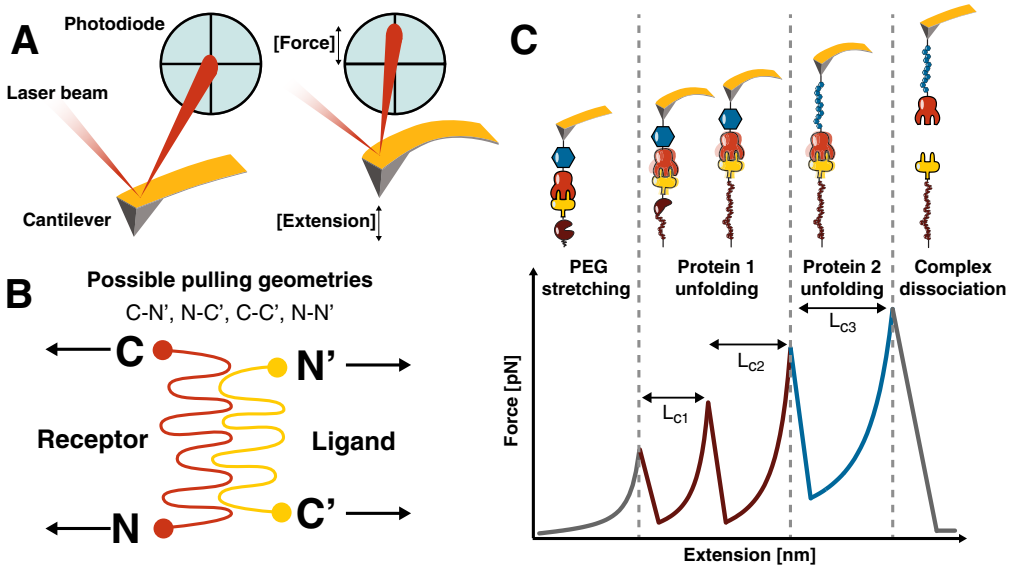

Fig. 1. Configuration for performing receptor–ligand SMFS with (poly)protein fingerprints. (A) Schematic of the measurement setup. The change of force is detected via the differential signal of the laser beam deflection on a quadrant photodiode. (B) For a protein complex consisting of two domains, 4 terminal pulling configurations are possible (N-N′, N-C′, C-N′, C-C′). (C) Fingerprints (brown and blue) are site-specifically and covalently attached to the cantilever and surface. Receptor (orange) and ligand (yellow) form a stable receptor–ligand complex. Note that the fingerprints can be individual sub-domains, or repetitive polyproteins in their own right. Shown is a typical force-extension trace with unfolding of the fingerprints, followed by rupture of the receptor ligand complex. In order to observe unfolding of the fingerprints in sufficient numbers, their most probable unfolding force should lie well below the most probable rupture force of the complex for the given loading rate.

Table 1
Overview of selected receptor–ligand pairs usable as specific handles for protein-based SMFS experiments. Rupture forces depend on immobilization sites for surface conjugation. Note that rupture forces can also vary depending on probe spring constants and loading rates. Abbreviations: NHS: N-hydroxysuccinimide; PEG: poly(ethylene glycol); Mal: maleimide; Cys: cysteine; CoA: coenzyme A; SFP: 4′-phosphopantetheinyl transferase; ybbR-Tag: peptide sequence DSLEFIASKLA; LF: low force unbinding path; HF: high force unbinding path. For the column 'immobilization method', the terminology X (Y) Z means: molecule X is attached to Z mediated by enzyme Y.

