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Single-Molecule Mechanochemical Sensing

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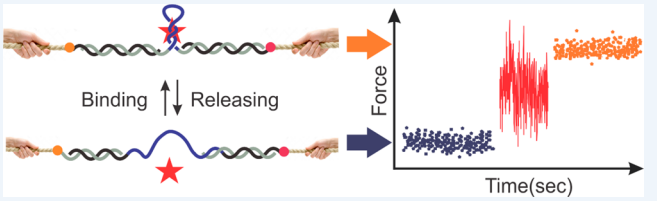
CONSPECTUS: Single-molecule mechanochemical sensing (SMMS) is a novel biosensing technique using mechanical force as a signal transduction mechanism. In the mechanochemical sensing, the chemical binding of an analyte molecule to a sensing template is converted to mechanical signals, such as tensile force, of the template. Since mechanical force can be conveniently monitored by single-molecule tools, such as optical tweezers, magnetic tweezers, or Atomic Force Microscopy, mechanochemical sensing is often carried out at the single molecule level. In traditional format of ensemble sensing, sensitivity can be achieved via chemical or electrical amplifications, which are materials intensive and time-consuming. To address these problems, in 2011, we used the principle of mechanochemical coupling in a single molecular template to detect single nucleotide polymorphism (SNP) in DNA fragments. The single-molecule sensitivity in such SMMS strategy allows to removing complex amplification steps, drastically conserving materials and increasing temporal resolution in the sensing. By placing many probing units throughout a single-molecule sensing template, SMMS can have orders of magnitude better efficiency in the materials usage (i.e., high *Atom Economy*) with respect to the ensemble biosensing. The SMMS sensing probes also enable topochemical arrangement of different sensing units. By placing these units in a spatiotemporally addressable fashion, *single-molecule topochemical sensors* have been demonstrated in our lab to detect an expandable set of microRNA targets. Because of the stochastic behavior of single-molecule binding, however, it is challenging for the SMMS to accurately report analyte concentrations in a fixed time window. While multivariate analysis has been shown to rectify background noise due to stochastic nature of single-molecule probes, a template containing an array of sensing units has shown ensemble average behaviors to address the same problem. In this so-called *ensemble single-molecule sensing*, collective mechanical transitions of many sensing units occur in the SMMS sensing probes, which allows accurate quantification of analytes. For the SMMS to function as a viable sensing approach readily adopted by biosensing communities, the future of the SMMS technique relies on the reduction in the complexity and cost of instrumentation to report mechanical signals. In this account, we first explain the mechanism and main features of the SMMS. We then specify basic elements employed in SMMS. Using DNA as an exemplary SMMS template, we further summarize different types of SMMS which present multiplexing capability and increased throughput. Finally, recent efforts to develop simple and affordable high throughput methods for force generation and measurement are discussed in this Account for potential usage in the mechanochemical sensing.



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novel single molecule topochemical analysis was used to detect miRNAs with subpicomolar detection limit.

1. INTRODUCTION

Molecular detection with high sensitivity and specificity is much desired in various areas including disease diagnosis, forensic analysis, and environmental monitoring. Biosensors using enzyme-linked immunosorbent assay (ELISA) and polymerase chain reactions (PCR) are widely used to detect molecules.[5−7] The World Health Organization (WHO) has used the ASSURED criteria, affordable, sensitive, specific, user-friendly, rapid and robust, equipment-free, and deliverable to end-users, as benchmarks for diagnostic tests.[8] We feel the same criteria can be adopted for ideal biosensors.[9] While the sensitivity and specificity describe the technical necessity of a biosensor, the rest features (affordable, user-friendly, rapid and robust, equipment-free, and deliverable to end-users) with an additional requirement of multiplex tasking can be summarized as the usability of the biosensor. Most biosensing devices use optical[10] and electrochemical[11] signals to report analytes. While electrochemical biosensors use redox-active mediators that may interfere with molecular recognition,[12] optical signals such as fluorescence often require organic synthesis and special instruments with increased complexity and cost.[13] Therefore, biosensors employing signals with improved *sensitivity*, *specificity*, and *usability* need to be developed.

Mechanical signals in mechanochemical sensing have shown these promising potentials and proved their complementarity to the optical and electrochemical signals.[14] Mechanochemical sensing uses mechanochemistry principles to detect chemical analytes. Mechanochemistry is an emerging discipline that investigates chemical processes from mechanical perspectives.[15] Its occurrence in nature has been summarized under the label of mechanobiology.[16,17] Mechanochemical sensing can be traced back to the work[18] in which change in surface stress of cantilevers was explored to follow hybridization of DNA strands. Later, the uptake of glucose in living cells was determined by the deflection pattern of an atomic force microscope (AFM) cantilever.[19] To induce detectable mechanical signals, enough chemical binding events should take place, limiting the sensitivity of the methods. We reasoned that the amount or size of detectable analytes scales with the mass or size of a sensing template:[14] the smaller the sensing template, the better the detecting sensitivity. By reducing the cantilever probe to single-molecule sensing templates, it is expected that individual molecules can be detected in the so-called single-molecule mechanochemical sensing (SMMS).[3]

Apart from the ultimate mass detection limit of individual molecules in SMMS, the response of an SMMS sensing template can be fast. Upon the analyte binding, the mechanical response ensues via the mechanochemical coupling between the chemical binding and the variation in mechanical properties of the template. The principle of using sensing templates to detect individual molecules can be readily extended to other techniques, such as single-molecule fluorescence sensing.[20] However, not all molecules have fluorescence signals, nor can they vary fluorescence upon analyte binding. In contrast, mechanical properties, such as tension and extension, have ubiquitous presence ranging from chemical bonds to macromolecular assemblies, which portend the wide applicability of the SMMS.

This Account focuses on our efforts in SMMS research using optical tweezers. Via selected examples, we establish that the SMMS not only renders ultimate single-molecule sensitivity but also offers strong specificity after incorporating recognition elements with high affinity and polyvalent arrangement. The *usability* of SMMS is then demonstrated by its small size and prudent use of materials, as well as its multipurpose units for multiplexing and high-throughput capabilities. For the SMMS to function as a viable approach acceptable to the biosensing community, it is necessary to reduce the complexity and cost of instrumentation. Recent efforts to generate force in affordable instruments and high-throughput force measurement at the ensemble level are discussed at the end of this Account.

2. BASIC ELEMENTS IN SINGLE-MOLECULE MECHANOCHEMICAL SENSING (SMMS)

Biosensing consists of two major steps, analyte binding and signal processing.[12] Whereas analyte binding determines the sensing specificity by selective recognition events, signal processing improves detection limits via amplifications. The main feature of SMMS is that signal amplifications can be omitted due to the ultrasensitive detection of single molecules, which is achieved by the following two elements (Figure 1).

