Review

Real-Time Single-Polymer Growth towards Single-Monomer Resolution

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Synthetic polymers are heterogeneous; a significant contributor is the asynchronous and nonequilibrium kinetics of multistep polymerization reactions. Understanding the polymerization mechanism and associated kinetic dispersion is important fundamentally and for applications; this necessitates real-time monitoring of the single-chain growth of synthetic polymers, which faces challenges distinct from biopolymerizations for which many single-molecule studies exist. Here, we review the latest advances in single-molecule studies of real-time polymer growth. We describe the principles of available methods, illustrating them with earlier applications in biopolymerizations, and highlight the breakthroughs in studying single synthetic polymers and the unique insights inaccessible from bulk studies. We discuss the strengths and limitations of each method and the challenges and opportunities in the emerging field of single-polymer growth studies.

The Nature of Synthetic Polymers Necessitates Real-Time Single-Chain Level Studies

Synthetic polymers are ubiquitously used in our daily life [1–5]. One salient feature is their heterogeneity: individual polymer molecules differ in length, tacticity, and/or topology; this heterogeneity influences the properties and processability of polymer materials [6]. The heterogeneity of polymer chain length was treated theoretically by Flory ~80 years ago, considering the probabilistic nature of monomer addition kinetics [7], which, however, significantly underestimates the dispersity observed experimentally. Many other aspects of polymerization reactions are now known to contribute to polymer heterogeneity [e.g., the activity of the catalyst(s)] [8,9]. Fundamentally, the heterogeneity of the molecular structure must result from the dispersion of polymerization kinetics among individual polymers when they are synthesized. This dispersion makes it necessary to monitor the growth of polymers at the single-chain level in real time, so as to shed light not only on the differences among individual polymers, but also on how each polymer changes temporally under reaction conditions, which are nonequilibrium in nature.

Advances in single-molecule methods have made it possible to measure the real-time growth and polymerization kinetics of single biopolymers, such as DNA, RNA, and proteins. These methods include: (i) single-channel recording, (ii) single-particle manipulation, and (iii) single-molecule fluorescence. These methods are based on different working principles and observables regarding polymer growth and have provided a unique window into the mechanisms of biopolymer growths, for example, DNA growth, which involves orchestrated actions of many enzyme subunits to perform biopolymerization reactions, typically comprising many intermediate steps (discussed later). The templated synthesis of these biopolymers also provides convenience for single-molecule measurements, where the template can be used for attaching tethers or labels. The stability of biopolymers in aqueous solutions and aerobic conditions is another convenient feature, as compared with synthetic polymers that are often synthesized in organic solvents and using water- and/or air-sensitive catalysts.

Highlights

The heterogeneity of synthetic polymers affects the properties and processability of polymer materials. A key contributor is the asynchronous and nonequilibrium kinetics of multistep polymerization reactions. Real-time monitoring of the single-chain growth of synthetic polymers is needed for understanding the mechanisms and achieving better controls.

Single-molecule techniques are powerful for probing dynamic, asynchronous, and nonequilibrium processes. Their application to polymerization reactions of synthetic polymers remains challenging, however.

Here, we introduce single-molecule techniques that have been used to visualize single biopolymer growth, review latest breakthroughs in studying single synthetic polymer growth, and highlight the unique insights inaccessible from bulk studies; we also discuss the challenges and opportunities in this emerging field.
Real-time single-chain-level information on synthetic polymers is also highly desirable. This information can provide insights critical for optimizing reaction conditions to make polymer materials with improved properties. For example, how does the conformation of a growing polymer chain affect its polymerization kinetics in situ? How are the sequence, tacticity, and mechanics of individual chains related to the crystallinity and mechanical properties of bulk polymer materials? Bulk-level measurements, which predominantly assess the average physicochemical properties, have provided many insights here, but real-time single-chain-level measurements are expected to yield higher-resolution, and likely unprecedented, information, as exemplified recently by the direct observation of concurrent polymerization and depolymerization processes [10] and the discovery of nonequilibrium conformational entanglements (see Glossary) that form and unravel stochastically during living polymerization reactions [11].