Protein handles Handle A:Handle B	Sizes (kDa)	Dissociation force (pN)	Immobilization method	Handle position (N/C)	References
Cohesin:dockerin I	15.4/8.3	122 ± 18.5	NHS-PEG5000-Mal/Cys	C:C	Stahl et al. (2012)
Cohesin:dockerin III	21.6/26.2	606 ± 54	NHS-PEG5000-Mal/Cys	N:C	Schoeler et al. (2015)
		111 ± 30 (LF)	NHS-PEG5000-Mal/CoA (SFP) ybbR	C:C	Schoeler et al. (2015)
		597 ± 67 (HF)	NHS-PEG5000-Mal/CoA (SFP) ybbR		
NiNTA:HIS6	0.2/0.8	153 ± 57	Gold-Cys	n.a.	Verbelen et al. (2007)
Avidin:biotin	66-69/0.2	160 ± 20	Biotinylated BSA	n.a.	Florin et al. (1994)
StrepTagII:streptavidin	1.1/52.8	253 ± 20	BSA/NHS-biotin	n.a.	Wong et al. (1999)
Streptavidin:biotin	52.8/0.2	200	Biotinylated BSA	n.a.	Rico and Moy (2007)
Calmodulin:CBP	16.7/1.1	16.5 ± 1.8	Pulldown via NI-NTA	n.a.	Junker and Rief (2009)
StrepTagII:mono-streptactin	1.1/58.4	116	NHS-PEG5000-Mal/Cys	C:C	Baumann et al. (2015)
		46	NHS-PEG5000-Mal/CoA (SFP) ybbR	N:C	
Anti-GCN4 sFv:GCN4(7P14P)	26.7/4.0	70	NHS-PEG5000-Mal/Cys	N:C	Morfill et al. (2007)
Anti-digoxigenin:digoxigenin	170/0.4	40	NHS-PEG6000	n.a.	Neuert et al. (2006)

AFM-SMFS, however, requires covalent immobilization of the two binding partners to the cantilever and surface, respectively, in order to avoid clogging of the molecules on the cantilever tip. Site-specific (i.e., residue specific) conjugation methods provide strict control over the pulling geometry and result in higher accuracy, precision and reproducibility, compared to conjugation methods resulting in a multiplicity of possible linkage sites (e.g., amine-targeting). Fig. 2 provides an overview of established surface chemistry strategies.

Another advantage of our modular system is the ability to use one construct (i.e., fingerprints with immobilization site) in all desired biochemical or biophysical assays, since immobilization relies on a PEG derivative, which is orthogonal to conventional specific pull down methods. It is compatible with a wide range of binding assays like Western Blotting, ITC, SPR, and ELISA.

The Ni-NTA:HIS_6-tag interaction can be used as force probe as well. This interaction has been employed as an adhesion sensor by probing a cell surface containing His-tagged protein. Since the His-tag is only located at one of the protein's termini, the insertion direction of the protein as well as it's position can be detected (Alsteens et al., 2013; Dupres et al., 2009; Pfreundschuh et al., 2015). This technique is especially useful since the His-tag can be used as a protein purification tag and simultaneously provides a single-molecule force handle.

5.1. Cysteines

Cysteines are relatively rare in proteins, making them attractive as a point mutation residue. The thiol side chain of cysteine is nucleophilic, and will spontaneously react with maleimide leaving groups at neutral pH. It can be used to site-specifically attach proteins to PEG coated surfaces for receptor–ligand AFM-SMFS. Alternatively, engineered cysteines can also be used as oligomerization sites to create disulfide-linked polyproteins, as was done for green fluorescent protein (GFP) (Dietz and Rief, 2006). However, cysteine/thiol-based protein conjugation has some drawbacks, including the tendency of cysteine-modified proteins to multimerize and ultimately aggregate over time, and incompatibility with proteins dis-

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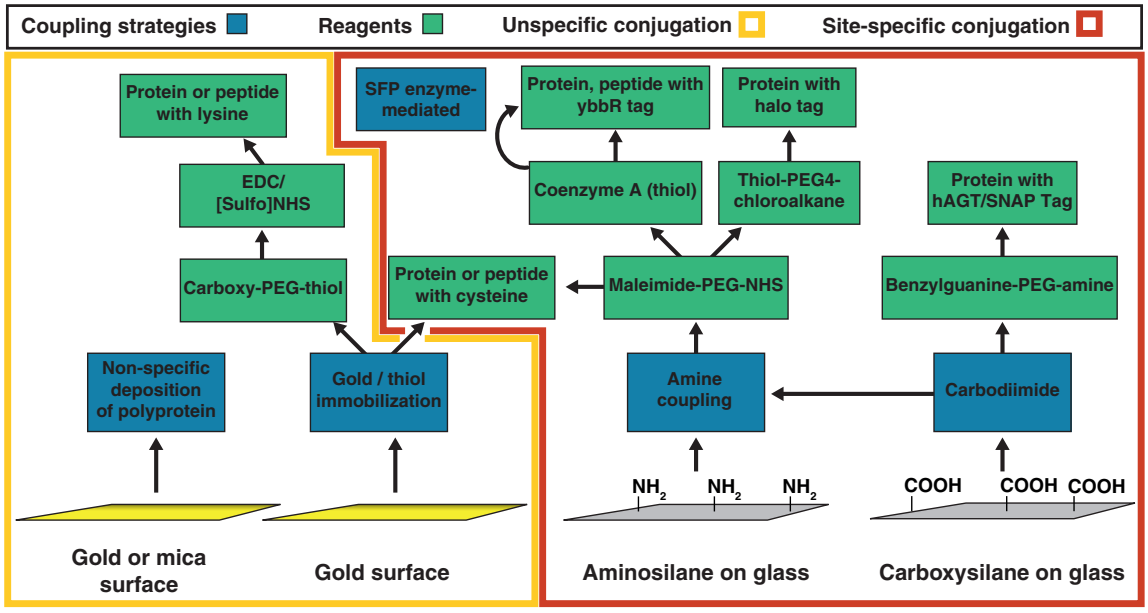

