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Extending the Capabilities of Molecular Force Sensors via DNA Nanotechnology

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Abstract

At the nanoscale, pushing, pulling, and shearing forces drive biochemical processes in development and remodeling as well as in wound healing and disease progression. Research in the field of mechanobiology investigates not only how these loads affect biochemical signaling pathways but also how signaling pathways respond to local loading by triggering mechanical changes such as regional stiffening of a tissue. This feedback between mechanical and biochemical signaling is increasingly recognized as fundamental in embryonic development, tissue morphogenesis, cell signaling, and disease pathogenesis. Historically, the interdisciplinary field of mechanobiology has been driven by the development of technologies for measuring and manipulating cellular and molecular forces, with each new tool enabling vast new lines of inquiry. In this review, we discuss recent advances in the manufacturing and capabilities of molecular-scale force and strain sensors. We also demonstrate how DNA nanotechnology has been critical to the enhancement of existing techniques and to the development of unique capabilities for future mechanosensor assembly. DNA is a responsive and programmable building material for sensor fabrication. It enables the systematic interrogation of molecular biomechanics with forces at the 1- to 200-pN scale that are needed to elucidate the fundamental means by which cells and proteins transduce mechanical signals.

Keywords

mechanotransduction; molecular biomechanics; mechanobiology; molecular tension sensor; DNA nanotechnology; DNA origami; force spectroscopy

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I. INTRODUCTION

Although the first evidence of force-mediated tissue remodeling in bone was published over 100 years ago,[1] it has only been in the past 20 years that the extent and importance of mechanical signaling in cell biology has been revealed. Rather than an isolated phenomenon, numerous studies in multiple cell types have shown the importance of exposure of cells to a range of physical forces—such as pressure, tension, and shear either directly or via modulation related to the material properties of the underlying substrate—as being significant in modulating cell response. For example, studies showing that mesenchymal stem cell (MSC) fate can be determined by substrate stiffness[2] and that shear stress modulates endothelial cell (EC) shape and immune response typify this behavior.[3,4] Forces allow cells to communicate loading information and regulate tissue function in both heavily loaded tissues as well as those that are ostensibly static, because in all tissues, cell-cell, cell-matrix, and cell-fluid forces provide a constant source of "mechanical" information about the extracellular environment. As reviewed by Mammato et al. and Eyckmans et al., forces are increasingly deemed to be as important as biochemical signals throughout embryonic development, tissue and organ formation, remodeling, homeostasis, and disease development.[5,6]

The communication of force is such an important factor in development that it has overturned a primary dogma in development biology. Embryonic patterning was formerly understood to be driven solely by spatiotemporal gradients of soluble factors.[7] However, recent studies have shown that mechanical forces provide equally important regulatory signals in development.[8–10] Tensional or lengthening forces generated by the cytoskeleton of one cell can be transmitted across membrane receptors to neighboring cells, and cells respond to these mechanical signals by altering their own signaling and mechanical structure and function (i.e., altering cell shape and internal tension and activating additional mechanotransduction pathways for sensing and responding to mechanical cues). These changes can ultimately switch cell fate to growth, differentiation, motility, or even apoptosis.[8,9] Cells are capable of sensing and responding to mechanical signals, but the extent to which mechanical and biochemical signals interact in cellular communication is unknown. Further, it is not well understood what portion of sensing and response is done purely at the molecular level, involving membrane and cytoplasm, without the involvement of the nucleus. To better understand how these complex spatiotemporal patterns of force application provide regulatory cues to guide cell structure, behavior, and organization, researchers can carefully control the mechanical environment (e.g., stiffness and shear stress profile) of cultured cells (Fig. 1).

As researchers have sought to understand the role of mechanics and forces in cell signaling, the toolkit for cellular biomechanics has expanded to include microfabricated tools, including traction force microscopy (TFM) and micropost arrays for mapping cellular traction forces, atomic force microscopy (AFM) for measuring ligand binding and protein force extension, and optical trap (OT) assays to investigate membrane tension and molecular motor mechanics. Reviews of tools for mechanobiology detail the history and application of these approaches.[6,11–13] Techniques for studying cell-scale biomechanics typically measure nanonew-ton to micronewton forces *in vitro* and include micropipette aspiration and

micropost force assays.[6,14–18] Cell-scale force assays are used to determine bulk properties of cells like cell stiffness and viscoelasticity as well as adhesion strength and contractile force. At the nanoscale, adhesion forces and contractile forces generated by individual proteins can be interrogated by single-molecule force spectroscopy (SMFS) techniques, including both OT assays and AFM.

Researchers in mechanobiology often employ multiple characterization methodologies to study forces at a variety of scales. For example, while the contraction of an individual myosin motor generates about 2 piconewtons of force and can be studied using OT assay,[19] a heart muscle cell or cardiomyocyte, containing millions of these molecular motors arranged in sarcomeric units, can generate between 0.7 and 12.6 micronewtons of force. This is six orders of magnitude more than an individual motor.[20–23] Selection of appropriate techniques for a given application is therefore based on the force and displacement sensitivities of each technique, as well as the unique bandwidths of its operation.[11,18,24] However, micro- and macroscale techniques are frequently limited by necessary trade-offs between force resolution and experiment throughput. Tension probes, often made using DNA, can provide both high force resolution and high throughput.