Herein, we review the state-of-the-art advances in this emerging area of single-polymer growth studies. We start by introducing the single-molecule methods that have been used to study real-time polymerization of single biological polymers, focusing on their working principles. We then highlight recent studies of real-time growth of synthetic polymers that apply some of these methods as well as the unique insights inaccessible from bulk studies; we also discuss their respective strengths and limitations. Finally, we call attention to the remaining challenges and opportunities. The single-molecule studies of the assembly of macromolecules are not discussed [12,13], because no covalent bonds are formed during these assembly processes. The atomic force microscopy-based studies of surface-grafted polymers are not discussed here, as the characterizations were performed on polymers postsynthetically and real-time polymerization kinetics were not available [14]. In addition to polymer growth, single-molecule techniques and super-resolution imaging methods have also been applied in studying single-chain polymer physics and characterizing polymer materials. These studies can be found in recent reviews elsewhere [15–23].

**Polymer Growth via Single-Channel Recording**

*Single-Nanopore Current*

This method is based on measuring the voltage-driven electrical current through a single nanosized pore within an insulating membrane [24]. This could be a channel protein, such as *Mycobacterium smegmatis* porin A (MspA) [25] or α-hemolysin (αHL) [26], embedded in a lipid membrane (Figure 1A), or a nanosized hole in a thin solid substrate, such as silicon nitride [27] or silicon oxide [28]. The current is proportional to the size of the nanopore, through which the charge carriers (i.e., ions) can migrate. When a molecule sits inside or passes through the nanopore, the current decreases due to the reduced effective pore size [29]. The amplitude of the decrease and its duration are related to the physical dimension, chemical structure, and concentration of the molecule and therefore can identify the molecule with appropriate calibrations [29,30]. This method can easily detect a single molecule passing through the nanopore with typical time resolutions of <1 millisecond [31]. However, only one nanopore can be operating for one set of electrodes at a time, making this measurement low throughput.

This nanopore approach has been applied to study the real-time polymerization of DNA by DNA polymerases (DNAPs) at the single-nucleotide resolution with concurrent sequence determination [32]. Gundlach and coworkers studied phi29 DNAP in complex with a DNA that has a single-stranded DNA overhang (Figure 1A). The single-stranded end of the DNAP-DNA complex is driven into the MspA nanopore by an electric field until the DNAP sits on the nanopore. The nanopore current is primarily determined by the four nucleotides near the narrowest region of the nanopore. During DNA polymerization, the single-stranded template moves towards the DNAP; this changes the nucleotides in the nanopore and thus the current, changes in amplitude...
and duration of which report the nucleotide identity and polymerization rate. Similar single-molecule DNA sequencing studies have also been done using αHL [33]. Recently, the Bayley group used the αHL nanopore to study polymerizations of synthetic polymer poly(disulfide)s, as discussed separately in the following text [34].

Growth of Single Poly(disulfide)s
Poly(disulfide)s are synthetic polymers in which the repeating units are linked by disulfide bonds. Bayley and coworkers [34] used αHL nanopores to study the real-time polymerization of poly(disulfide)s in aqueous electrolytes (Figure 1B). The growth of a poly(disulfide) molecule is initiated from a unique cysteine thiol group (-SH) inside a heteroheptameric αHL channel (Figure 1B). Two types of disulfide compounds act as reagents for the polymerization reaction, provided on separate sides of the membrane to avoid their direct reaction. One is 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB), an activating reagent, which first undergoes disulfide exchange with the cysteine to form an activated mixed disulfide (step b<sub>n</sub> to a<sub>n</sub>; Figure 1C). This activation step is needed because terminal -SH group cannot directly react with another -SH group to form a disulfide unless some oxidant is present. The other reagent is (mercaptoethyl)-ether (MEE), the actual monomer for the polymerization, which then reacts with the mixed disulfide via another disulfide exchange and links to the cysteine (step a<sub>n</sub> to b<sub>n+1</sub>; Figure 1C). This two-step disulfide exchange completes one cycle of monomer addition for the polymer chain growth,
effectively transferring the disulfide bond from one DTNB molecule to the growing chain; the cycle then repeats for the continual polymer growth.

Real-time recording of the nanopore current registers the intermediate activated state and chain growth with single-monomer resolution. In the activation step, the current decreases due to the DTNB group addition to the polymer chain (as, Figure 1C,D). In the following monomer incorporation step, the current increases slightly because the bulky DTNB is replaced by the smaller monomer MEE (bn+1, Figure 1C,D). Overall, the current decreases linearly with increasing chain length (Figure 1D). Furthermore, the rates and rate constants of chain activation and single-monomer incorporation are directly obtainable from the durations of current steps. The rate constants are lower than those measured at the bulk level, likely because the reagent access to the reaction site is limited by the nanopore.