Fig. 2. Surface chemistry and bioconjugation strategies for single-molecule force spectroscopy. The diagram is by no means exhaustive and is roughly divided into site-specific conjugation methods that provide a single anchoring point for proteins to surfaces/cantilevers (right), and unspecific conjugation methods that provide a heterogeneity of loading configurations (i.e., a multiplicity of pulling points) (left).

playing cysteines on their surfaces in their wild-type form. Hence several other conjugation strategies were developed to overcome this challenge. Most of the newer techniques rely on N- or C-terminal attachment sites because the length of the requisite peptide tags or fusion domains makes inclusion into internal sites of a folded protein domain more challenging.

5.2. HaloTag

The active site of the haloalkane dehydrogenase (HaloTag) has been used to covalently immobilize proteins on chloroalkane surfaces. The unfolding forces of the HaloTag depend on its loading geometry (N-terminus: 131 pN; C-terminus: 491 pN). The domain provides an unfolding fingerprint of defined contour length, which also depends on the pulling geometry (N: 66 nm, C: 26.5 nm) (Popa et al., 2013).

5.3. hAGT/SNAP tag

The DNA repair protein O^6-alkylguanine–DNA-alkyltransferase (hAGT, SNAP-tag) binds benzylguanine covalently as a substrate, which can be attached to glass surfaces via an amino-polyethylene glycol (Kufer et al., 2005). With 22 kDa, the SNAP-tag is slightly smaller compared to the HaloTag (34 kDa).

5.4. SpyTag/Catcher

The versatile SpyTag/Catcher system can also be employed for site-specific surface immobilization. The linkage between SpyTag and Catcher is based on an internal protein interaction, which forms an isopeptide (covalent) bond. Based on this observation, the interaction was further developed and engineered, and now consists of a 13 amino acid large SpyTag and the binding domain Spy Catcher (Zakeri et al., 2012).

5.5. ybbR/SFP

The ybbR-Tag is an 11 amino acid protein sequence that is enzymatically linked to coenzyme A (CoA) by 4′-phosphopantetheinyl transferase (SFP) enzyme (Pippig et al., 2014; Yin et al., 2006; Yin et al., 2005). Both ybbR-Tag and the SpyTag/Catcher system have been shown to be N- and C-terminally active. Both tags can also be inserted internally, if the structure of the protein allows it, however, proper folding is not guaranteed and must be evaluated on a case-by-case basis.

5.6. Surface chemistry

Like the modular design of fingerprints and site-specific immobilization tags, surface chemistry can also be modularized to improve workflow. We note that the type of surface chemistry goes hand in hand with the design of the bioconjugation tags for protein production. Our standard approach follows the protocol described by Zimmermann et al. (2010): amino-silanized glass slides and cantilevers are functionalized with a hetero-bifunctional poly (ethylene glycol) (PEG) polymer with an N-hydroxysuccinimide group and a maleimide group at opposing ends. PEG coating provides a passivated surface that resists nonspecific protein adhesion, reducing background and artifacts during measurement. The entropic elasticity behavior of PEG (i.e., persistence length) is similar, although not equal to that of protein backbones, making it a suitable choice for surface conjugation in AFM-SMFS, without interfering too strongly with data interpretation. The maleimide group can then either be modified with CoA containing an inherent thiol group to proceed with ybbR/SFP chemistry, or alternatively directly be reacted with a protein domain displaying a reduced cysteine residue. The PEG incubation can be modified or extended depending on the requirements of the linker and the end group.