II. A REVOLUTION IN PICONEWTON FORCE SENSING WITH FLUORESCENT TENSION PROBES

A. Genetically Encoded Tension Sensing

Over the past 12 years, the need to measure pN-scale forces experienced by single molecules and complexes in a highly parallel manner drove the development of fluorescent probes whose optical properties change on loading because of distance-dependent and rotation-dependent energy transfer. This modulation is typically achieved using Förster resonance energy transfer (FRET), quenching, and emission modulation.[25–27] Two early FRET molecular tension sensor (MTS) approaches were not genetically encoded, but they demonstrated the potential of this approach. Kong et al. demonstrated a novel approach for simultaneously investigating the clustering of adhesion proteins and measuring the magnitude of cell-material forces applied between integrin receptors and adhesion ligands.[28] In this work, hydrogels were adhesion peptides, some of which were decorated with Alexa Fluor 488 and others with Alexa Fluor 546. FRET studies with MC3T3-E1 preosteoblasts on these gels measured nanometric displacements due to clustering of FRET-pair–labeled peptides as well as traction forces exerted by cells. Smith et al. used FRET studies to demonstrate that cellular traction forces are capable of stretching the extracellular protein fibronectin.[29] The fibronectin itself was labeled with the FRET pair fluorophores Alexa Fluor 488 and Alexa Fluor 546. Cell traction studies revealed that fibronectin can be both straightened and unfolded, while FRET studies showed the extent of unfolding of FnIII modules. These FRET approaches demonstrated the power of approaches that allow the measurement of forces as well as nanometric displacements.

The first fluorescent genetically encoded MTSs built by Meng et al. drew on these prior concepts to create FRET cassettes that could be inserted into structural proteins.[30] This FRET-based sensor approach, called stFRET, consists of a stable alpha-helix domain flanked

by GFP-like fluorophores, Cerulean and Venus. In this system, fluorescence changes as a function of chromophore separation and relative chromophore rotation, and this change acts as a proxy for stress.[31] Because MTS constructs can therefore be expressed in living cells, they can provide information about the mechanical state of proteins *in vivo*. A key advantage of the cassette-based MTS approach is that no direct connection to an external probe like a microcantilever or a functionalized microbead is needed; the MTS approach is noninvasive, and because the probe is inserted in a protein of interest, this assay is protein-specific by design. In early genetically encoded MTSs, Grashoff et al. made the approach highly quantitative by introducing a powerful new fluorescent sensor module whose force-FRET signal had been calibrated using OT experiments.[32] This sensor, the tension sensor module (TSM), contained the FRET pair mFTP1 and Venus flanking an extensible peptide based on spider silk. TSMs are sensitive to forces in the range of 1 to 6 pN. For the majority of these systems, fluorescence can be used as a spectroscopic force-gauge technology whose displacement is approximately linear (analog) with force over a given range of sensitivity.

This functionality is achieved by fluorophore selection and placement. MTS sensors like the TSM that are flanked by fluorescent protein FRET pairs undergo FRET as a function of stretch and rotation.[33–35] FRET occurs between fluorophores with spectral overlap between "donor" emission and "acceptor" absorbance. When FRET pair fluorophores are close (typically less than 8 nm apart), separation-dependent nonradiative emission occurs between the donor and the acceptor, allowing the acceptor to fluoresce when the donor is activated.[35] This radiative energy transfer is also a function of the alignment of the fluorophores. In a molecular tension probe, when the elastic spring element is stretched or deformed, the change in separation and/or orientation of the flanking fluorophores results in a change in the amount of energy transferred. The acceptor-to-donor ratio of fluorescence can therefore be used to report the stretch, and with a force-to-fluorescence–calibrated sensor like the TSM, fluorescence can be used to quantify piconewton-scale forces in real time across a cell. By inserting a tension sensor "spring" in series with a protein of interest (see Fig. 2), this approach enables cell-wide measurement of the forces that proteins experience. However, it can also fundamentally change the stiffness of that protein. Therefore, numerous controls must also to be employed to verify that insertion of a genetically encoded MTS sensor does not alter the function of the protein being studied for a given cell type.[36–38]

Recently reported TSMs that utilize a ferredoxin-like (FL) linker peptide have expanded the capability of TSMs; these sensors can exhibit nearly digital force response with increased sensitivity.[39] They allow the quantitative interpretation of low-pN ensemble force measurements with the capability to multiplex force measurements from probes with a range of sensitivities. In this way, the FL-TSM method has revealed an intramolecular tension gradient across talin-1 due to integrin-mediated cell adhesion. Lemke et al. showed that TSM sensors can also be used *in vivo* to study mechanical force transmission in *Drosophila* muscle attachment sites.[40] These studies found that the molecular forces across talin were in the range of those experienced in cell culture, but less than 15% of talin molecules *in vivo* experienced detectable levels of load at the same time.

Fundamentally, the conceptual change that the MTS revolution introduced was the manufacture of spring-like force probes for mechanobiology at the same scale as native

mechanosensitive proteins. Hoffman et al. extended the capabilities of these sensors by providing a framework for generating expressed nanosensors of tunable stiffness.[36,41] This enables single-molecule force spectroscopy for forces from 1 to 200 pN.[42] Access to a diversity of probe stiffnesses allowed Hoffman's group to identify that extension (strain) rather than force regulates vinculin loading.[41] Notably, they also found that sensor function in the crowded cellular environment is different from sensor function outside—that is, free of cells—highlighting an important and often unrecognized challenge for molecular mechanobiology studies. In other words, expressed probes successfully minimize perturbation of the biological systems under study, but their force extension properties can be affected by the solution environment.