More recently, Bayley and coworkers used cyclic disulfides as monomers for the polymerization [10]. The chain growth occurs between a free -SH end and the cyclic monomer via a ring-opening disulfide exchange reaction (Figure 1E), regenerating a free -SH at the chain end. The incorporations of individual monomers appear as stepwise current drops due to the increase of chain length (Figure 1F). Meanwhile, the chain could also be degraded by cyclization and release of the terminal subunit, leading to stepwise current increases (Figure 1F). One unique advantage of this measurement is that the rate constants of both polymerization and depolymerization were determined; they are challenging to deconvolute in bulk measurements where the polymerization and depolymerization are convoluted.

Owing to the high sensitivity of the blocking current to the size of the molecule inside the nanopore, the nanopore method offers desirable features for monitoring polymer growth. First, the polymerization or depolymerization reaction can be cleanly dissected at single-monomer resolution and the monomer incorporation sequence could also be obtained when the monomers have different structures. Second, the microscopic reaction kinetics of every single-monomer insertion can be determined; it can thus directly probe potential dynamic disorder of the polymerization process, inaccessible to bulk experiments. Third, the electrical current is not influenced directly by the monomers in the electrolyte solution, the polymerization can be studied at high monomer concentrations (tens of mM in the reported work [10,34]), highly relevant to practical synthesis of synthetic polymers.

However, several experimental challenges exist, hampering the broader application of this method in studying the growth of synthetic polymers. First, only limited events of monomer incorporations can be observed due to the small nanopore size. Once the polymer grows long enough to block the nanopore, the nanopore current becomes zero; no further incorporations could be detected. The poly(disulfide)s in the aforementioned work only grew up to approximately ten monomers, a size typically viewed as oligomers instead of polymers [10,34]. Second, the protein-based nanopores and the associated lipid membranes can only be used in aqueous solutions, whereas most synthetic polymers are made in organic solvents. Solid-state nanopores, made of inorganic materials, could address this solvent issue [27,28,35], but the incorporation of a single active site in the nanopore will become more challenging.

**Polymer Growth via Single-Particle Manipulation**

Many single-molecule methods are based on mechanical manipulation of a particle tethered by a single molecule. These include optical tweezers, flow stretching assays, tethered particle motion assays, and magnetic tweezers (Box 1). In these methods, one end of the growing (bio)polymer is tethered to the surface; the other end is tethered to a microparticle. By tracking the movement of the particle, the growth of the tethered (bio)polymer can be visualized in real-time.
Optical Tweezers
In optical tweezers [36,37], a tethered dielectric particle (e.g., polystyrene microparticle), is trapped optically by a focused laser beam (Figure 2A), where the trapping force scales with the particle’s displacement from the trap center [36,38–40]. However, measurements on individual molecules are serial, with low throughput. The high laser intensity at the focus can cause heating that could influence biological phenomena and affect local dielectric properties of the medium and thus the exerted force.

Flow Stretching
The force can be tuned by the solution flow rate and particle size [72], ranging from sub-piconewton to tens of piconewtons. The time resolution, primarily determined by the imaging camera, is typically milliseconds to microseconds.

Tethered Particle Motion
As the determination of $\rho_{\text{rms}}$ requires sampling the particle position over a period of time, this method has limited time and length resolutions in tracking tether length changes, even though the particle position can be determined accurately at high imaging rates. Nevertheless, this method allows for multiplexed measurements and is particularly suited for studying shorter polymers (~0.1–1 μm) undergoing variations in extension or mechanics [46,73].

Magnetic Tweezers
The pulling force can be tuned from sub-piconewton to tens of piconewtons by adjusting the distance between the magnets and the tethered particle. It has nanometer spatial resolution and microsecond to millisecond temporal resolution [39]. As the magnetic field is rather homogeneous over an area of tens to hundreds of microns square [74], magnetic tweezers can manipulate many tethered particles in parallel. Coupled with wide-field imaging, this parallel measurement provides high data throughput. The torsional manipulation is readily applied by rotating the magnets, a unique feature that is typically inaccessible to other methods. However, the permanent magnet configuration cannot manipulate objects in three dimensions and the electromagnet configuration requires unwieldy feedback control, results in hysteresis in the magnetic field, and may generate substantial heat, which could affect the behavior of the molecule under study [39].