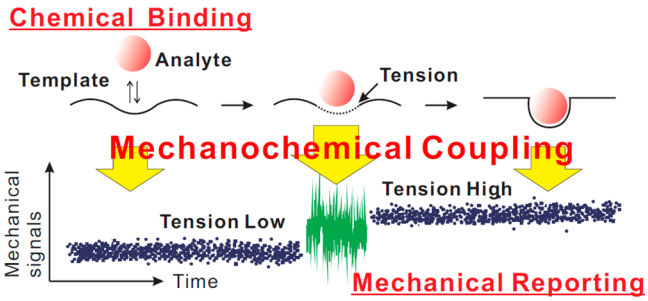

Figure 1. Transduction of chemical energy of analyte (orange sphere) binding to mechanical energy of the sensing template (black trace) via mechanochemical coupling. Left: Sensing probe in a relaxed state before the analyte binding. Middle: Analyte touches the sensing unit, causing the unit to vibrate (lower middle). Right: Analyte is tightly bound in the sensing unit, increasing the tension of the probe by rearranging its conformation.

2.1. Single-Molecule Probes for Chemical Binding

Single-molecule detection has been demonstrated by techniques such as fluorescence[21] and force spectroscopies.[22] The single-molecule sensitivity requires careful experimental design to reduce background noise. As sensor surfaces may compromise individual binding events in sensing, the recognition element should be placed away from the surface. This can be achieved by using biopolymer spacers, such as polysaccharide[23] or DNA molecules, to isolate the probes. Because of its cost-effectiveness and precisely defined sequence that can extend over several micrometers when stretched, DNA becomes a material of choice to serve as both sensing probes and isolating spacers.

The versatility of DNA structures, such as hairpins, H-DNA (a triplex[24]), and tetraplexes, including G-quadruplex[25] and i-motifs,[26] allows DNA to recognize a variety of analytes. Screened by in vitro selection method, SELEX,[27] these secondary structures are also involved in DNA aptamers to bind analytes with nanomolar K_d, achieving high specificities.

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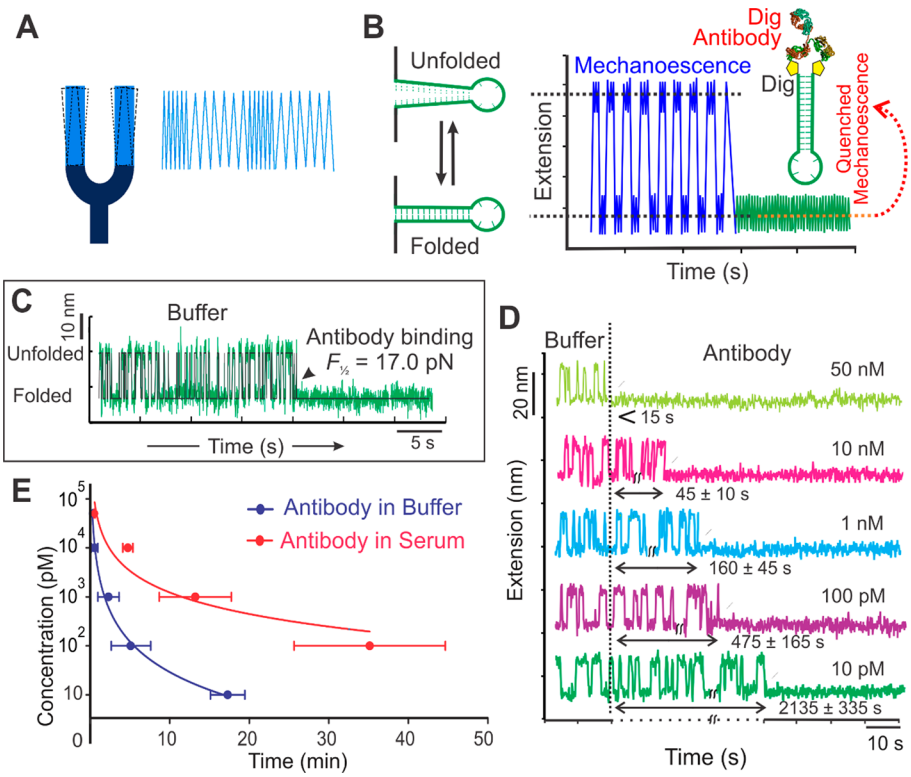

Figure 2. Molecular tuning fork for antibody detections. (A) Tuning fork with its prongs vibrating at two frequencies. (B) DNA hairpin serves as a molecular tuning fork. Binding of the digoxigenin (Dig) antibody (yellow diamond) locks the hairpin, stopping its hopping. (C) Hopping of the hairpin probe without (left) and with (arrow) the Dig antibody in a 10 mM Tris buffer with 100 mM K^+ at pH 7.4. The sensing probe is maintained at 17 pN force. (D) Real-time detection of the Dig antibody in the Tris buffer. Addition of the Dig antibody is marked by the dotted line. (E) Semilog plot of the antibody concentration versus detection time in the Tris buffer (blue) or serum (red). Reproduced with permission from ref 1. Copyright 2015 John Wiley and Sons.

Table 1. Characteristics of Different SMMS Methods

sensing probe	detection limits	dynamic ranges	specificities	advantages	disadvantages
Molecular tuning fork design[1]	10 pM (buffer, 30 min)	10 pM−50 nM	Antibody	fast detection	complicated preparation
	100 pM (serum, 35 min)			flexible design	materials inefficiency
Single DNA hairpin[2]	100 pM (30 min)	100 pM−1 μM	DNA or miRNAs	fast detection	complicated preparation
				flexible design	materials inefficiency
Multivalent design[34]	1 fM (20 min)	1 fM−100 pM	Hg(II)	fast detection	complicated preparation
					materials inefficiency
Poly(T) repeats[40]	3 pM (20 min)	3 pM−10 μM	Melamine	fast detection	multistep preparation (RCA)
				high-throughput material efficiency	
DNA origami[3]	10 pM (10 min)	10 pM−50 nM	PDGF	fast detection	low/medium throughput
				multiplexing	material inefficiency
Multiple DNA hairpins[43]	1 fM (20 min)	1 fM−100 nM	DNA or miRNAs	high throughput	multistep preparation (RCA)
				multiplexing	
				fast detection	
Topochemical design[4]	100 fM (buffer)	100 fM−50 nM	DNA or miRNAs	multiplexing	complicated preparation
	1 pM (serum) (25 min)			fast detection	
DNA hairpin with multivariate reporters[44]	0.1 pH unit	5.0−7.4	pH	10 nm spatial resolution	materials inefficiency
				precise and accurate measurement	limited pH range
					slow detection

2.2. Mechanical Properties as Reporting Signals (Mechanical Reporting)

2.2.1. Tension and Extension. Mechanical properties include tension and extension, as well as elasticity, pliability, and plasticity,[28] among others. Compared to electric signals or photons, mechanical signals have reduced background noise especially when the system is isolated from environment by optical traps for example. In addition, tension and extension are universal variables for almost all materials. These two factors have made mechanical signals widely applicable with

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high signal-to-noise ratios. Most of the SMMS use force and extension as reporting signals. In a typical experiment, an SMMS template can be mechanically extended, resulting in increased tension of the template.