B. Extracellular Molecular Tension Sensors

While genetically expressed MTS systems have benefits for intracellular forces, extracellular molecular tension sensors, introduced by Stabley et al., have complementary functions. They can be synthesized outside of the cell and can be readily bound to variety of cells over a range of concentrations.[43] These immobilized tension sensors must incorporate (1) an extensible domain, (2) synthetic fluorophore FRET pairs or quencher pairs, and (3) additional adhesion moieties such as the cyclo-Arg-Gly-Asp motif.[44,45] This approach is called molecular tension fluorescence microscopy (MTFM), and the benefits of this nongenetically encoded approach are as follows: (1) the native biological function of force-transducing proteins like vinculin and talin are not perturbed by the addition of a tension-sensing moiety, (2) the forces between the cell and the extracellular environment can be measured, and (3) fluorophores with high quenching efficiency and high quantum yield can be used in conjunction with any adhesion peptide for extremely sensitive traction force detection. While elastic linkers like polyethylene glycol (PEG) polymers provide a more analog signal, immobilized tension sensors that incorporate DNA can offer more digital behavior, which has important implications for force studies.

The unzipping of complementary DNA or a DNA hairpin is highly cooperative and occurs over a narrow range of forces (see Fig. 3). This means that DNA binding tends to unzip in a digital manner, with the force to unzipping increasing with the number and guanine-cytosine (GC) content of bases.[46,47] The critical force above which a DNA tension probe unfolds is referred to as the $F_{1/2}$ value, and the binary transition between the on and off states renders these probes highly quantitative. For example, a hairpin sensor can enable a turn-on system that will abruptly unfold and unquench upon loading above the $F_{1/2}$ value, and twice the fluorescence indicates that twice as many tension probes are open. When the loading is reduced, this system can return to its folded hairpin condition.[48] Hairpin sensors can be formed using multiple distinct DNA strands, which simplifies decoration to require one chemical modification per DNA strand. Zhang et al. demonstrated a tension probe switch capable of generating a 20-to 30-fold increase in fluorescence upon loading with a threshold piconewton force.[49] Zhang et al. utilized these spectrally encoded probes, which could be spectrally multiplexed, to simultaneously measure forces of different magnitudes exerted by cellular receptors.[49] Building on this work, the Salaita lab adapted the previous probe system to investigate the activation of platelets, a highly mechanosensitive cell type critical to blood clotting.[50]

DNA systems also enable the creation of irreversible sensors that record readout for later observation. Tension gauge tether (TGT) systems contain DNA duplexes that unzip and detach upon loading. In 2013, Wang and Ha demonstrated ligand-bound TGTs that rupture at a given force, or tension tolerance, which could be used to investigate the force at a single integrin-ECM ligand bond required for cell adhesion.[51] Murad et al. contributed significantly on the theoretical side of TGTs by developing a computational approach to determine receptor forces of serially connected sensors with high accuracy.[52] However, TGTs are not capable of capturing weak or short-lived events. To address this limitation, Ma et al. utilized highly sensitive stem-loop DNA hairpin switches to create probes with irreversible oligonucleotide locks capable of measuring the transient and infrequent pN-level forces in mechano-immunology.[53] This platform can store molecular tension history for short-lived events like a T cell's sampling of antigens that would be impossible to visualize using reversible probes. In addition to reporting the accumulation of events equaling or exceeding the $F_{1/2}$ value of the probe, the introduction of an unlocking strand can erase the signal by triggering a toehold-mediated strand displacement reaction.

Using the similar hairpin-based DNA tension motif, Ma et al. used ratiometric tension probes to quantify the forces T cells apply to fluid membrane junctions upon activation, highlighting the connection between receptor tension, signaling, and mechanotransduction.[54] Brockman et al. adapted DNA-based tension probes to measure both the magnitude and direction of receptor forces, bridging a remaining gap between the capabilities of molecular tension probes and traction force microscopy.[55] Glazier et al. introduced molecular tension–fluorescence lifetime imaging (MT-FLIM) to overcome the quantitative limitations of ratiometric techniques.[56] MT-FLIM and force orientation analysis enable the measurement of receptor tension exerted by podosomes on the supported lipid bilayer model system.[56]

One key distinguishing feature of nucleic acid–based force probes is that they can incorporate a broad range of functionalizations, including fluorophores, quantum dots, biotin, and tags for conjugation to proteins. For example, Liu et al. developed an MTFM sensor that utilized gold nanoparticles (AuNPs) decorated with cyclic arginylglycylaspartic acid (RGD).[57] Thiol-decorated DNA in the sensor binds the AuNPs to enable anchoring as well as stretch-dependent quenching behavior. Further, this form of AuNP-based quenching, called nanometal surface energy transfer (NSET), is less dependent on fluorophore orientation than FRET.[58] Lipid-modified DNA tension probes that can stably self-assemble on cell membranes allow measurement of intercellular tensile forces.[59,60] Therefore, DNA-based systems can be used to create more robust fluorescent sensors than genetically expressed molecular tension sensors and can incorporate cutting-edge chemical modifications as they become available.

However, Morimatsu et al. demonstrated that genetically expressed molecular tension sensors can be decorated with organic fluorophores after expression to create MTSs with higher brightness and photostability.[61] This hybrid approach complements the genetically expressed molecular tension sensor approach by enabling decoration with a wider range of fluorophores, adhesion moieties, and nanoparticles. Morimatsu et al. later showed that a hybrid MTS created using an extensible (GPGGA) 8 peptide linker flanked by Alexa 546

and ATTO647N can be used with super-resolution microscopy to enable investigations into focal adhesion component arrangement in space and time as a function of applied force.[62]

Recent work by Zhao et al. utilizes synthetic DNA mimics, peptide nucleic acids (PNAs), and modified RNAs to develop TGT tension sensors that can withstand degradation by both soluble and membrane-bound DNase.[63] Specifically, while dsDNA sensors were degraded and unquenched by both soluble and membrane-bound DNase on cells, the PNA/DNA, dsRNA (modified), and PNA/RNA sensors successfully resisted enzymatic degradation. Of those DNase-resistant constructs, only the PNA/DNA sensor retained its force-reporting capability with high a signal-to-noise ratio and specificity. This study demonstrated that the incorporation of PNA into MTSs can broaden the application of tension sensors for cell mechanobiology.

