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PERSPECTIVE

Together We Are Stronger: Protein Clustering at the Nanoscale

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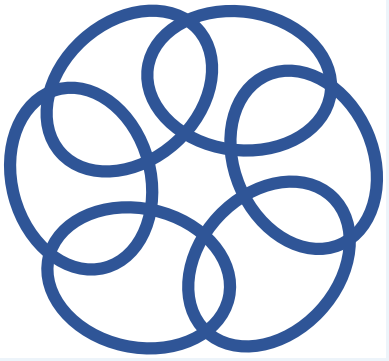
ABSTRACT: **Cell surface proteins are known to assemble into nano- and microscale domains in order to govern biological processes, including cell adhesion, endocytosis, and immune responses. The small size and ephemerality of these structures have made their direct observation and functional analysis challenging. In this Perspective, I discuss recent progress made in applying nanotechniques to study protein clustering, emphasizing the use of state-of-the-art single-molecule atomic force microscopy, as reported by Strasser *et al.* in this issue of *ACS Nano*.**



Living cells are highly heterogeneous systems composed of multicomponent protein structures and machineries that control key cellular functions. A hot topic in current molecular and cell biology is understanding how surface proteins assemble into functional domains ("clusters").[1] Due to the small size (typically, in the 10−500 nm range) and dynamic nature of protein clusters, their direct visualization and analysis have long been difficult to achieve. Since the late 1990s, there have been tremendous advances in applying nanoscopy techniques for the characterization of individual proteins and their assemblies in physiological conditions.[2,3] Super-resolution microscopy,[4] also called optical nanoscopy, has enabled researchers to track the real-time position, distance, distribution, and dynamics of surface proteins, such as the viral envelope (Env) glycoprotein of HIV-1 viral particles,[5] and the soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE)[6] and syntaxin[7] membrane proteins. Optical nanoscopy breaks the diffraction limit barrier of light microscopy by switching on and off the fluorescence of subpopulations of molecules that are separated in time. However, two issues with optical nanoscopy are that fluorescent staining may alter the function of the target molecules and high lateral resolution requires lowering the temporal resolution.

By contrast, force nanoscopy techniques exert and sense forces to probe single molecules in a label-free manner. The most powerful of these methods is atomic force microscopy (AFM), in which the small forces acting between a sharp tip and the specimen are sensed to contour its surface (Figure 1a).[3,8] Atomic force microscopy images complement electron and X-

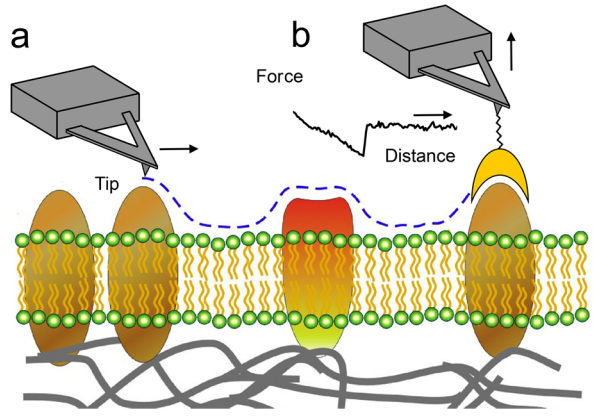


Figure 1. Atomic force microscopy analysis of cell-surface proteins. (a) In the imaging mode, a sharp tip follows the contours of the cell surface with nanometer resolution. Shown here is the lipid membrane in which proteins are embedded. (b) In single-molecule force spectroscopy, the tip is labeled with ligands (*e.g.*, antibodies) to detect, to localize, and to manipulate individual surface proteins.

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ray crystallography data in that they provide insights into the oligomeric state and supramolecular assembly of surface proteins at subnanometer resolution and in physiological buffer conditions.[8]

In single-molecule force spectroscopy (SMFS), the AFM tip is used as an ultrasensitive force probe to quantify the interactions, dynamics, and assembly of single molecules (Figure 1b).[9] Force−distance curves are recorded by measuring the deflection of the cantilever while the tip is moved up and down. In addition to AFM, other ultrasensitive force techniques to measure forces in single biomolecules include microneedles, the biomembrane force probe, and optical and magnetic tweezers.[10] These techniques cover a wide range of forces (0.1 pN to >10 nN), ranging from small intermolecular interactions to strong covalent bonds. Notably, AFM is the only method that can spatially localize where force is measured, which is an important asset since interactions are linked to structure in biological systems.