2.2.2. Mechanical Hopping. Like a tuning fork that resonates at a constant pitch, a molecular tuning fork can undergo transitions between folded and unfolded states of a DNA hairpin ("hopping", see Figure 2).[1] Because of the stochastic nature at the single-molecule level, the folding and unfolding occur randomly. However, the average of the folding or unfolding rate constant should be constant in a sufficiently long time. When a molecular tuning fork contacts an analyte, it changes its hopping transition. Similar to the fluorescence emitted by a fluorophore, the hopping transition by a mechanophore[15] such as DNA hairpin is called mechanoescence.[1] Mechanoescence can reveal structural, thermodynamic, and kinetic information on specific chemical and biochemical processes with little environmental interference.

To test the efficiency of using the hopping transition to report binding events, two digoxigenin molecules were attached to the two ends of a DNA hairpin stem. The binding of the digoxigenin antibody to either of the digoxigenin drastically changed the hopping pattern of the hairpin due to the proximity effect when the antibody is located close to the DNA hairpin. When an antibody bound to both digoxigenins, hopping ceased as the two strands in the hairpin stem were locked by the antibody (Figure 2). This molecular tuning fork allowed to detect 100 pM antibody in human serum around 30 min (Figure 2, see Table 1 for summary).

2.3. Mechanochemical Coupling

Chemical binding of an analyte to the DNA probe can be transformed to a mechanical signal through mechanochemical coupling (Figure 1). Mechanochemical coupling has been harnessed in cellular processes such as replication, transcription, and translation,[29] in which the chemical energy stored in molecules, such as ATP, is converted to the mechanical energy that drives motor proteins such as DNA/RNA polymerases and ribosomes. These motor proteins move along DNA/RNA templates while incorporating monomers to elongate newly synthesized biopolymer strands. The process is a mimic of a locomotive moving along the railway track (a form of mechanical energy) by burning coals, which releases chemical energy. In the sensing, the chemical energy released by binding of an analyte to the DNA template is utilized to change the conformation of the template, which generates detectable mechanical signals.

3. PREPARATION OF SINGLE-MOLECULE TEMPLATES

As discussed in section 2, to avoid detrimental surface effect on single-molecule sensing, biopolymer spacers such as DNA have been used to isolate sensing probes. In single-molecule biophysics, duplex DNA strands serve as handles to tether biomolecules.[30] To be easily captured by the optical tweezers, these DNA handles were anchored to optically captured particles through affinity interactions or covalent linkages.[31] By replacing biomolecules with analyte recognition units, these constructs become SMMS templates. To ensure sensing specificity, recognition units should selectively bind analytes. These recognition elements can be made of DNA, RNA, peptide, or their analogues. To be compatible with DNA spacers, however, DNA has been used as recognition elements sandwiched between two dsDNA handles in SMMS templates, which are then tethered between two optically captured particles to monitor changes in mechanical properties (tension, extension, or hopping) of the template upon analyte binding. With an appropriately adjusted concentration, a single DNA polymer can be tethered between two optically captured particles with a 100% success rate (Figure 3).

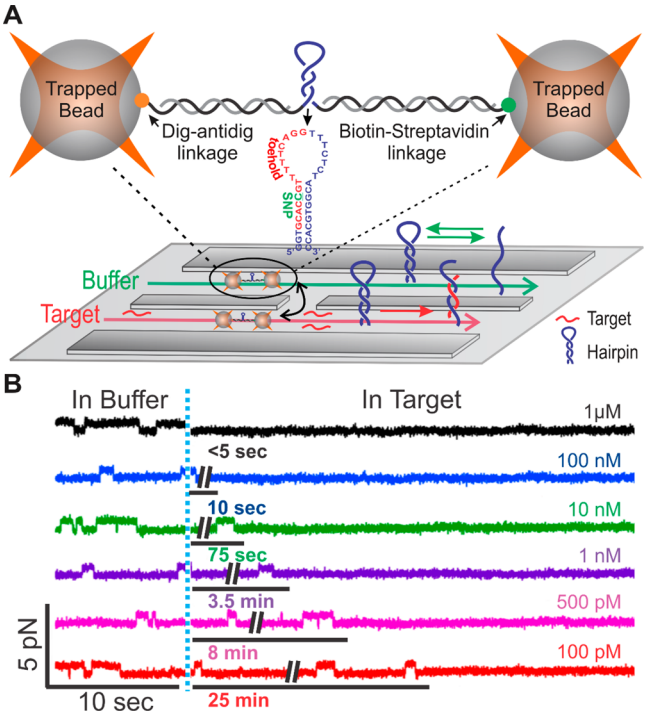

Figure 3. Single-Nucleotide Polymorphysim (SNP) detection by using a DNA hairpin. (A) Hairpin probe is tethered between two optically trapped beads. The hairpin loop serves as the toehold to bind to an SNP fragment. The sensing template is transported between buffer and target microchannels. (B) Hopping traces for the hairpin probe at different concentrations of SNP targets. The vertical dotted line indicates the transfer of the probe from the buffer to the target channel. Average time is marked before hopping ceases into unfolded hairpin because of the binding of the SNP target. Reproduced with permission from ref 2. Copyright 2011 American Chemical Society.

3.1. General Preparation Scheme for Single-Molecule Sensing Templates

Such a preparation process is illustrated in a single-unit SMMS template to detect single-nucleotide polymorphism (SNP) (Figure 3),[2] which is a common genetic variation involved in many human diseases.[32] In this SMMS (Figure 3A), a tethered DNA hairpin serves as an SNP recognition unit.[2] Part of the hairpin loop works as a toehold[33] to bind to the SNP fragment by Watson–Crick hybridization. In addition, part of the hairpin stem close to the loop will bind to the rest of the SNP fragment. The toehold binding of the SNP fragment triggers its invading into the hairpin stem, generating mechanical signals corresponding to the unfolding of the hairpin.

Without addition of an SNP fragment, the hairpin hops between folded and unfolded states (bistate hopping, Figure 3B) at a fixed force. After the SNP target was bound, the hairpin ceased the hopping into the unfolded state. This SMMS platform detected the SNP target as low as 100 pM without using any amplification in 30 min. However, the