Both genetically encoded MTSs and extracellular molecular tension sensors enable force studies at the single-protein scale in a highly parallel manner. Further details about applications and quantification for both genetically encoded and immobilized fluorescent tension sensors are available in several excellent reviews.[27,44,64] In the following section, we discuss how structural DNA nanotechnology can extend DNA-based immobilized tension sensors to incorporate novel force-displacement behaviors, novel transduction mechanisms, targeting, and improved stability in biological media.

III. DNA ORIGAMI STRUCTURES AND SENSORS

A. Structural DNA Nanotechnology

Structural DNA nanotechnology involves self-assembly of nanostructures from single-stranded DNA (ssDNA). In the 1980s, Seeman pioneered the field of structural DNA nanotechnology by using DNA to form lattices and junctions, which are the building blocks of larger structures.[65] DNA origami is a simple and predictable scaffolding approach in structural DNA nanotechnology that demonstrated by Rothemund.[66] In DNA origami, nanostructures are composed of a long single-stranded scaffold DNA, typically from the M13 bacteriophage, in combination with hundreds of short synthetic staple oligonucleotide strands. The short staple strands are uniquely complementary to a specific region along the scaffold, and they serve to clamp, or staple, the scaffold into a preprogrammed arrangement. After combining the scaffold strand and staple strands in a single pot, the mix is annealed from 95°C and cooled to 20°C for 2 to 24 hours.[66,67] Scaffolded DNA origami has been extended to successfully build a variety of solid, hollow, and wireframe two- and three-dimensional objects.[68–72] Increasingly, DNA origami is being used to create dynamic, responsive machine-like structures, which are reviewed in DeLuca et al. and Ijäs et al.[73,74]

In this section, we introduce DNA origami nanostructures and nanomachines that have been used to load biomolecules and measure biomolecular forces. However, it is important to note that that scaffolded DNA origami approach is not the only modular way to build DNA-based nanostructures. Nonscaffolded methods like the single-stranded tile approach (SST) can create fixed-length and periodically repeating structures that are made entirely from short, multi-domain ssDNA strands.[75,76] This approach can be used to form two- and three-dimensional nanostructures with as many as 10,000 subunits.[77,78] These structures can be

decorated, synthesized, and purified, similarly to DNA origami, and nonorigami structures can offer unique advantages over scaffolded origami. For instance, unlike DNA origami, often a range of SST structures can be formed by using specific oligomer subsets of a master structure. Additionally, the modularity of SST systems allows easier design of micrometer-scale periodic structures.[76,79] However, it can be more difficult to sever the scaffolded DNA structure due to the covalent bonds of the scaffold backbone that run through and connect the entire structure. To date, force-sensing studies using structural DNA nanotechnology have typically been carried out using DNA origami platforms.

B. DNA Origami to Augment Measurement Accuracy and Precision

In microscopy and biophysics, the mechanics and spatial control of DNA origami can be used to improve existing approaches. For example, in 2013 Pfitzner et al. demonstrated that elastic linkers used in optical trap studies were contributing substantial thermal noise to single-molecule experiments.[80] The introduction of more rigid DNA origami linkers enabled unprecedented noise reduction in the measurement of conformational changes in small DNA secondary structures. Additionally, DNA origami systems allow the precise placement and orientation of biological systems for testing. Kilchherr et al. demonstrated how a two-part tethered DNA origami system could be used to interrogate the forces and lifetimes of DNA base-stacking interactions.[81] While OT enabled the measurement of pN-scale forces and nm-scale displacements, the DNA origami tethered beam platform enabled the relative positioning and tethering required for weak base-stacking interactions to be systematically characterized.

In the field of structural biology, DNA origami has enabled molecular structural determination of proteins in cryo-electron microscopy.[82] Martin et al. created a hollow three-dimensional cylinder to address sample preparation complications during this imaging procedure. Here, hollow DNA origami, while controlling molecule orientations, served as a shield to protect the sample from physical forces and unwanted air-water interactions. This aided the reduction of background noise in the images to provide a more comprehensive structural analysis of the examined protein.

DNA origami's nanoscale building resolution has been used to address the lack of length standardization in super-resolution microscopy. Schmeid et al. precisely arranged fluorescent dyes on DNA origami rectangles, bundles, and nanopillars to form nanorulers.[83,84] The known distances between the fluorescent dyes on each DNA structure served as a calibration standard to validate the resolution of new super-resolution microscopy techniques. The calibration standard also served to associate possible experiment failures to the microscope or the sample preparation process.

Structural DNA nanotechnology approaches can be used to improve the signal-to-noise ratio in existing FRET-based and quencher-based approaches for measuring forces and displacements. As demonstrated by Selnihhen et al., DNA origami can be used to create high-sensitivity "beacon" biosensors containing upwards of 100 fluorophores per origami device.[85] The opening or closing of this "beacon" device was driven by strand-displacement reactions, and actuation resulted in a detectable shift in FRET efficiency at concentrations as low as 100 pM. This beacon approach reduces the minimum necessary concentrations of

sensors and targets while preserving or exceeding the FRET efficiency of single FRET-pair systems.

These applications illustrate that DNA origami can fully complement and extend the capabilities of existing tools for single molecular force measurement and quantitative microscopy. In the following sections, we detail applications for standalone DNA origami force sensors for pN and sub-pN force sensing.

C. DNA Origami Sensors for Biological Force Sensing

Designer DNA origami mechanisms also allow for force studies that investigate mechanical parameters beyond simple peak force and instantaneous spring extension. For example, Hudoba and colleagues developed a bistable DNA origami sensor that transitioned between its open and closed states in response to molecular crowding forces with a sensitivity on the order of 100 fN (Fig. 4A).[86] In this case, the programmability of DNA origami and length-controlled stability of DNA hairpins allowed highly parallel measurement of sub-pN forces.