6. Advances in measurement techniques

Current advances in measurement resolution, instrument stability and accessible dynamic ranges open up new opportunities for measurements of biomolecules. Here we highlight recent innovations aimed at improving quality and precision of AFM-SMFS measurements.

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6.1. Improved time resolution

In general, the timescales relevant for protein un-/folding and the corresponding timescale for thermally induced crossing of energy barriers are not fully detectable by common SMFS techniques, which typically resolve slower than 50 μs. Early on, the importance of developing high-speed AFM imaging and force spectroscopy through miniaturization of cantilevers with high resonance frequencies and low viscous drag coefficients was appreciated (Viani et al., 1999a,b). Nonetheless, only recent studies were able to overcome timescale limitations to observe, for example, extraordinarily slow protein misfolding transitions (~0.5 ms) using optical tweezers (Yu et al., 2015). Furthermore, advanced statistical methods extended optical tweezers SMFS time resolution to the ~10 μs range (Žoldák et al., 2013), and optimization of AFM cantilevers for SMFS has pushed the limit toward resolution on the microsecond timescale (Edwards et al., 2015). These developments allow experimentally accessible ranges to approach the lower limits of fast folding transition dynamics (Chung et al., 2012; Schuler and Hofmann, 2013), resolving short-lived intermediate states and yielding important insights into other fast conformational dynamics.

6.2. Bridging the timescale gap to steered molecular dynamics simulations

Recently, experimental measurements were brought into proximity (Dong and Sahin, 2011; He et al., 2012; Schoeler et al., 2015) or even overlap (Rico et al., 2013) with all atom steered molecular dynamics (SMD) simulations. Depending on the size and thus complexity of the simulated system, it has so far been possible to achieve SMD simulation timescales in the nanosecond to mid-microsecond range (Freddolino et al., 2008; Heymann and Grubmüller, 2001; Lee et al., 2009). Rico *et al.* developed a high speed force spectroscopy AFM based on an Ando-type high speed imaging AFM (Ando et al., 2001), with a high resonance frequency (600 kHz) miniature multilayer piezoelectric actuator (calibrated before each experiment and run in open loop mode), and a short cantilever with a high resonance frequency (550 kHz in liquid), and low viscous damping. This system was used to record protein unfolding data at extremely high speeds. To reduce hydrodynamic drag, the sample surface was tilted against the direction of the movement. With these improvements and data acquisition in the megahertz range, they were able to record meaningful and interpretable data at pulling speeds of up to 4000 μm/s, which is about 2–3 orders of magnitude faster than conventional methods and starts overlapping with the range of SMD simulations (Rico et al., 2013). Despite these successes, care must be taken because underdamped or 'ringing' cantilevers like the ones used here are not in agreement with the basic assumptions of the traditional SMFS framework, but can be improved by custom cantilever optimization procedures at the cost of time resolution (Edwards et al., 2015).

6.3. Long-term stability and force precision

Sophisticated measurements of complex biological systems or single molecules often require extraordinarily stable low-drift instruments, capable of continuous long-term data acquisition to gain sufficient and reliable statistics. Active stabilization techniques were developed to enable routine long-term stability and Ångstrom scale precision at room temperature for optical trap setups: differential sample position was measured and regulated with two independently stabilized and MHz modulated lasers, backscattered on sample and probe, and recorded separately on a single photodiode using lock-in amplifiers (Walder et al., 2015). This method is deemed applicable to surface-based and dual-beam optical traps, magnetic tweezers, AFM setups and optical microscopy, including super-resolution techniques.

AFM cantilever long-term stability and force precision can be increased even further by partially removing the reflective gold coating from the cantilever to dramatically reduce cantilever bending caused by the bimetallic effect (Churnside et al., 2012). Stability and precision improvements, which still retain high measurement bandwidths, enable and improve on picoscale force and sub-nanoscale motion measurements of molecular properties and dynamics in various biological systems. These may include groundbreaking investigations like the observation of single RNA polymerase base pair stepping (Abbondanzieri et al., 2005; Zhou et al., 2013), base pair unwinding of helicases (Cheng et al., 2011) and prion misfolding pathways (Yu et al., 2015, 2012). More details on long-term stability measurements and force precision are covered in the recent review of Edwards and Perkins (2016).