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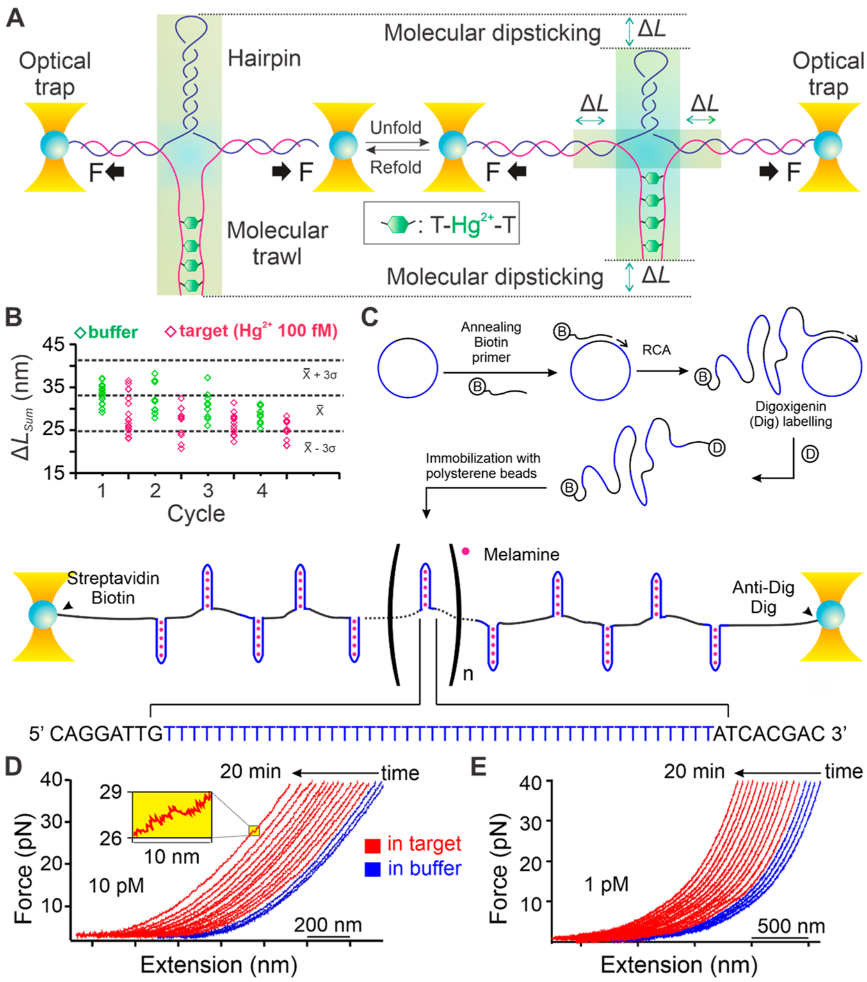

Figure 4. SMMS templates with multiple sensing units. (A) SMARTS device contains a molecular trawl with multiple recognition elements (thymines) in each of the two ssDNA strands, which serve as two pincers to catch analytes (green hexagons, Hg^{2+}). Two top DNA hairpins serve to report the amount of bound analytes via mechanical unfolding experiments. (B) Recycling of the poly$(T)_{10}$ probe between the Tris buffer channel and 1 nM Hg^{2+} channel demonstrates reversible sensing. (C) Schematic of the RCA to prepare poly$(T)_{44}$ repeats flanked by two 8-nt spacers. Unfolding F−X curves at 10 pM (D) and 1 pM (E) melamine (MA) in a 10 mM Tris buffer (pH 7.4) with 100 mM KCl. Reproduced with permission from refs 34, 40. Copyright 2016 American Chemical Society and Copyright 2020 Springer Nature.

throughput of this SMMS strategy was limited because each sensing template contained only one probing unit to recognize only one analyte (see Table 1 for summary).

3.2. Single-Molecule Sensing Templates with Increased Throughput

3.2.1. Polyvalent Design. The detection throughput can be improved by introducing tandem recognition units in the single-molecule template. Mandal et al. constructed single-molecule mechanoanalytical real-time sensing (SMARTS) devices that contained multiple units for Hg^{2+} binding[34] (Figure 4A, Table 1). These poly thymines (poly(T)) were arranged in a molecular trawl format, which worked like pincers to catch Hg^{2+} ions by forming T−Hg^{2+}−T pairs. The molecular trawl served as a molecular dipstick to report the binding Hg^{2+} via reduced change-in-contour-length (ΔL) values during mechanical unfolding experiments. Within 20 min, the detection limit of 1 fM (2×10^{-4} ppt) Hg^{2+} was achieved, which is 2 orders of magnitude lower than the best reported Hg^{2+} detection method.[35] The polyvalent binding between thymines and Hg^{2+} allowed a detection limit of 9 orders of magnitude lower than the K_d for Hg^{2+} binding.

3.2.2. Sensing Probes with Atom Economy Design. DNA handles have been used to isolate the sensing template from the surface, which can be detrimental to the binding events between analyte and sensing units. However, the long handles reduce the efficiency in materials usage as most of the handles do not contribute directly to the analytie recognition. Similar to the Atom Economy concept in Green Chemistry that represents the percentage of the product mass over the mass of all reactants,[36] we propose the atom economy concept in sensing by calculating the mass percentage of the recognition units over the entire sensor or the sensing process. In one of the most used binding materials, IgG antibody, the atom economy is merely ∼1.1% (paratope 15 amino acids; M_w for IgG, 150 kDa[37]). This is because the paratope binding unit only constitutes a fraction of the entire antibody, most of which serves for a structural purpose. For a typical sandwich immunoassay in which signal amplification is not considered, the atom economy is about half of this level (0.55%). For the ELISA using enzyme catalyzed reactions for amplifications, this value decreases to 0.36%, assuming the same molar mass between the reporting enzyme and the recognition antibody. If amplification chemicals are accounted, this atom economy is

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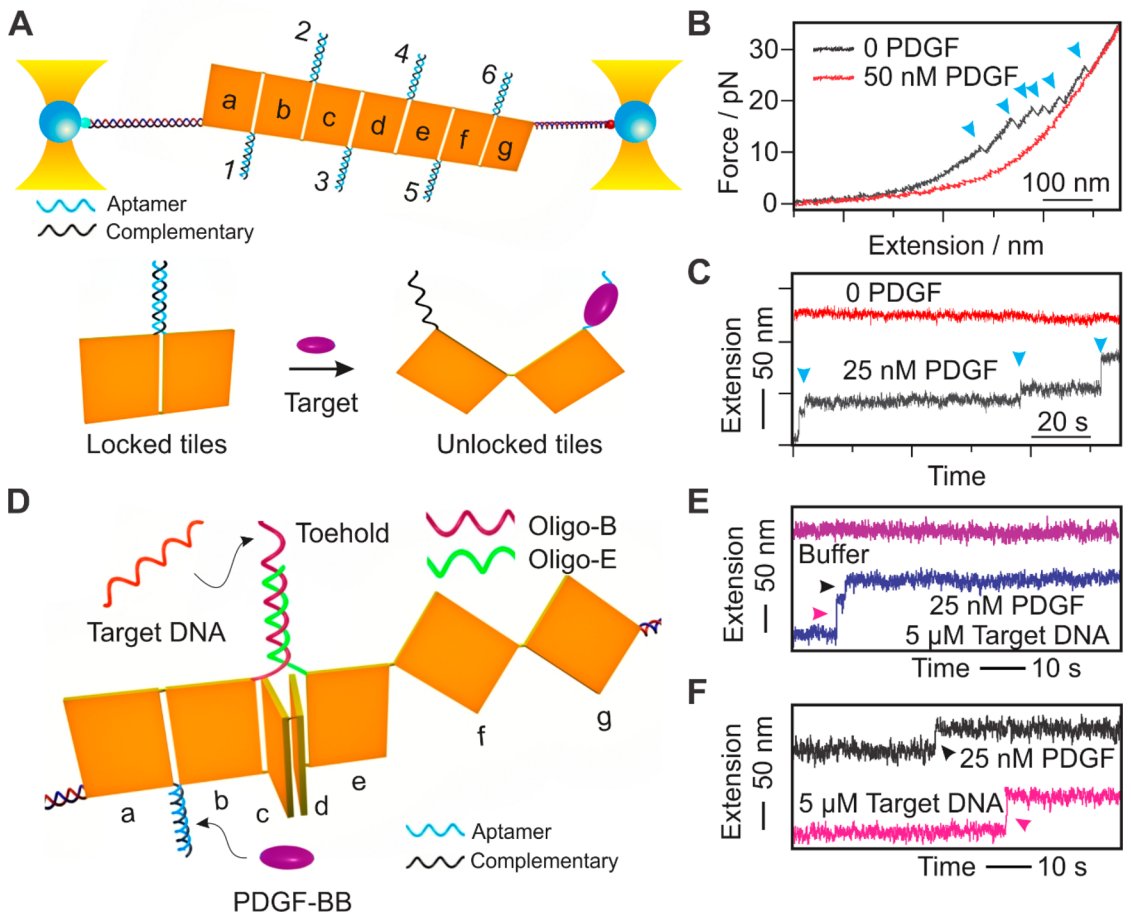