Nickels et al. developed a force clamp system using ssDNA as an entropic spring whose end-to-end distance was tightly controlled by a DNA origami test structure (Figs. 4B and 4C). By incorporating elastic ssDNA domains with stiff DNA origami structures of known size and shape, these force clamps enabled massively parallel studies of tension-induced bending that could be performed without the need for expensive equipment like an optical trap or atomic force microscope.[87] With this platform, Nickels et al. demonstrated the mechanosensitivity of the tension-induced bending of a central DNA duplex by TATA-binding protein. This work demonstrates how complex structures under prestress can be used as high-throughput force sensors, and it suggests, for example, that previously published DNA origami tensegrity structures by Liedl et al.[88] could be incorporated into future mechanosensing applications.

Kuzuya et al. demonstrated that DNA origami could be used to create nanomechanical pinching devices.[89] These devices consisted of two levers and a fulcrum, which could snap closed upon binding to a desired analyte. Conformation changes to the DNA pliers were read out using AFM. Other DNA origami lever–based measurement tools include nanoscopic calipers of known torsional stiffness for investigating forces due to stacking interactions between nucleosomes[90] as well as the conformation of DNA-wrapped nucleosomes for readout using AFM, TEM, and FRET (Figs. 4D and 4E).[91,92] Funke et al. took advantage of this lever concept to create a versatile FRET-based readout system that theoretically could study a wide range of biophysical systems. They positioned a FRET pair near the fulcrum of their nanocaliper to achieve fluorophore displacements in the peak range of FRET sensitivity. They also controlled where to attach the biomolecules of interest in order to accommodate larger-scale motions without sacrificing fluorescence signal.

Subnanometer placement of biomolecules using DNA origami allows for controlled studies of spacing- or crowding-mediated interactions of biomolecules. Early studies of molecular motors used DNA origami templates to enable tug-of-war events between motor populations[93,94] and biomimetic sarcomere contractile studies.[95] Using helical DNA origami nanosprings, Iwaki et al. demonstrated that an OT-calibrated nanospring could be used to

study the stepping dynamics of human Myosin VI under tension (Figs. 4F and 4G).[96] This system enabled low-cost, nanometer-level precision and single-molecular fluorescence imaging of individual molecular motors with a DNA origami tool system that could be inexpensively recreated by labs without OT facilities. Precision placement of antigens on DNA origami templates has also allowed the first studies of the spatial tolerance of antibodies.[97] It is difficult to imagine any approach other than DNA origami for achieving highly parallel and reliable arrangement of numerous molecules with subnanometer precision, and this is particularly useful as increasing numbers of biological systems are understood to be sensors of displacement rather than force.[41,98]

In 2018, Dutta utilized DNA origami to create multivalent DNA hairpin probes for mapping the force experienced by human blood platelets during activation and adhesion (Fig. 4H).[99] This proof-of-concept approach revealed that hairpin unfolding is semicooperative and orientation-dependent. The ability to control sensor placement and orientation at the nanoscale once again highlights the unique strength of structural DNA nanotechnology for mechanobiology studies.

Finally, DNA origami nanopores and nanocages can be used to spatially confine and detect molecules of interest. Custom DNA origami nanopores allow detection and filtration as well as interfaces with electronics.[100–103] Custom nanocages can confine molecules of interest with subnanometer resolution; such a nanocage was used to demonstrate for the first time a long-held hypothesis that confinement of G-quadruplexes—four-stranded DNA structure motifs—enhances structure stability.[104]

D. Mechanosensor Functionality from DNA Origami Nanobiosensors

As an add-on or standalone tool for force spectroscopy, DNA origami can integrate a vast array of chemical functionalizations and mechanical elements to create versatile biocompatible mechanosensors with unmatched capabilities. These sensors can be described as a special subset of DNA origami nanobiosensors (Fig. 5). As described by Liu et al.,[105] DNA origami nanobiosensors can detect a range of bioanalytes and report binding events by means of a physical transduction mechanism to create a detector-specific output. DNA origami mechanosensors detect their target and upon binding apply or measure a load that they report. As shown in the previous examples, these outputs can include changes in topography, conformation, fluorescence, or impedance.

DNA origami force sensors have primarily been used *in vitro* in the presence of reconstituted protein systems rather than in living cells. These first demonstrations have shown the potential of DNA nanotechnology for mechanobiology. Building off the success of genetically encoded tension sensors *in vivo*,[40] the next frontier of study remains application of DNA origami nanosensors to living cellular systems *in vitro* and *in vivo*. Indeed, as Shaw et al. demonstrated with a DNA origami nanocaliper system for controlling ligand spacing,[106] biological "realities" such as clustering and crowding of the cellular environment can alter receptor function, and cell-compatible sensors must be able to measure and deliver pN forces with high precision control of both placement and orientation of ligands.

IV. CONCLUSION AND FUTURE CHALLENGES

DNA-based force sensors offer an exquisite capability, largely unparalleled by other technologies, to measure forces in complex biologic systems rapidly and in a high-throughput manner over a force range of hundreds of piconewtons down to tens of femtonewtons. To date, most demonstrations of DNA-based force sensors have been conducted *in vitro* over periods of minutes to hours. The next steps for this technology involve practical advances to transition from short-term proof-of-concept *in vitro* studies to longer-term studies in cellular environments. Studies *in vivo* may present challenges for fluorescence imaging; however, significant strides have been made in this area.[40] As the use of these sensors broadens throughout the research community, mass bioproduction of platforms will be necessary to keep costs down and standardize platforms.