6.4. Mapping molecular recognition events: multiparametric imaging modes

The idea of mapping molecular recognition by simultaneously measuring surface topography and force–extension data ('force volume mapping' or 'affinity imaging') was introduced early (Hinterdorfer et al., 1996; Ludwig et al., 1997), and refined to remarkable temporal and spatial resolution. While these molecular recognition imaging techniques turned out to be a valuable tool for detecting and locating specific binding sites on surfaces, their development into dynamic recognition force imaging (Hinterdorfer and Dufrêne, 2006; Raab et al., 1999; Zhang et al., 2014) greatly increased temporal and spatial resolution, while still yielding information about surface elasticity and adhesion, as well as identifying biomolecules at the same time.

Multiparametric imaging modes can simultaneously detect physical properties of the surface and forces exerted on specific biomolecular binding sites. The AFM cantilever oscillates with amplitudes around 100 nm at sub- or low kilohertz frequencies to measure force–distance data, and simultaneously records image topography and other surface properties at sub- or low hertz line-scanning frequencies. The recorded force and topography data is collected orders of magnitude faster compared to force volume mapping methods, yielding imaging speeds comparable to conventional AFM imaging methods (Alsteens et al., 2012; Pfreundschuh et al., 2014). Another benefit of this method is that a large range of loading rates for receptor–ligand dissociation events can be probed in a single experiment, due to the largely varying cantilever tip velocities. Recently, this method was applied to gain nm-scale resolution imaging data of a G protein-coupled receptor (PAR1) in proteoliposomes while characterizing their ligand-binding energy landscape (Alsteens et al., 2015) from loading rates ranging between 1e3 and 1e6 pN/s, already two orders of magnitude higher than conventional force–distance based SMFS. Another recent study demonstrates the ability of this technique to distinguish two different binding events on opposite sides of engineered PAR1 by their unbinding force, and thereby determine their orientation within the lipid bilayer (Pfreundschuh et al., 2015).

6.5. Lateral force sensors

A slightly different approach developed a T-shaped cantilever (Dong et al., 2009; Dong and Sahin, 2011) to drive it at its flexural resonance frequency (~9 kHz) and record force data from cantilever torsion, resulting in a lateral laser deflection signal that was acquired while imaging the sample in conventional tapping mode. Due to the cantilever's high torsional resonance (~115 kHz), unbinding dynamics could be measured at the

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microsecond timescale and at extraordinarily high loading rates of up to nearly 1e9 pN/s (Dong and Sahin, 2011), about four orders of magnitude faster than conventional SMFS. Force curves and therefore unbinding events and their corresponding force values could be mapped with high spatial and temporal resolution, while providing AFM images that were simultaneously recorded as surface topography. Mechanical elasticity properties of the substrate were also detected in the phase signal.

7. Theory and data analysis

7.1. The data analysis problem

Technical advances greatly increasing the throughput of AFM-SMFS measurements have made automated data analysis protocols an essential requirement. In practice, researchers face the problem of extracting meaningful single molecule signal from large datasets that contain an abundance of unusable data. The use of well-defined fingerprint domains with known unfolding patterns facilitates this procedure greatly. To avoid tedious and time-consuming manual sorting of thousands of data traces, and potential introduction of bias into the data analysis procedure, algorithms which identify the fingerprint unfolding length increments and classify the data correspondingly have been developed and implemented with success (Bosshart et al., 2012; Jobst et al., 2015; Kuhn et al., 2005; Puchner et al., 2008).

7.2. Polymer elasticity models and contour length transformations

Single molecule force measurements generally only gain access to a protein's extension under a given force. The stochastic nature of domain unfolding or complex dissociation under force as well as the non-linear elastic behavior of the polymer backbone chain makes analysis in force-extension space difficult. The same unfolding event is observed over a range of different positions in force–extension curves for multiple measurement cycles as shown in Fig. 3B i.