Optical Tweezers
In optical tweezers [36,37], a tethered dielectric particle (e.g., polystyrene microparticle), is trapped optically by a focused laser beam (Figure 2A), where the trapping force scales with the particle’s displacement from the trap center [36,38–40]. By changing the relative position of the optical trap and the sample stage, one can manipulate the tethering molecule. The tethering surface can also be replaced by another trapped particle in the double trap configuration [41]. Block and coworkers used this method to monitor the real-time synthesis of single RNA molecules by RNA polymerase from a DNA template [42]. There, a polystyrene particle at the downstream end of a DNA template is optically trapped (Figure 2A). As the surface-immobilized RNA polymerase transcribes the DNA to polymerize nucleotides to make RNA, the trapped particle is pulled and its displacement reports the underlying polymerization rate of transcription. The single-molecule displacement trajectories display periods of steady transcriptional elongation interrupted by pauses. More important, the force-velocity curves show highly convex shapes, indicating that the rate-limiting reaction is not load dependent, in contrast to those of myosin and kinesin, two motor proteins. More recently, Katranidis, Hegner, and coworkers used dual-trap optical tweezers to monitor real-time synthesis of single polypeptides by ribosome [43]. They observed single polypeptide growths that occur concurrently with folding (i.e., cotranslational folding) as well as those that are decoupled from folding, a feature unique to structured biomacromolecules.

Flow Stretching
In a flow stretching assay, the polymer-tethered microparticle is manipulated by the laminar fluid drag force inside a microfluidic cell (Figure 2B); the position of the particle is optically imaged and...
tracked [40,44]. With **wide-field imaging**, many molecules within the cell can be manipulated and measured simultaneously, giving multiplexed data collection, for example, in the force-extension measurements of single DNA [44]. Van Oijen and coworkers used flow stretching to study DNA replication by *Escherichia coli* DNA polymerase III holoenzyme on a stretched single-stranded DNA template (Figure 2B) [45]. **Template-directed nucleotide polymerization** by the DNA polymerase converts the single-stranded template DNA into a double-stranded DNA. At pN drag force, double-stranded DNA is longer in end-to-end extension than the corresponding single-stranded DNA. Consequently, this polymerization leads to a tether lengthening. They observed single DNA lengthening trajectories with steps and interstep pauses, resulting from repeated cycles of single extension events and stochastic dissociation of polymerase from DNA. The direct observation of single extension events provided more precise polymerization kinetics compared with bulk measurements, which are complicated by the occurrence of multiple consecutive events of binding, synthesis, and dissociation.

**Tethered Particle Motion**

Without applying any types of force, the confined diffusive motion of a particle tethered by the polymer molecule of interest is tracked by optical microscopy (Figure 2C) [46,47]. The reach of the particle’s diffusive motion depends on the tether length; it is typically quantified by the lateral...
root-mean-squared displacement \((\rho_{\text{rms}})\) around the surface anchor position \([48]\). Cooperman, Goldman, and coworkers used the tethered particle motion method to monitor real-time peptide synthesis by single ribosomes \([49]\). They attached a micron-sized fluorescent particle to the 3’ end of an mRNA molecule that is bound to a surface-immobilized ribosome (Figure 2C). While the ribosome synthesizes the polypeptide encoded by the mRNA, it pulls the 3’ end of mRNA toward the ribosome, shortening the tether and restricting the particle’s diffusive motion range. By quantifying the temporal changes of \(\rho_{\text{rms}}\) of the particle, they determined the apparent peptide synthesis rates for individual ribosomes.

**Magnetic Tweezers**

In magnetic tweezers, the magnetic field from a magnet(s) is used to apply pulling and torsional forces to micron-sized superparamagnetic particles tethered by a single chain (Figure 2D) \([39]\). The magnetic tweezers method has been extensively utilized to study the conformational dynamics of torsionally strained, supercoiled DNA molecules; also, the mechanics of biomacromolecules and synthetic polymers at the single-molecule level \([38]–[40]\). Croquette and coworkers used magnetic tweezers to measure the real-time replication rate by a single DNAP via monitoring extension changes resulting from the elasticity difference between single- and double-stranded DNA \([50]\). They found the replication rate to be strongly dependent on the force applied on the template and determined that the biochemical steps limiting replication to be coupled to the movement. Saleh and coworkers used magnetic tweezers to monitor the extension increase of a single linear polysaccharide hyaluronan (HA) when proteoglycan aggrecans bind noncovalently to the backbone of HA as side chains, to form a bottlebrush structure increasing the backbone rigidity. It should be noted that there is no monomer addition along the HA backbone and the side chain binding is via noncovalent interactions \([51]\). Recently, the Chen group applied this method to visualize the real-time polymerization dynamics of synthetic polymers \([11]\), as discussed later.