As described in this issue of *ACS Nano*, Strasser *et al.*[11] used state-of-the-art high-speed AFM (HS-AFM) and SMFS to study how immunoglobulin G (IgG) binding induces clustering of monomeric target molecules in lipid membranes. They tracked the dynamic oligomerization of IgGs and captured the molecular interactions involved.

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IMAGING PROTEIN OLIGOMERIZATION

The slow temporal resolution of conventional AFM imaging limits its use to studying molecular and cellular dynamic processes. However, with HS-AFMs, improved cantilevers and electronics enable millisecond time resolution on biological structures.[12] As a prominent example, Shibata *et al.* used HS-AFM for dynamic imaging of the light-driven proton pump bacteriorhodopsin. They resolved individual trimers at a speed of 100 ms per image and observed structural changes in response to light.[13] Casuso *et al.* used HS-AFM to characterize the diffusion and interaction dynamics of trimeric OmpF, a channel-forming protein found in the *Escherichia coli* outer membrane.[14]

In this issue of *ACS Nano*, Strasser *et al.* describe their use of HS-AFM to follow the time evolution of IgG oligomerization on antigenic membranes. IgG molecules interacting with surface-bond antigens may form ordered hexamers *via* specific interactions between fragment crystallizable (Fc) domains (Figure 2a). Therefore, they used a supported lipid bilayer containing dinitrophenyl (DNP)-labeled lipids as a model for an antigen-containing cell membrane. The addition of chimeric human IgG1 anti-DNP antibodies for increasing time spans resulted in distinct distributions of differently sized IgG oligomers on the membranes (Figure 2b). The populations of higher oligomers increased with incubation time, with the first