From a physicist's point of view, mechanical stretching of an unfolded protein domain is described by polymer elasticity models such as the worm-like chain (WLC) (Bustamante et al., 1994), the freely jointed chain (FJC) (Ortiz and Hadziioannou, 1999), or the freely rotating chain (FRC) model (Livadaru et al., 2003). These models contain the free contour length L of the polymer, including surface tethers and unfolded protein backbone, as a parameter. The free contour length is simply the length of the polypeptide along the contour of the biopolymer chain, given a specific folding state (e.g., Fig. 3A). Under a set of physically relevant constraints (L, x, $F > 0$, $x < L$), these elasticity models provide one-to-one mappings from force–extension space into force-contour length space. The models can be solved for the contour length parameter (Jobst et al., 2013; Puchner et al., 2008), yielding an expression for the contour length as a function of force and extension $L(F,x)$. This function can be used to transform force–extension traces from constant speed or force clamp/ramp experiments into contour length space (Fig. 3B ii). The calculated contour length then can be binned (Fig. 3B iii), aligned, and subsequently averaged to precisely locate energy barriers (Fig. 3B iv) along a protein's unfolding pathway, and to classify data sets based on unfolding patterns. This idea was first proposed by Puchner et al. (2008) and has been successfully applied in multiple AFM-SMFS studies (Jobst et al., 2015, 2013; Otten et al., 2014; Schoeler et al., 2014; Stahl et al., 2012; Thoma et al., 2015).

7.3. Worm-like chains, freely-rotating chains and beyond

The WLC model accurately describes a protein's stretching response for forces up to approximately 150 pN. While many protein unfolding or dissociation events take place well within this force regime, some interactions like titin Ig domain unfolding (Rief et al., 1997a), cohesin unfolding (Valbuena et al., 2009), disso-

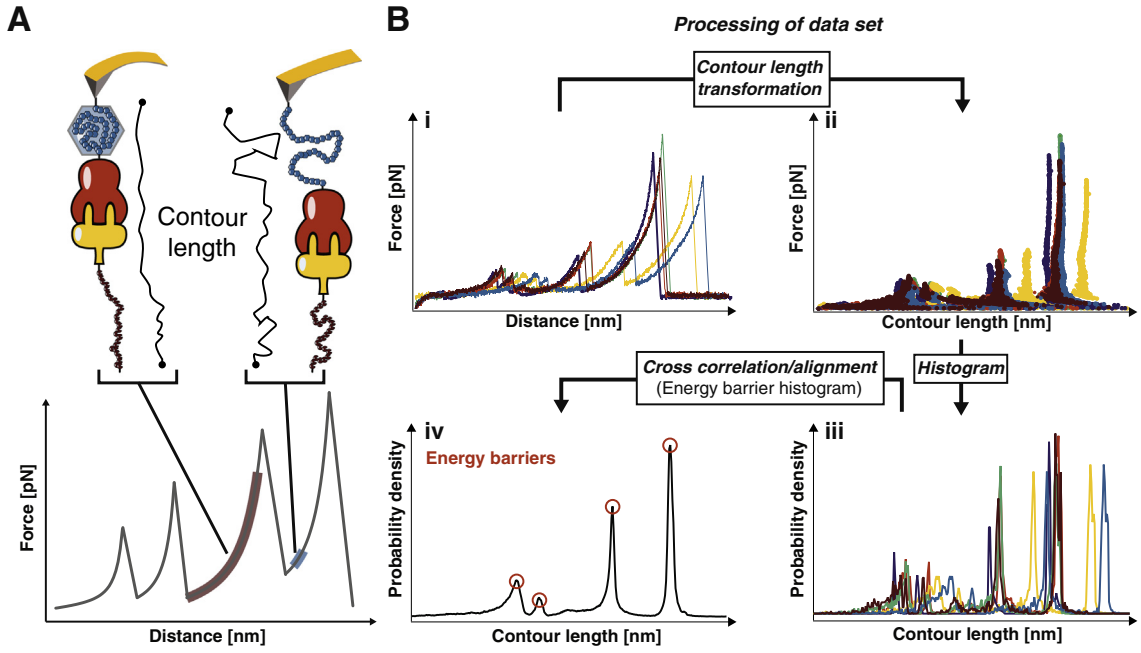

Fig. 3. Assembly of contour length histograms for screening AFM-SMFS datasets. (A) Force–extension traces are transformed into contour length space using an appropriate polymer elasticity model. The choice of the model depends on the force range. (B) Following transformation, the data (i) are plotted in force-contour length space (ii). Force and contour length thresholds are applied and the data are histogrammed (projected onto contour length axis) with an appropriate bin width, i.e., nanometer scale, to obtain the diagram in (iii). Each trace analyzed this way can be searched for a specific contour length increment (distance between two peaks in the probability density vs. contour length plot) corresponding to one of the fingerprints. To obtain a master histogram describing all the observed increments in a dataset, individual histograms reflecting a specific unfolding pathway are aligned by cross-correlation and offsetting along the contour-length axis (iv).