Figure 5. Sensing using 2D and 3D origami nanostructures. (A) Seven-tile 2D DNA origami nanoassembly is tethered between two optically trapped beads through dsDNA handles. Each origami tile (marked a–g) has a dimension of 39.5 × 27 nm^2. Adjacent tiles are locked (marked 1–6) by an aptamer DNA (gray) and its complementary strand (black). (B) Representative F-X curves of the 7-tile DNA nanoassembly without (gray) and with (black) 50 nM PDGF. (C) Real-time sensing strategy with a constant force of 8 pN. (D) 3D origami nanoassembly for multiple targets. The lock between tiles a and b contains a PDGF aptamer whereas that between tiles b and e contains a toehold DNA strand. All other tiles remain unlocked. (E) When both targets were present, two extension jumps reflecting the breaks of the two locks were observed. (F) In a solution with only one target, one extension jump was observed. Arrow heads depict transitions. Reproduced with permission from ref 3. Copyright 2014 John Wiley and Sons.

further reduced dependent on amplification cycles. DNA based probes have significantly better atom economy. In molecular beacon strategy for example,[38] the atom economy is above 30%, ~2 orders of magnitude improvement compared to the antibody binding. Single-molecule sensing, such as SMMS, has inherently better atom economy since amplification may not be required. However, for the SMMS strategy that contains only one probing unit (~20 nt DNA) sandwiched between long DNA handles (~2000 nt), the atom economy is around 1%.

To increase the atom economy, we introduced recognition units throughout the entire sensing template by rolling circle amplification (RCA[39]). This approach has been demonstrated in the sensing of melamine[40] (MA) by forming T-MA-T triplet pairs (Figure 4C, Table 1). First, the RCA-synthesized DNA construct with many copies of poly(T)$_{44}$ were interspersed with 16-nt spacers with an atom economy of 73%. The DNA construct was, then, anchored between two optically trapped beads via affinity linkages, while MA was added to the microfluidic chamber. As low as 3 pM melamine was detected in 20 min in this SMMS platform, which is a 1000-fold improvement compared to previous melamine sensing.[41,42] These findings provided convincing evidence that SMMS can detect low concentrations of chemicals while maintaining a high atom economy in sensing.

4. MULTIPLEXING CAPABILITY

By detecting many different analytes each time, multiplex sensing increases detection throughput, while providing more complete and accurate results. For example, in the COVID-19 (SARS-CoV-2) diagnosis,[9] false positives or false negatives will be reduced if both nucleic acids and proteins from the virus can be evaluated. In addition to these, if anti-SARS-CoV-2 antibody can be assessed at the same time, the infection history of a patient can be revealed.[45]

The multiplex sensing requires prudent incorporation of multiple recognition units in a template with limited size such as the SMMS sensor. The atom economy of 74% in the RCA template for melamine detection in section 3.2.2 represents the highest materials efficiency among known biosensors. RCA, however, works best when a circular template contains only one repeating unit. This is because multiple repeating units need larger circular DNA to accommodate, which becomes difficult to circularize because of the side reactions, such as

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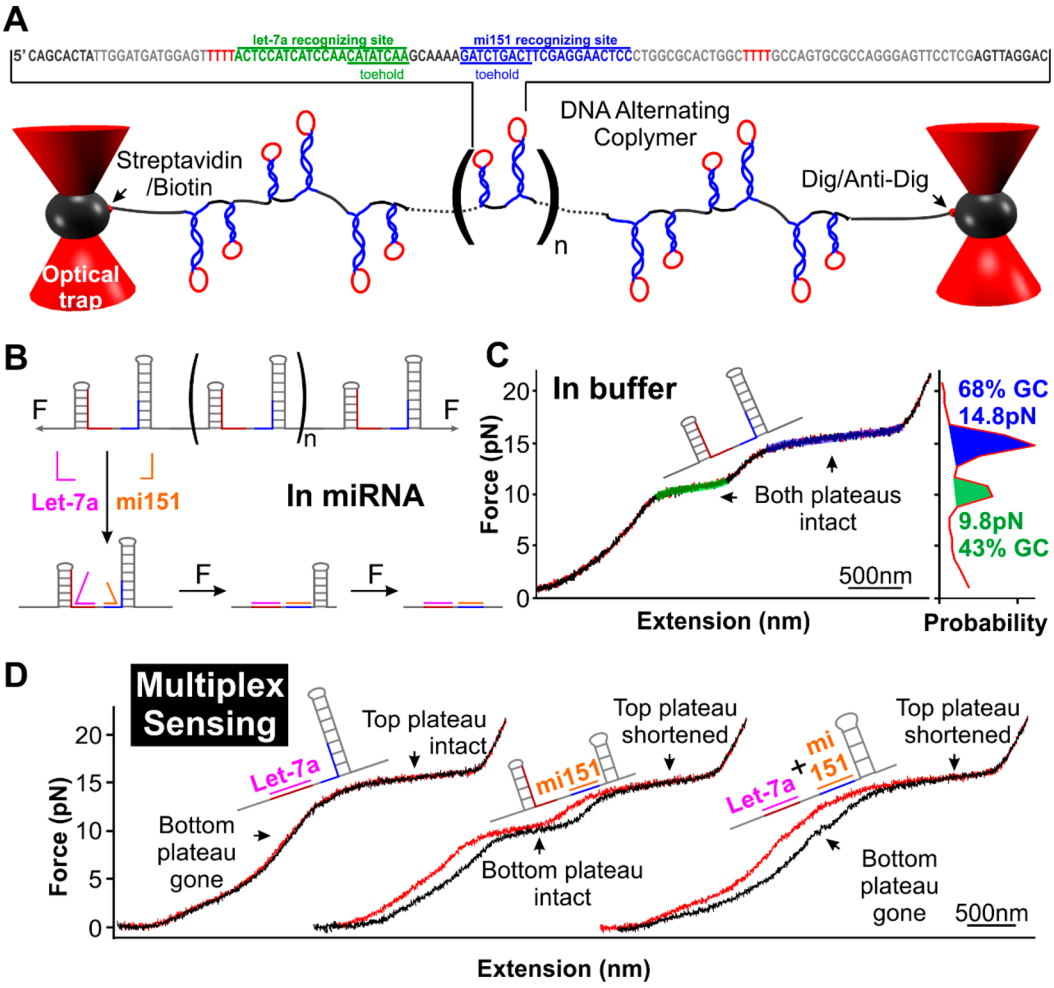