**Growth of Single Poly(cycloolefins) via Magnetic Tweezers**

Chen and coworkers’ study using magnetic tweezers \([11]\) focused on the ring opening metathesis polymerization (ROMP) of norbornene and cyclooctene, catalyzed by the Grubbs second-generation catalyst (i.e., G2 catalyst). In these experiments (Figure 2E, inset), one end of the growing polymer is tethered to a surface via silane chemistry, which is stable in organic solvents. The other end is connected via a C=Ru bond to the G2 catalyst, the \(N\)-heterocyclic carbene ligand of which is functionalized with silane groups to attach to a magnetic particle, which has been coated with silica before use. During ROMP, new monomers, supplied at high concentrations (e.g., 1 M) under N\(_2\) protected atmosphere, insert into the C=Ru bond, leading to the growth and lengthening of the tethered polymer (Figure 2E, inset). The torsional manipulation in magnetic tweezers identifies single polymer tethers: they rotate freely.

This experiment uncovered surprising polymerization dynamics of single synthetic polymers. Above all, the authors discovered that the extension of a growing polymer did not increase continually but exhibited stochastic wait-and-jump steps (Figure 2E,F). These steps are attributable to the formation and unraveling of conformational entanglements (called ‘hairballs’) from newly incorporated monomers (Figure 2F, inset). The formation of these hairballs appears to be caused by the torsional strains introduced by the monomer incorporation during living polymerization, a nonequilibrium phenomenon not previously observed. From the real-time growth trajectories, the microscopic properties of each hairball are measured in terms of its size, kinetic stability, and structural looseness, as manifested by the jump length \(j\), waiting time \(\tau\), and waiting period slope \(s\), respectively (Figure 2F). These microscopic properties show strong correlations with the polymerization rates of individual polymers, suggesting that these hairballs play key roles in controlling the polymerization kinetics and dispersion. Real-time single-molecule measurement of polymer
growth made it possible to uncover these nonequilibrium dynamic events. These results also suggest that relevant reaction conditions, such as a shearing solution flow, might be used to manipulate polymer conformation to alter polymerization kinetics and, potentially, chain-length distribution.

Moreover, the syntheses of biopolymers, including peptide synthesis on ribosomes, nucleic acid synthesis on polymerases, and polysaccharide synthesis in the cell wall, are also, or could be, living polymerization reactions. Such nonequilibrium conformational entanglements may also play a role in biopolymerization kinetics as well. One related behavior is the collapsed conformation of nascent peptides during protein synthesis on ribosomes [43].

Despite the important scientific insights here, the single-particle method still suffers from technical limitations. First, both ends of the growing polymer chain are tethered in the experiment and the polymer growth must proceed by insertion polymerization. Many polymerization reactions do not involve insertion chemistry, however. Second, the z-resolution of magnetic tweezers is typically several nanometers, corresponding to approximately ten monomers in monitoring single-polymer growth. High resolution measurements are needed to resolve single-monomer insertions. Using a super-luminescent diode illumination and high-speed camera detection, magnetic tweezers measurement can be achieved at 1 nm precision [52]. Optical tweezers can also achieve a resolution of ~1 nm [39]. This resolution would be close to resolving single-monomer incorporation, for example, in using larger cyclic olefins as monomers for ROMP.

**Polymer Growth via Single-Molecule Fluorescence**

Single-molecule fluorescence imaging is perhaps the most widely used single-molecule method. Molecules can also be identified by labeling with different fluorophores. In addition, the position of a single fluorophore can be localized with nanometer precision in three dimensions, providing super-optical resolution of typically ~10–20 nm, which can be advantageous if spatial information is relevant. Wide-field imaging further provides high data throughput. This method has been applied to study the real-time growth of single DNA through labeling either the monomer (i.e., nucleotide) or the catalyst (i.e., enzyme) and these labeling strategies have also been adapted toward studying the polymerization of single synthetic polymers; but experimental gaps and challenges still remain.

