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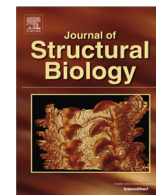

Single-molecule force spectroscopy on polyproteins and receptor–ligand complexes: The current toolbox

Wolfgang Ott [a,b,1], Markus A. Jobst [a,1], Constantin Schoeler [a,1], Hermann E. Gaub [a], Michael A. Nash [a,c,d,*]

[a] Lehrstuhl für Angewandte Physik and Center for Nanoscience, Ludwig-Maximilians-Universität, 80799 Munich, Germany
[b] Center for Integrated Protein Science Munich (CIPSM), Ludwig-Maximilians-Universität, 81377 Munich, Germany
[c] Department of Chemistry, University of Basel, 4056 Basel, Switzerland
[d] Department of Biosystems Science and Engineering, ETH-Zürich, 4058 Basel, Switzerland

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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.

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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.,

* Corresponding author.
E-mail address: michael.nash@lmu.de (M.A. Nash).
[1] Theses authors contributed equally to this work.

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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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