Longer-term studies require DNA origami sensors that have been stabilized against nuclease degradation and low salt denaturation. This can be achieved using protection strategies such as base-specific crosslinking,[107] lipid bilayer encapsulation,[108] electrostatically coating with polymers,[109,110] and using a combination of nuclease-resistant biomaterials with DNA.[63] Nanostructure stabilization is an active area of research and has recently been summarized in excellent reviews by Stephanopoulos and Ramakrishnan et al.[111,112]

Delivery of sensors to the cells or receptors of choice will require precision targeting as well as the potential for triggered stimulation or “remote activation” of the sensors. To accomplish these feats researchers can borrow from the drug delivery toolkit to incorporate targeting molecules on DNA origami sensors. For example, DNA aptamers that bind to membrane receptors have been used to direct DNA origami that carry gene therapies to cells of interest,[113] and the geometry of origami itself has been shown to effect cellular uptake.[114,115] Techniques for targeted delivery of DNA origami to cells are described in the excellent review by Balakrishnan et al.[116]

Tissues absorb and scatter light, which can greatly reduce fluorescence signal to noise. Therefore, new strategies will need to consider microscopy techniques and fluorophores that are capable of overcoming these limitations and are compatible with imaging *in vivo*. For example, FRET with near-infrared fluorophores can be used to detect DNA interactions deep in tissue.[117]

Finally, as the use of DNA-based sensors grows, and as larger quantities are needed for *in vivo* studies, large-scale biomanufacture of DNA origami components will become critical for standardization and cost control. Praetorius et al. recently reported a novel approach to generate macroscopic quantities of scaffold and staple strands using shaker-flask cultures. This utilizes a custom plasmid containing the scaffold and staples, each flanked by self-excising DNase cassettes.[118] This approach reduces cost by three orders of magnitude compared with traditional chemical synthesis, enabling low-cost biotechnological production of DNA origami products.

DNA-based mechanosensors provide a powerful complement to macro-, micro-, and nanoscale tools for experimental mechanobiology. Their unmatched versatility and precision

promise to offer exciting opportunities as they move from proof of concept to workhorse tools in biomedical research.

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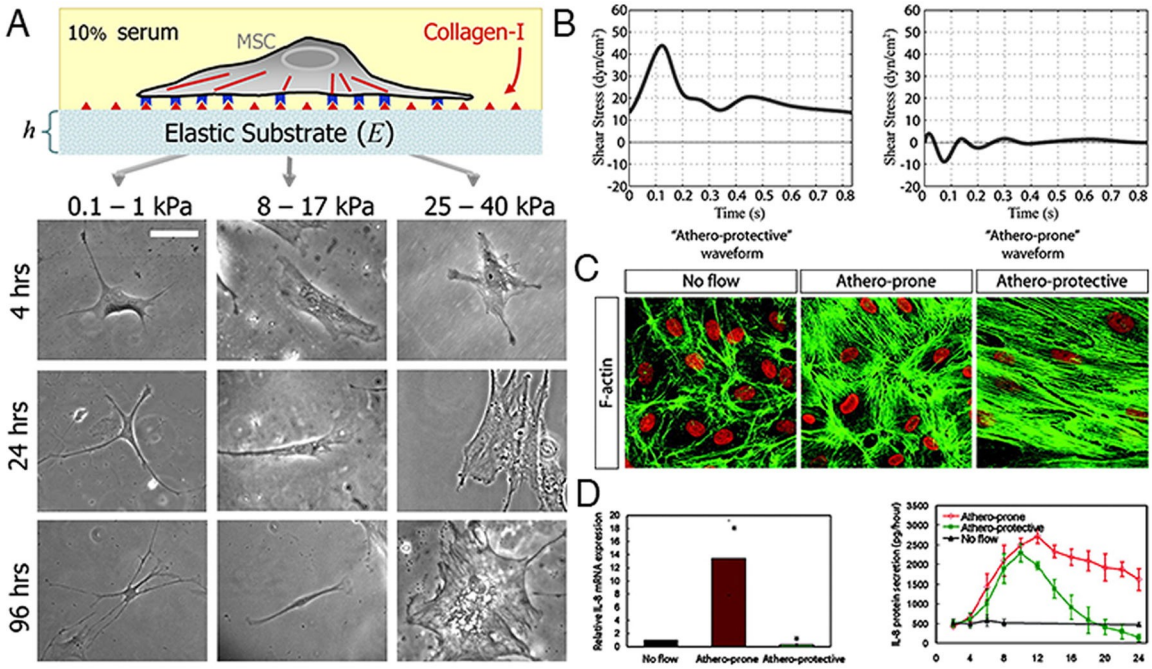


FIG 1:

Cells act as mechanosensors. (A) When grown on substrates mimicking the stiffness of brain (0.1–1 kPa), muscle (8–17 kPa), and stiff crosslinked collagen (25–40 kPa), naive MSCs develop from a rounded phenotype into branches, spindle, and polygonal shapes, respectively. These MSCs also demonstrate respective upregulation of neurogenic, myogenic, and osteogenic markers. Reprinted with permission from Elsevier, Copyright 2006.[2] (B and C) Endothelial cells (ECs) exposed to no flow, an athero-prone waveform, and an athero-protective waveform respond to the athero-protective flow by aligning to it, (D) while ECs exposed to the athero-prone flow respond within 24 hours by expressing IL-8, a marker of inflammation. Reprinted with permission from the National Academy of Sciences, Copyright 2004.[3]