**Labeled Monomers**

Korlach, Turner, and coworkers used fluorescently labeled deoxynucleotides as monomers to image single DNA polymerizations in real time at single-monomer resolution [53]. There, the DNAP in complex with a primed single-stranded DNA template is immobilized at the bottom of a zero-mode waveguide (ZMW) (Figure 3A). This ZMW confines the laser excitation and reduces the observation volume by more than three orders of magnitude compared with conventional confocal fluorescence microscopy, so that the fluorescently labeled deoxynucleotide can be supplied at micromolar concentrations to ensure processive DNA polymerization by the polymerase. The fluorescent label on the deoxynucleotide is designed to be cleaved during polymerization and thus does not alter the DNA structure. Each monomer incorporation is reported by a sudden appearance of fluorescence inside a ZMW well; its subsequent disappearance results from label cleavage (Figure 3B). The duration of the fluorescence signal of a cognate deoxynucleotide binding is much longer than, and thus readily differentiable from, the diffusive motions of free monomers and free labels in the ZMW. By using different fluorescent tags for the four different deoxynucleotides and using spectral differentiation, they achieved single-DNA sequencing at the single-monomer resolution (Figure 3B). Alternatively, Xie and coworkers developed a fluorogenic labeling scheme on the deoxynucleotide monomers; the
label on the monomer is not fluorescent until it becomes cleaved off upon incorporation into the growing DNA [54].

Labeled Catalyst (Enzyme)
During DNA polymerization, DNAP moves along the single-stranded DNA template and stays at the end of the growing strand. Therefore, van Oijen and coworkers visualized the polymerization of single DNA by tracking the movements of the fluorescently labeled DNAP on the template DNA [55], which was tethered on the surface at the 5′-end and stretched out by a solution flow (Figure 3C). While the other DNA strand is polymerized as part of rolling-cycle DNA replication, the DNAP moves away from the tethering point (Figure 3D), and the length of the growing strand is measured through the distance between the polymerase and the tethering point on the surface. This approach directly measures the catalyst action, allowing them to measure replication rate from the DNAP moving velocity and discover that DNAP exchanges rapidly during the polymerization, but the spatial resolution is limited by the thermal fluctuations of DNA conformation, incapable of reaching single nucleotide resolution.

Polymerization Chemistry via Single-Molecule Fluorescence
Using fluorescently labeled monomers, Blum and coworkers used single-molecule fluorescence microscopy to image monomer incorporations at the single-monomer level into polymer aggregates during ROMP reactions catalyzed by Grubbs’s second generation catalyst (Figure 4A–C) [56–60]. The incorporation of labeled monomers into the polymer chains was observed through the fluorescence bursts (Figure 4B) [56]. To achieve single-monomer level observation, ultra-low concentrations (10⁻¹³ to 10⁻¹⁰ M) of the labeled monomers were used (e.g., mixed with tens of
mM unlabeled monomers), which is necessary for minimizing the fluorescence background caused by the free labeled monomers in solution. Although the visualization of single-polymer growth remains to be achieved, temporal activity changes and activity heterogeneity were observed in these polymer aggregates [57,58]. More recently, Blum and coworkers further
investigated the competition between chain elongation and chain termination by adding 10^{-14} M labeled monomer and labeled terminator simultaneously (Figure 4C), which provided new insights into how the chain propagation and termination kinetics could influence the molecular weight distribution of the polymer product [60].

Using fluorescently labeled catalyst, Goldsmith and coworkers investigated the initiation process of single palladium catalysts [51]. The catalyst is fluorescently labeled on the pyridine ligand (Figure 4D). Upon the initiation of the surface-immobilized catalyst, the labeled ligand will fall off from the catalyst and diffuse away, leading to sudden fluorescence intensity drops in single-molecule fluorescence trajectories (Figure 4E). The initiation rate is directly calculated based on the fluorescence loss time after introducing the reactant. By analyzing the distribution of the fluorescence loss time, they found that the initiation rates are heterogenous between individual catalysts, attributed to the difference in the microenvironment of individual catalysts. Although polymerization has not been studied, this work demonstrates that the labeled catalyst strategy can be used in studying organometallic complex catalyzed polymerizations. For example, fluorescently labeled Grubbs-Hoveyda catalyst has been synthesized (Figure 4F); it can potentially be used in imaging single-polymer growth during ROMP [62].