Figure 6. Multiplex SMMS by alternating DNA copolymers. (A) Schematic of sensing for two miRNA targets. Recognizing sequences are shown at the top. Toehold regions are marked for each miRNA. (B) Bindings of the Let-7a (pink) and mi151 (orange) miRNAs to the colored sites in hairpins, which lead to changes in the mechanical stability of the hairpins. (C) F-X curves of the copolymer in buffer. Unfolding force plateaus for the hairpins with low (43%) and high (68%) GC contents are plotted in force histograms (right). (D) F-X curves in the presence of either one or both miRNAs. Reproduced with permission from ref 43. Copyright 2020 American Chemical Society.

dimerization and oligomerization. For multiplex sensing in which different recognition units are required, this task can be accomplished when templates with larger frameworks, such as DNA origami[46] and DNA copolymer templates, are used. Alternatively, innovative coding schemes involving both spatial and temporal factors can be explored to achieve this goal.

4.1. SMMS Using DNA Origami Templates

Invented by Rothemund in 2006,[46] DNA origami is made of a long ssDNA template self-assembled into a desired topology by strategically hybridizing short DNA staples at different locations. The technique catapults one-dimensional DNA to 2D or 3D materials. Therefore, DNA origami can serve as an expanded framework onto which multiple recognition units are placed. In an example demonstrated by Koirala et al. (Figure 5, Table 1),[3] two neighboring tiles in a 7-title DNA origami construct were connected with a dsDNA bridge. Since an aptamer for platelet-derived growth factor (PDGF) was contained in one of the bridging strands, binding of the PDGF to the aptamer compromised the dsDNA bridge and separated connected tiles. Without PDGF, typical force–extension (F-X) curves showed 6 unfolding features corresponding to the separation of 7 tiles (Figure 5B). With 50 nM PDGF, no unfolding events were observed, indicating a fast PDGF binding to separate tiles. When 8 pN force was maintained in the template, real-time variations in extension were observed in 20 nM PDGF buffer channel (Figure 5C). Compared to the 100 pM detection limit in the single-unit sensing template within 30 min (Figure 3), the detection limit was reduced to 10 pM within 10 min in this origami template.[3]

To demonstrate the multiplex sensing, a 3D DNA origami nanostructure with multiple detection probes was synthesized (Figure 5D). Tiles A and B were locked by the same PDGF aptamer containing dsDNA bridge. Tiles B and E were connected by another dsDNA bridge, which contained a toehold region to recognize a DNA target. Real-time detection was performed by monitoring extension over time at a constant tension (8 pN, Figure 5E and F). Two extension jumps were observed that respectively corresponded to the disassembly's of the two bridges. By adopting special bridging arrangements in expandable 3D DNA origami sensing templates, this strategy can differentiate binding targets through the magnitude of extension jumps.

4.2. Ensemble Single-Molecule Sensing Using DNA Copolymers

Another strategy to incorporate multiple sensing units is to prepare a circular DNA which contains more than one

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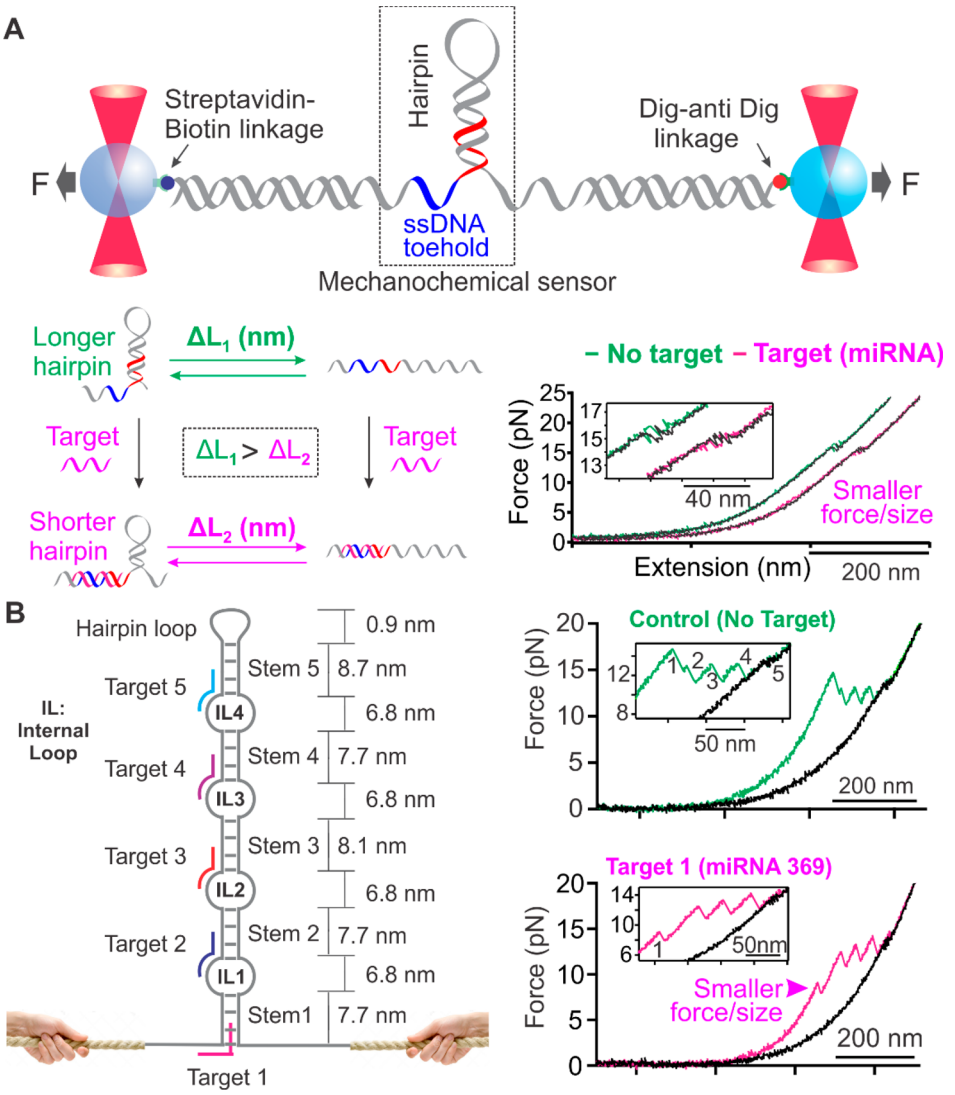