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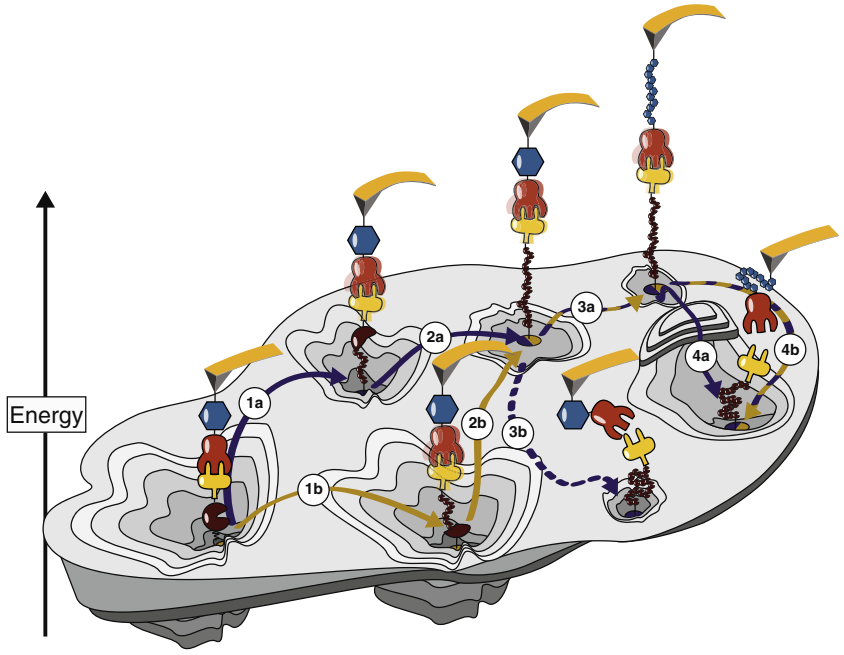

Fig. 4. Schematic depiction of an (un)folding energy landscape. The bound state of a protein receptor–ligand complex can be thought of as a Brownian particle confined to a complex multidimensional energy landscape. At equilibrium, the system can escape the bound state driven by thermal fluctuations. This escape can occur along any pathway on the energy landscape. When measuring the thermal off-rate with bulk assays such as surface plasmon resonance biosensors, a weighted average of all thermally accessible pathways is obtained. In a single-molecule pulling experiment, however, a small subset of pathways is selected, which is defined by the projection of the energy landscape onto the pulling coordinate as illustrated by paths 1–3. Caution is required when comparing data obtained from single molecule techniques with bulk data. In cases where SMFS probes a steep pathway with a high free energy barrier, the fitted zero-force off rate may greatly differ from values obtained by bulk techniques. Path 4 illustrates the thermal escape (4b) versus the forced pathway across an additional energy barrier (4a) by the AFM cantilever.

ciation of skeletal muscle titin–telethonin bonds (Bertz et al., 2009) or dissociation of cellulosomal adhesion complexes (Schoeler et al., 2015, 2014) exhibit much higher unfolding or rupture forces. To adequately describe the elastic response of polymers in such high force regimes, models beyond the standard WLC are required. To address this shortcoming, Hugel et al. (2005) developed quantum mechanical corrections for polymer elasticity models to account for polypeptide backbone stretching at high forces. These corrections can be applied to obtain the contour length at zero force L_0 (Puchner et al., 2008).

Livadaru et al. proposed a more sophisticated model exhibiting three distinct regimes for a protein's stretching response as a function of the applied force (Livadaru et al., 2003). For AFM based SMFS, however, mainly the medium to high force regimes are relevant. The medium force regime of protein stretching, roughly between 10 and 125 pN, exhibits classical WLC stretching behavior, whereas the high force regime shows the behavior of a discrete chain, where the stretching response is independent of the persistence length. This model is most suitable for studying high force interactions, especially when combined with the aforementioned quantum mechanical corrections for backbone stretching.