The aforementioned work has established the feasibility toward visualizing single-polymer growth with single-monomer resolution using the single-molecule fluorescence method. Compared with the single-nanopore current method, which also has single-monomer resolution, the single-molecule fluorescence measurement is in principle not limited in the polymer chain length and therefore is more suitable for investigating synthetic polymers that can have thousands of repeating units. However, many technical challenges are present. First, high monomer concentrations, usually above millimolar level, are needed for the synthesis of synthetic polymers. With fluorescently labeled monomers, the fluorescence background would be too high to allow detection of individual molecules. Here the ZMW approach could be a solution. Second, it is in general problematic to label the monomers or catalysts with fluorescent tags. The bulky tag may affect the activity of the monomer or the catalyst and the functional groups of the tag may interfere with the catalytic chemistry. Third, the impurity levels in organic solvents are generally higher than in water and difficult to remove, causing a higher fluorescence background and false detections, further hindering accurate single-molecule imaging [61].

**Concluding Remarks**

Single-molecule studies have led to many scientific breakthroughs in understanding biopolimerizations. The associated technological advances also made it possible to examine real-time growth of synthetic polymers at the single-polymer level, leading to novel insights, such as the deconvolution of polymerization and depolymerization processes [10] and the discovery of nonequilibrium conformational entanglements and their functional roles [11].

Real-time single-polymer growth experiments also allow for accessing molecular information or experimental conditions that are not possible at the bulk level. For example, one can design experiments to gain additional molecular insights into the functional roles of hairballs. A simple experiment might be to select solvents that can enhance or impair intra-hairball van der Waals interactions. One can also derivatize the monomers with OH groups to introduce intra-hairball hydrogen bonding, with polar groups to enhance dipole–dipole interactions, or with charged groups to introduce electrostatic attractions/repulsions. Capitalizing on the recent advances of cryogenic electron microscopy (cryo-EM) [63–65], one can perhaps use rapid freezing to trap these hairballs and use cryo-EM to examine their structures. Moreover, because single-polymer growth measurements are performed at extremely low polymer concentrations, they...
are amenable to studying polymers of poor solubility, such as underivatized conjugated polymers, polymerization kinetics of which have been a long-standing challenge to bulk studies [66–68].

Many technical challenges remain for real-time single-polymer growth studies (see Outstanding Questions), but they also provide ample prospects for further technological and scientific developments. Compatible and less perturbative linkage chemistry and labeling schemes will always be needed. This is especially relevant considering the diversity of synthetic polymer chemistries that can involve different solvents, functional groups, and catalysts. More sophisticated sample cell designs will be needed to provide environmental control, for example, for air- and moisture-sensitive chemical reactions and catalysts (e.g., organometallic complexes) or for those that require higher reaction temperatures. Techniques that can measure single molecules without extreme dilution will be advantageous because catalyst-monomer affinity is often low for polymerization, requiring high monomer concentrations to reach appreciable polymerization rate.

Each technique also offers unique advantages in measuring single-polymer growth: single-channel recording can provide the information about the intermediate state of the reaction and achieve monomer sequencing of copolymers. Single particle manipulation allows the application of mechanical force to control the chain conformation during growth, which is particularly powerful for studying mechano-chemistry coupling and effect of chain conformation on polymerization chemistry. Single-molecule fluorescence imaging is applicable to all types of polymerizations and meanwhile can achieve single-monomer resolution and the sequencing of polymers.

With the existing technical capabilities (Table 1), one can envision many new scientific opportunities and challenges (see Outstanding Questions). One strategy to better tackle these scientific challenges technically is to combine different techniques. For example, combining fluorescence imaging and single-channel recording made it possible to achieve parallel measurements of multiple channels and high data throughput [69]. Combining single-particle force manipulation and single-molecule fluorescence has enabled the investigation of force effect on the conformation dynamics of DNA [70,71].

With many inspiring examples in studying biological systems and the latest advances in studying synthetic polymers, the authors’ view here is optimistic: understanding polymerization reactions of synthetic polymers at the single-chain level in real time and with the ultimate single-monomer resolution presents a myriad of opportunities to make novel and likely surprising discoveries.

Acknowledgments
We thank the support by the Army Research Office (grant W911NF-18-1-0217) (P.C.) and the University of Akron (C.L.). We also thank Dr Felix Alfonso for comments and Prof. Timothy Swager for suggesting the solution shear flow for manipulating polymer conformation in solution.

Declaration of Interests
No interests are declared.