Figure 7. Topochemical sensing. (A) Sensing mechanism of one miRNA in laser tweezers. The miRNA target first binds to the ssDNA toehold region (blue) and invades to the DNA hairpin stem (red). Representative F-X curves in the Tris buffer without (green, bigger ΔL_1) and with (pink, smaller ΔL_2) 100 nM miRNA Let 7a. (B) Topochemical sensor to recognize 5 miRNA targets. Target 1 (pink) binds with the toehold at the base of the probe and invades into stem 1. Targets 2−4 bind with corresponding internal loop toeholds and half of the adjacent stems. Without a target, the DNA construct produces five well-separated unfolding features (top right). With target 1, both RF and ΔL of the first feature are expected to be smaller (bottom right). Reproduced with permission from ref 4. Copyright 2019 American Chemical Society.

recognition unit. The circular DNA then serves as a template from which multiple repeats of these recognition units are synthesized by RCA (see Figure 4C). We call these materials structural or functional DNA copolymers, which achieve multiplex sensing with high atom economy. To demonstrate this method, Jonchhe and co-workers first prepared a DNA copolymer that contains a tandem array of DNA structures (hairpins) by RCA.[43] It was found that as few as 10 tandem DNA hairpins were needed to show ensemble unfolding behaviors in which unfolding of one hairpin was indistinguishable from others. Using these so-called ensemble single-molecule behaviors, 1 fM miRNAs were detected in 10% serum solution within 20 min, which was about 6 orders of magnitude better than the detection limit of the single-hairpin SMMS sensing template.

By placing two different miRNAs (Let-7a and mi151 miRNA) recognizing units in a circular DNA template, alternating DNA copolymers were prepared by RCA (Figure 6A and B). Without any miRNA targets, two plateaus were shown in F-X curves, corresponding to the collective (ensemble) folding/unfolding of two different hairpins, respectively. In the presence of only one miRNA, either the 10 pN Let-7a plateaus disappeared or the ∼14−15 pN relaxing plateau for the mi151 became shortened (see Table 1). Both variations were observed when both miRNAs were added. The results from alternating DNA copolymer templates demonstrated that design of copolymers, such as structural DNA block copolymer, is a viable approach to expand the multiplexed sensing capability.

The multiple recognition units in a sensing template can also address the stochasticity problem in the sensing. Because of the stochastic nature, binding of an analyte to the recognition unit in a sensing template occurs randomly at any given time, which may bring false negative results. By incorporating many identical recognition units, the likelihood of analyte binding

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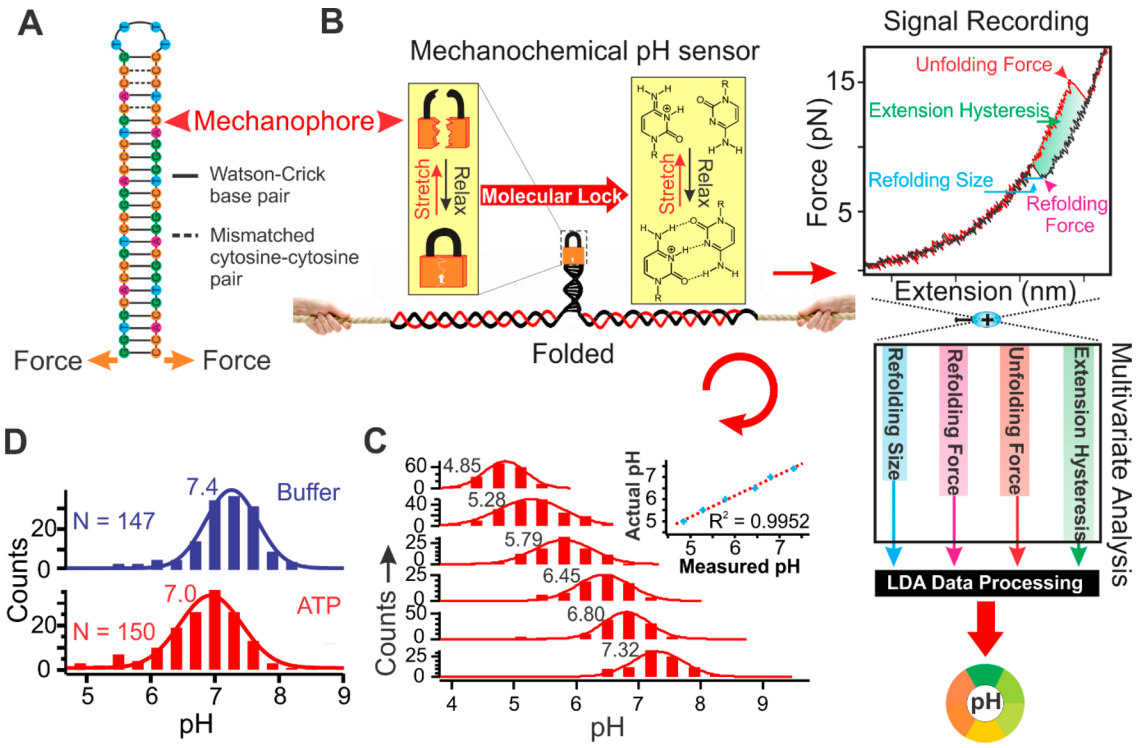

Figure 8. Multivariate pH sensing. (A) Mechanophore with three pairs of cytosine (C)-cytosine bases in a DNA hairpin. At low pH, the hemiprotonated C−C pairs strengthen the DNA hairpin. (B) Mechanical properties (top right) of the DNA hairpin mechanophore vary with pH. These properties are analyzed by multivariate analyses LDA to produce pH measurement (bottom right). (C) Histograms of measured pH in different buffers (pH 5.0−7.4). Inset shows excellent correlation between measurement with actual pH. (D) pH profiles with (red) and without (blue) ATP obtained by the SMMA method ~10 nm away from an alkaline phosphatase. Reproduced with permission from ref 44. Copyright 2017 American Chemical Society.

increases at a particular moment, rendering more accurate results.

4.3. Spatiotemporal Coding to Expand Multiplexity: Topochemical Sensing Strategy

Spatial arrangement of multiple sensing units in an SMMS template can be drastically expanded into time domain by sequential interrogation of these sensing units. This spatiotemporal coding-decoding strategy has been called as topochemical analyses[4] to reflect its position-dependent binding and dissociation processes.