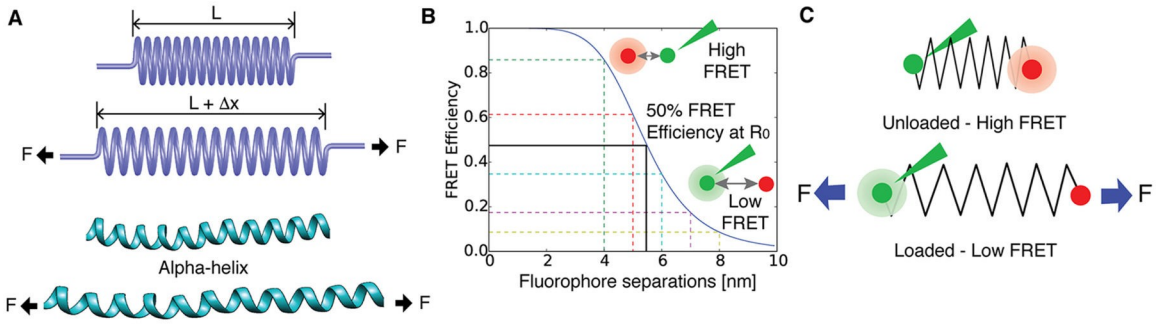
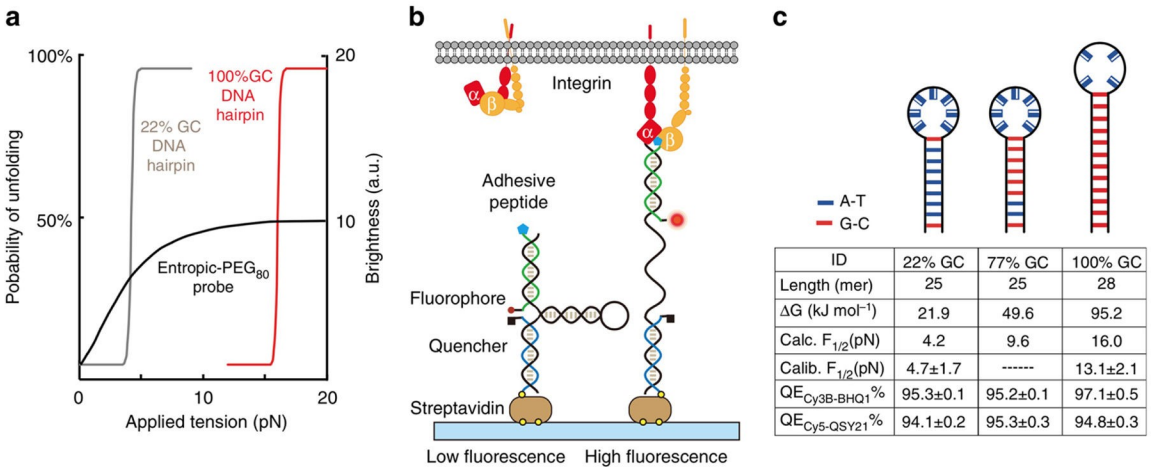


FIG. 2:

Biological analogs of mechanical springs. (A) Biological molecules like peptide alpha-helices can act like simple mechanical springs that deform under loading force F. Other protein domains including coiled coils, beta sheets, and random coils can be treated as springs. Adapted and reprinted with permission from Mary Ann Liebert, Inc., Copyright 2014.[38] (B) Efficiency of FRET decreases as a function of fluorophore separation according to the relationship $\%Eff = R_0^6/(R_0^6 + r^6)$. (C) As FRET-based tension sensors are loaded and stretched, FRET efficiency decreases.



ID	22% GC	77% GC	100% GC
Length (mer)	25	25	28
ΔG (kJ mol^{-1})	21.9	49.6	95.2
Calc. $F_{1/2}$(pN)	4.2	9.6	16.0
Calib. $F_{1/2}$(pN)	4.7±1.7	------	13.1±2.1
$QE_{Cy3B\text{-}BHQ1}$%	95.3±0.1	95.2±0.1	97.1±0.5
$QE_{Cy5\text{-}QSY21}$%	94.1±0.2	95.3±0.3	94.8±0.3

FIG. 3:

Digital tension sensing with DNA hairpins and dsDNA. (A) The unfolding force is digital in nature as compared to more analog entropic spring probes and increases with GC content. (B) When the binding force exceeds the tension tolerance, the hairpin unfolds, allowing the previously quenched fluorophore to fluoresce. (C) Example $F_{1/2}$ values, free energy, length, and GC content for hairpin probes used in the study. Reprinted with permission from Springer Nature, Copyright 2014.[49]