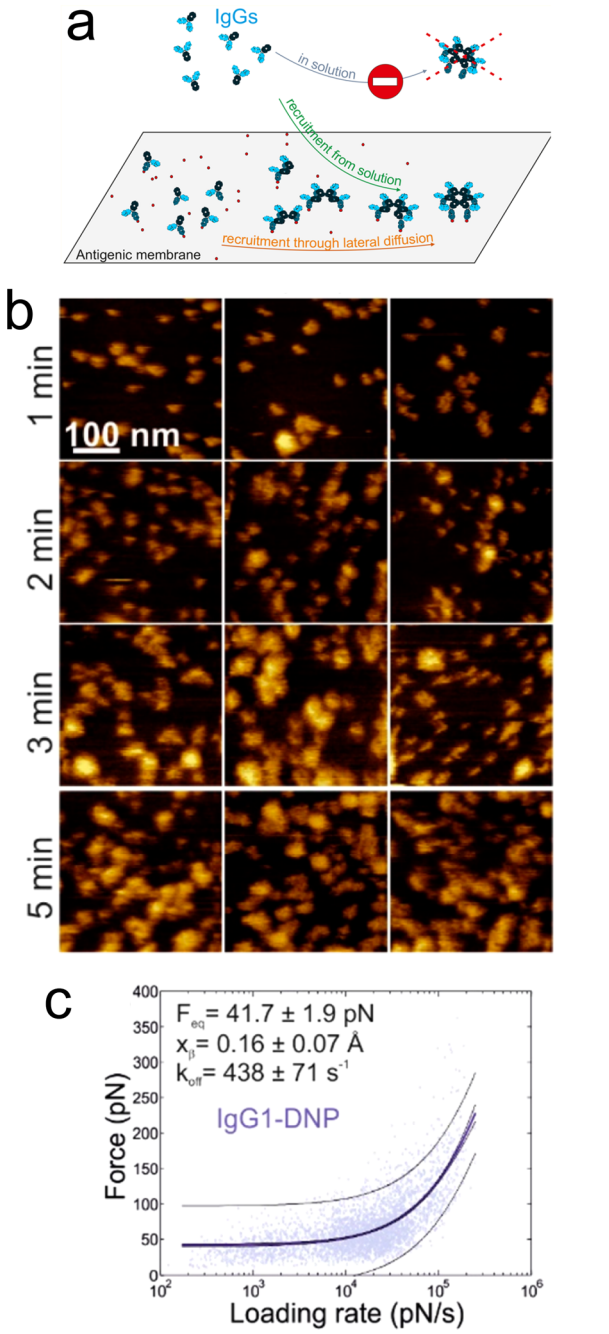

Figure 2. Unravelling the mechanism of IgG oligomerization on antigenic surfaces. (a) IgG molecules interacting with surface-bond antigens form ordered hexamers *via* specific interactions between fragment crystallizable (Fc) domains. Strasser *et al.* use (b) high-speed atomic force microscopy and (c) single-molecule force spectroscopy to study the dynamic oligomerization of IgGs and the molecular interactions involved. Reprinted with permission from ref 11. Copyright 2020 American Chemical Society.

hexamers appearing after 5 min. Large protein assemblies were more abundant with a Fc−Fc interface single-point mutant, meaning it was more efficient in inducing oligomerization than the wild-type IgG.

Although HS-AFM can routinely observe well-defined purified membranes at nanometer resolution, the resolution on living cells is often limited to 50 nm, and so studying protein clustering in cellular samples is challenging. Single-molecule

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force spectroscopy-based imaging with biologically modified tips enables researchers to map the distribution of single molecules on cell surfaces.[8,9] For example, this mode has enabled researchers to capture the dynamic clustering of adhesion proteins on living bacteria and yeasts.[15,16] These studies suggest that protein clustering in response to mechanical stress might be a widespread mechanism for activating cell adhesion in living cells. Recently developed multiparametric imaging methods[17,18] have offered a means to map the structure and interactions of biosystems at high spatiotemporal resolutions, opening up new avenues for studying the dynamic clustering of proteins on cell surfaces.

BINDING FORCE OF SURFACE PROTEINS

In SMFS, the AFM tip is often modified with specific molecules, such as antibodies, and then continuously moved toward and withdrawn from the sample surface while measuring the interaction force. This process provides direct information on the strength and dynamics of the interaction between receptors and their ligands (heterophilic binding) or between receptors (homophilic binding). By means of this approach, Strasser *et al.* measured the specific binding forces between individual IgGs, from which they then determined associated energies and chemical rate constants (Figure 2c). They used poly(ethylene glycol) linkers to attach the antibodies to the AFM tip and to a solid substrate, enabling the binding partners to rotate freely as in aqueous solution. The authors recorded force−distance curves at a wide range of loading rates and analyzed the data using the Friddle−Noy−de Yero model. The results revealed an equilibrium rupture force of ∼50 pN and a dissociation constant in the mM range. This finding indicates that homotypic Fc interactions are weak and suggests that oligomer formation in solution is precluded. It also explains why mass spectrometry experiments in solution detected monomers rather than higher oligomers.

To achieve further insights into IgG oligomerization, Strasser *et al.* performed quartz crystal microbalance experiments in which they monitored the resonance frequency change of an antigenic membrane covered quartz crystal resonator upon IgG binding. They found that weak Fc interactions induce IgG oligomerization upon antigen binding. From there, they developed a detailed mechanistic model of IgG oligomerization and used the experimentally determined kinetic rates to perform numerical simulations of the complex oligomerization process. They developed a complete kinetic scheme depicting the combination of pathways for functionally monovalent IgGs to complete hexamerization.

In summary, this elegant study unravels the mechanistic details of IgG oligomerization on antigenic surfaces, which may find useful applications in immunotherapy. The weak homotypic Fc−Fc interactions prevent IgG oligomerization in solution, whereas they drive the dynamic assembly of IgG oligomers upon antigen recognition. Fc−Fc interactions enable two different oligomerization pathways, which, depending on the epitope density and the resulting preferential monovalent or bivalent binding state of the IgGs, lead to membrane-bound IgG oligomers. This work nicely illustrates the power of combining nanoscopy techniques with traditional approaches to understand the mechanisms guiding the assembly of cell surface receptors into functional clusters. As nanoinstrumentation continues to develop, I am confident that many crucial questions will be addressed in cell surface biology. A grand challenge will be to establish combined optical and force nanoscopy platforms[19] in order to identify and to analyze receptors simultaneously on cell surfaces.

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Notes

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