To demonstrate this principle, a template consisting of a DNA hairpin with a toehold for miRNA recognition was prepared (Figure 7A). Binding of the miRNA led to a smaller change-in-contour-length (ΔL) or decreased rupture force (RF). To recognize more targets, internal loops were used to dissect the hairpin into multiple segments to report corresponding RF and ΔL. Each internal loop contained a toehold region for a particular miRNA target. Figure 7B showed detection of five different miRNAs. Binding of a particular miRNA reduced the RF and ΔL of the corresponding hairpin segment. The sequential unfolding pattern pinpointed the location of each hairpin segment. The variation in RF and ΔL for a particular segment was used to indicate whether or not a corresponding miRNA was bound to that segment. Because of the similarity of such a strategy to the topochemical reaction,[47] we called this method topochemical sensing (see Table 1). In principle, this spatiotemporal topochemical sensing can be expanded to detect a massive set of miRNA targets by adding a corresponding array of internal loops into a hairpin. This spatiotemporal sensing detected five miRNAs in the proof-of-concept experiments.[4]

4.4. Single-Molecule Multivariate Analyses (SMMA)

Since different analytes can be detected, multiplex sensing renders a more complete and accurate picture for targets. To achieve detection accuracy, multivariate analyses on different sensing observables can also be performed. Such a multivariate analysis in SMMS has been demonstrated in a sensor,[44] in which environmental pH is deduced from mechanical properties of a pH sensitive mechanophore. These mechanical signals were collected in SMMS and analyzed using chemometric methods. First, three hemiprotonated cytosine(C)−C pairs[48] were introduced as pH sensitive mechanophores in a hairpin stem (Figure 8A). During mechanical unfolding of this hairpin, four sensing signals (extension hysteresis(Δx), unfolding force, refolding force, and refolding size) were analyzed by the multivariate linear discriminate analysis (LDA[49]) to deduce the pH (Figure 8B). In addition, three signals (the change-in-contour-length during cooperative refolding ($\Delta L_{refolding}$), extension hysteresis (Δx), and the difference between the unfolding and refolding forces (ΔF)) were used in a training set at pH 5.0−7.4. For a balance of accuracy and easy visualization, two principal factors (factors 1 and 2 in the Principle Component Analysis[49]) were used to construct a 3D pH calibration plot. The pH detected by this single-molecular multivariate analysis (SMMA) correlated rather well (R^2 = 0.997) with the actual pH (Figure 8C). Because of the nanometer size of this SMMS pH meter, it allowed to measure a decrease of 0.4 pH unit ~10 nm away from an active alkaline phosphatase (see Table 1 and Figure

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8D). Such superior spatial resolution and sensitivity overshadowed existing pH sensors.

5. CONCLUSION AND PROSPECT

In summary, by using mechanochemical coupling principles, single-molecule mechanochemical sensing (SMMS) converts chemical recognition events to mechanical signals, which offers real-time analyte detections. In traditional ensemble sensing formats, sensitivity has been achieved via chemical or electrical amplifications, which are materials intensive and time-consuming. Single-molecule sensitivity eliminates these extra amplification steps, considerably reducing materials consumption while reducing the size of sensing templates. A Green Chemistry concept, Atom Economy, has been proposed in this Account to describe the efficiency of a sensing practice (Atom Economy Sensing). By placing many probing units throughout sensing templates, SMMS has shown orders of magnitude better efficiency in the materials usage with respect to the bulk sensing.

In many advanced biosensors, multiplex tasking is essential to rapidly and reliably profile different analytes. The minute recognition units in SMMS templates facilitate the accommodation of an array of these units. Both recognition elements and sensing templates can be made of DNA materials, which have been rapidly burgeoning recently.[46,50−52] We have proposed in this Account a new material, structural DNA copolymer, to serve as SMMS templates with multiplexing capabilities. The single-stranded DNA templates employed in SMMS are quarter-copolymers[53] because they contain four deoxynucleotide monomer units, A (adenosine), T (thymidine), C (cytidine), and G (guanosine). The RCA product amplified from a circular DNA template is a periodic copolymer with many repeating segments. Secondary structures such as hairpins and quadruplexes form within these repeats. We call these DNA (quater) copolymers that contain DNA secondary structures as *structural DNA copolymers*, which contain secondary structures as the basic units instead of monomer units in regular copolymers. When different secondary structures (A, B, or C) are arranged in an alternating fashion, that is, $(ABC)_n$, we call it as an *alternating DNA copolymer* (Figure 6). Likewise, when the same secondary structures are clustered together in blocks, that is, $(A)_m(B)_n(C)_o$, we call it as *structural (tri) block DNA copolymer*. Previously, the name of DNA block copolymer has been used to refer to the polymers that contain a DNA section as one of the blocks in block copolymers, whereas other blocks are made of non-DNA materials.[54] Our structural DNA copolymers are exclusively made of DNA. This concept can be expanded to prepare copolymers made of other structural units such as RNAs or proteins.

The SMMS probes also facilitate the topochemical arrangement of different recognition units. By placing these units in a spatiotemporally addressable fashion, single-molecule *topochemical sensors* have been demonstrated to detect multiple microRNA targets. Because of the low throughput and stochastic behavior of single-molecule binding events, it is often difficult for the SMMS to accurately report analyte concentrations in a short time. While multivariate analysis has shown to rectify background noise due to the stochastic nature of single-molecule probes, an array of recognition units in sensing templates can behave collectively, improving the accuracy by averaging stochastic noises. Similar idea has been employed in the construction of single-molecule clocks as a result of serial chemical reactions.[55] We called such a detection as *ensemble single-molecule sensing*, which combines the single-molecule sensitivity with accurate measurement in ensemble assays.

Although DNA origami, structural DNA copolymers, and topochemical arrangement have been used to improve the throughput and multiplexing capabilities in the SMMS, the complicated instrumentation and cost of the sensing templates become concerns. Recently, Wong group has applied centrifugal forces on hundreds of single-molecules simultaneously.[56,57] In addition, Hu and co-workers have invented a shear flow platform to investigate mechanical unfolding of a massive set of molecules.[58] Up to 50 pN force was applied on an i-motif DNA structure. Gratifyingly, the shear force increased with the size of the analyte molecules, suggesting that higher shear force can be generated by attaching elongated or bigger handles (e.g., polymers or nanoparticles) to the analyte. This novel shear force platform can transform single-molecule force spectroscopy into an *ensemble force spectroscopy*, which serves as a future development for the SMMS.

Despite the advantages and promising future of the single molecule mechanochemical sensing, efforts are needed to expand its *usability*. First, although DNA is stable and easy to work with, it only contains four nucleotides with a limited chemical space. This feature reduces the binding capacity (affinity and binding kinetics) of the DNA. To increase the chemical diversity, non-natural DNA has been used.[59] However, these non-natural DNAs may not be compatible with current molecular biology tools. Second, although single-molecule sensitivity in SMMS does not require amplifications, amplification strategies performed on the sensing template is beneficial to improve the signal-to-noise ratio and reduce the concentration detection limit. Third, mechanical signals in SMMS often come from compromised DNA secondary structures upon analyte binding. These turn-off signals are not as sensitive as turn-on mode.[60] DNA secondary structures with increased mechanical force upon ligand binding (turn-on) should be explored in the future for better sensitivity. Finally, the instruments of the SMMS are often expensive and sophisticated, low-cost instruments with high sensitivity and easy usage will make SMMS more accessible to the community. Shear force method using benchtop homogenizers represents a promising direction[58] to reduce the cost. Another approach to achieve high throughput with reduced cost is to perform mechanochemical sensing on DNA chips. As demonstrated by Gaub,[61,62] force can be determined for arrays of DNA on chip. Such a setup can be harnessed for massively parallel detection of analytes. Other affordable approaches are needed for mechanochemical sensing to be used in a household setting.

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Notes

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