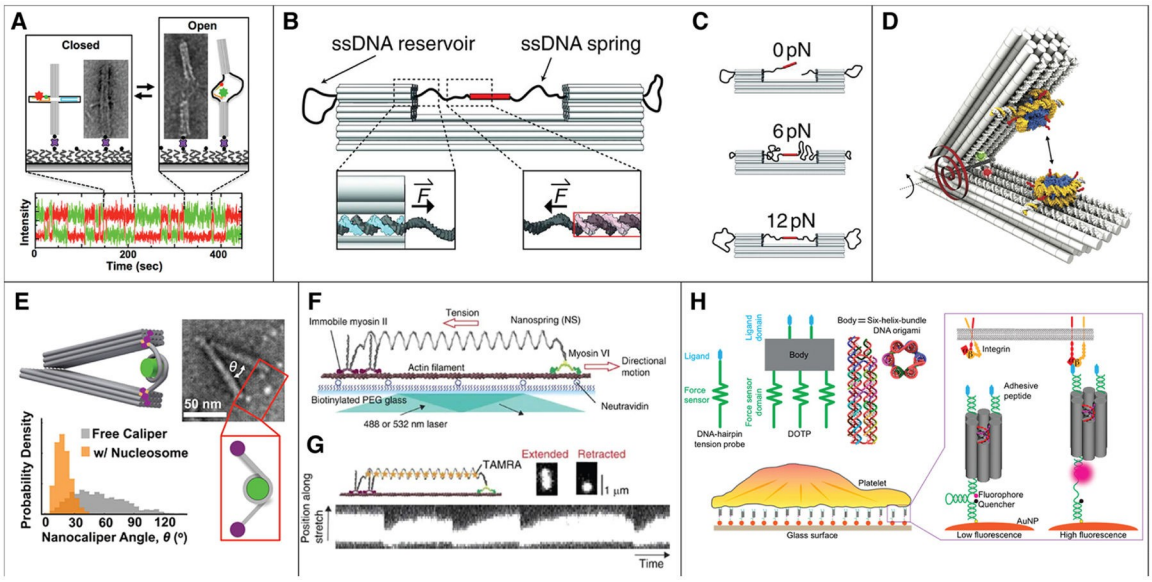


FIG. 4:

DNA origami sensors for measuring and applying pN and sub-pN force and nm-scale displacements. (A) Device for measuring molecular crowding forces with 100-fN force resolution. Reprinted with permission from the American Chemical Society, Copyright 2017.[86] (B) Nanoscale force clamp device can apply constant tension force to central DNA duplex. (C) At the central DNA duplex, the entropic spring force is set by the length of the ssDNA and end-to-end distance set by the DNA origami fixture. Reprinted with permission from the American Association for the Advancement of Science, Copyright 2016.[87] (D) Schematic of the DNA force spectrometer featuring a spring-loaded hinge with two attached nucleosomes. The torque generated by the hinge is illustrated with a torsional spring. Spheres indicate positions of fluorescent dyes (Atto647N and Atto550) that form a FRET pair. Reprinted with permission from the American Chemical Society, Copyright 2016.[92] (E) Nanocaliper for probing nucleosome stability provides a sensitive measure of nucleosome disassembly and can read out transcription factor (TF) binding to its target site within the nucleosome. Reprinted with permission from the American Chemical Society, Copyright 2016.[91] (F) Myosin VI tethered to a two-helix bundle (2HB) nanospring moves unidirectionally along actin against the load of the nanospring. (G) Stretch/compression dynamics of the nanospring by myosin VI at 2-mM ATP + 100-μM ADP. The kymograph shows repetitive stretching and compressing of the carboxytetramethyl-rhodamine (TAMRA)-labeled nanospring. Reprinted with permission from Springer Nature, Copyright 2016.[96] (H) DNA-origami-based tension probes contain three components: a ligand-presenting domain, an origami body, and a force-sensing domain. The body is composed of a six-helix-bundle DNA origami (side and top view), in which six parallel double helices are packed on a honey-comb lattice. Upon receptor (integrin) engagement to the adhesive peptide (cRGDfk) and application of sufficient tension, the hairpin unfolds, separating the fluorophore from the AuNP and organic quencher and dequenching the dye. Reprinted with permission from American Chemical Society, Copyright 2018.[99]

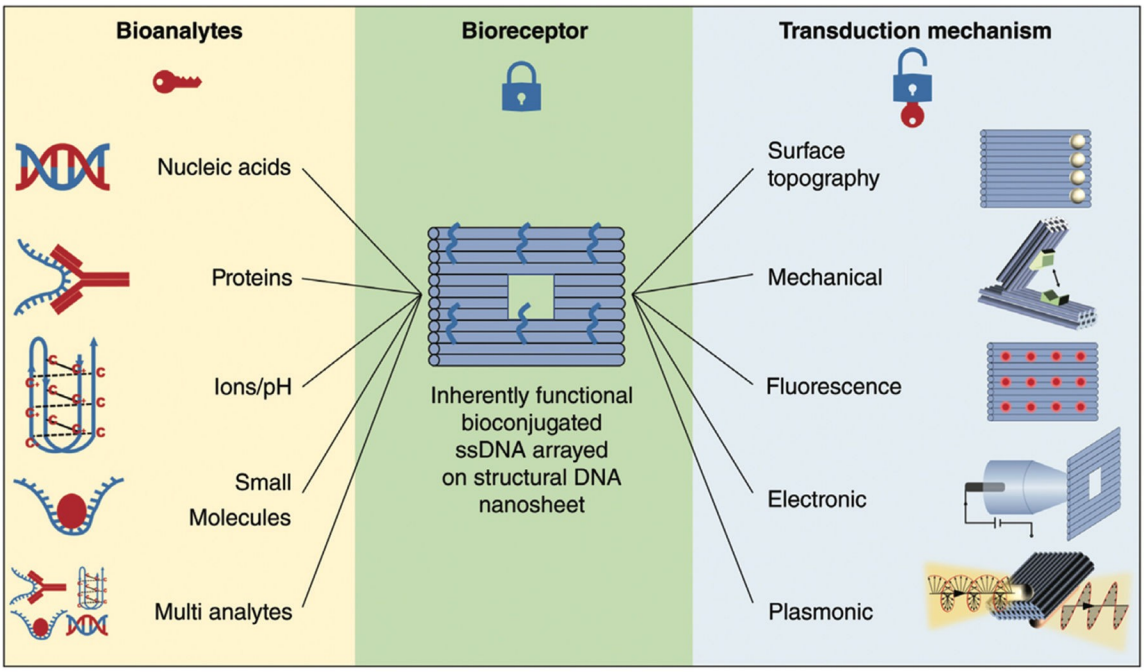


FIG. 5:

Nanosensors made using DNA origami can be designed to independently detect the binding of analytes like nucleic acids or small molecules and use custom-designed transduction mechanisms to report binding events via changes in, for example, structural conformation (mechanical), fluorescence, and impedance (electronic). Reprinted with permission from John Wiley and Sons, Copyright 2018.[105]