8. Kinetic and energetic parameters

In dynamic force spectroscopy of receptor–ligand pairs, kinetic and energetic parameters of the complex are of interest. The method most prominently used to extract this information from SMFS experiments is to vary the loading rate by measuring the rupture forces at different pulling speeds in constant speed mode (Baumann et al., 2015; Schoeler et al., 2014; Stahl et al., 2012), or with different slopes in force ramp mode (Oberhauser et al., 2001). The obtained rupture force data are then assembled into a dynamic force spectrum, a plot of most probable rupture forces against their corresponding loading rates. In their comprehensive guide to analysis of SMFS data sets, Noy and Friddle (2013) explain the basic physics of bond stretching. An SMFS measurement corresponds to the stretching of multiple elastic components in series, including the projection of the bond potential onto the pulling axis, the cantilever modeled as a harmonic spring and potential linker molecules with nonlinear elasticity deviating from those under investigation. Such a scenario gives rise to bound and unbound states separated by free energy barriers. By pulling on the harmonic spring, this energy landscape is constantly modulated. Since thermal fluctuations are orders of magnitude faster than changes in the external force, the transition from a bound to an unbound state is thermally driven in common loading rate regimes, as described by Bell (1978), Evans and Ritchie (1997), Izrailev et al. (1997). These models describe a linear dependence of the rupture force on the natural logarithm of the loading rate and give access to the zero force off rate k_0 (exponentially amplified under force) and the distance to the transition state Δx. Theoreticians extended this framework and accounted for modulation of Δx by the applied force (Dudko et al., 2006), and the possibility of rebinding at slow loading rates (Friddle et al., 2012). These newer models predict a nonlinear dependence of the most probable rupture force on the loading rate and give the height of the free energy barrier to unbinding ΔG as an additional parameter. Such non-linear trends were observed experimentally, and a comprehensive list of such data sets is given in Friddle et al. (2012). Joint experimental and computational data sets were also analyzed in recent studies (Rico et al., 2013; Schoeler et al., 2015). As Noy and Friddle (2013) point out, these models should only be used if the force spectrum of interest indeed exhibits a non-linear trend. If this is not given, fitting non-linear models results in non-meaningful fit parameters and the phenomenological model should be used instead.

Although in both bulk measurements and single molecule force measurements at common loading rates, the unbinding process is

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thermally driven, caution is required when comparing their data. While at unbiased equilibrium, all thermally accessible pathways from the bound state are sampled and the off rate is consequently measured as a weighted average, single molecule force measurements select only a small subset of these pathways due to the defined pulling geometry, as illustrated by paths 1–3 in Fig. 4. In cases where the energy landscape is highly asymmetric and the pulling experiment probes a steep pathway, the off rates obtained from single molecule *vs*. bulk measurements might differ greatly (see Fig. 4, paths 4a vs 4b).

9. Summary and outlook

We highlighted recent advances in experimental design, molecular design, sample preparation, measurement and analysis methods for AFM-SMFS on polyproteins and receptor–ligand complexes. We summarized site-specific bioconjugation strategies to obtain well-defined pulling geometries for improved reliability and reproducibility of experiments. We also highlighted receptor–ligand pairs with high mechanical strength (e.g., cohesin–dockerin), and their application as specific pulling handles in AFM-SMFS for improving experimental throughput and curve yield. Finally, we touched on recent innovations in positional control and cantilever microfabrication for improving time and force resolution and stability of the measurement, on emerging techniques for mapping force responses of surfaces to their topologies, and we discussed theoretical considerations for analyzing large numbers of curves.

In the future, there remain several technical challenges that need to be addressed. One of the limitations of AFM is that it covers a relatively high force range, yet there exist a multitude of biological interactions in the low-force regime that are of interest. Further technical advances in instrument design, cantilever fabrication, and feedback control might further improve force resolution and thereby enable such experiments. A second area for improvement involves sample throughput and parallel screening. With the development of more elaborate, sophisticated and well defined surface immobilization strategies and protein handles, significant gains in throughput can be envisioned. Innovations of the chemistry in combination with efficient data analysis protocols and state of the art instrumentation may pave the way towards in depth study of complex, multi-domain protein systems.

These advances in experimental design and throughput would greatly benefit from refined theoretical frameworks that account for parameters such as cantilever stiffness and ringing whilst maintaining analytical tractability. Consequently, with improved methodology we anticipate the community will be able to address an even wider range of questions about mechanical adaptations of proteins and protein complexes in the future.

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