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Reversible Addition−Fragmentation Chain Transfer Polymerization in DNA Biosensing

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Reversible addition−fragmentation chain transfer polymerization is employed here to allow detector-free visualization of specific DNA sequences for which dynamic polymer growth is used in signal amplification. In particular, surface-initiated polymer growth was regulated by the immobilization of chain transfer agents on the Au surface where DNA hybridization occurred. A linear polymer growth was observed as a function of the reaction time, characteristic of "living" polymer reactions. Significant improvement in assay sensitivity was realized in comparison to the previously reported polymerization-based sensing method by enhancing polymer growth rate and reducing background noises caused by nonspecific adsorption. Direct visualization of fewer than 2,000 copies of a short oligonucleotide sequence was demonstrated in a detector-free fashion.

Sophisticated technologies to recognize specific DNA sequence patterns with high sensitivity and accuracy have been developed in the past decades.[1] Current state-of-the-art sensors are reportedly capable of detecting a few DNA copies in complex biological matrices, quantifying DNA sequences through a dynamic range of several orders of magnitudes and simultaneously detecting tens and hundreds of genes in an array format.[2–5] To achieve such performances, however, most reported techniques rely on specialized equipments to carry out assays and inevitably have experienced limited mobility. The challenge nowadays in DNA biosensing therefore lies in simplifying signal readouts and building field-friendly sensors that are amenable to point-of-need applications without compromising achieved sensing sensitivity.[6]

Taking advantage of advancements in materials chemistry, we have recently developed an "*amplification-by-polymerization*" approach based on effective mass growth upon DNA recognition using controlled/"living" radical polymerization reactions.[7–9] In particular, a purposely selected polymerization reaction is activated upon DNA binding that subsequently leads to the formation of polymer brushes tethered to a solid support. The formed thin layer of polymer film is readily distinguishable as an opaque spot on the surface by the naked eye. This reported approach offers four unique advantages over the conventional signal amplification methods: (1) the use of a point-initiating polymerization reaction allows direct correlation of biological binding events to polymer growth; (2) the information, i.e. the "signal", contained in a small monomer is enhanced by hundreds of folds through polymer chain propagation; (3) based on the amount of analytes the degree of amplification is tunable by adjusting reaction time and monomer concentrations; and (4) the directly visible results eliminate the need for sophisticated readout equipments and, thus, enable field analysis outside of well-equipped laboratories.

Atom transfer radical polymerization (ATRP), the most popular controlled/"living" radical polymerization method,[10–12] was used initially in our concept-proof experiment for DNA detection. However, several inherent problems in ATRP have limited exploration for broader applications. For example, background noise from nonspecific adsorption is often observed due to the formation of complexes between positively charged metal ions and DNA molecules; the use of toxic transitional metal catalysts during assay detection also raises concerns for future clinical applications. To eliminate these concerns and broaden the application scope of the "*amplification-by-polymerization*" approach, a new control/"living" radical polymerization method, reversible addition−fragmentation chain transfer (RAFT) polymerization, is explored in this report to allow signal amplification in a catalyst-free fashion.

RAFT polymerization was first reported by Chiefari et al. in 1998.[13] It operates on the principle of a reversible chain transfer process, facilitated by chain transfer agents (CTAs) containing thiocarbonylthio groups.[14–17] The initial radicals formed using conventional thermal, photochemical, redox, or γ-irradiation methods attack the monomers and form $P^{\cdot}$ that start chain growth.

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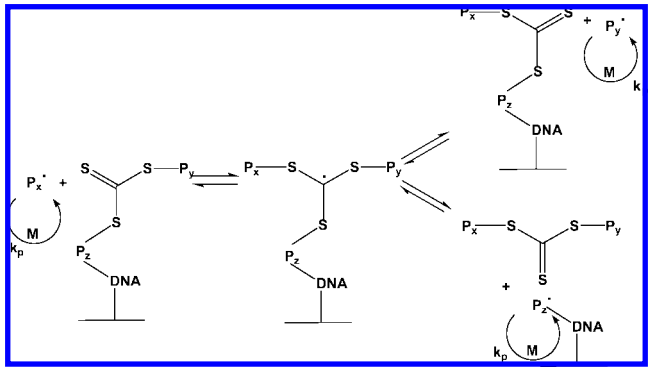
Scheme 1. Schematic Illustration of RAFT Reaction Mechanism on the Surface



P• interacts with CTA preferably and CTA-containing Px•, Py•, or Pz• are formed in solution and on the surface simultaneously (Scheme 1). Conventional chain termination events such as radical coupling and chain transferring end the polymerization reaction. Unlike in ATRP, the oscillating nature of CTA plays a critical role in RAFT that enables the "controlled" growth of polymer chains without compromising the growth rate.[18–20] It also offers additional benefits over ATRP, such as lower nonspecific grafting background and elimination of toxic transition metal catalysts. With a broad selection of reaction monomers and a good control over molecular weights of final products, RAFT is one of the most versatile polymerization reactions used in polymer synthesis. Here we describe the use of RAFT polymerization reactions in DNA sensing that exhibited improved sensing throughput, reduced background noises, and enhanced detection sensitivity.

EXPERIMENTAL SECTION

Materials. Au substrates (50 Å chrome followed by 1000 Å gold on float glass) were purchased from Evaporated Metal Films (Ithaca, NY). Dithiothreitol (DTT), triethylamine (TEA), 6-mercapto-1-hexanol (MCH), *N*-hydroxysuccinimide acid (NHS), 1-ethyl-3-(dimethylaminopropyl) carbodiimide hydrochloride (EDC), 3-hydroxypicolinic acid (3-HPA), diammonium citrate, dichloromethane, dioxane, *N*,*N*-dimethylformamide (DMF), azobisisobutyronitrile (AIBN) were purchased from Sigma-Aldrich (St. Louis, MO), and used as received. 2-(1-Carboxy-1-methylethylsulfanylthiocarbonylsulfany l)-2-methylpropionic acid (CMP) was a gift from Noveon (Cleveland, OH). Monomethoxy-capped oligo(ethylene glycol) methacrylate (OEGMA; mean degree of polymerization, 7 to 8) was a gift from Cognis Ltd. (Hythe, U.K.). OEGMA was purified by removing the methyl hydroquinone inhibitor. The column packing materials was purchased from Sigma-Aldrich, and the column was packed in house. All oligonucleotides were purchased from Integrated DNA Technologies (Coralville, IA), with the sequences listed (Supporting Information Table 1). T4 DNA ligase was purchased from Stratagene (La Jolla, CA). Micro Bio-Spin 30 columns were purchased from Bio-Rad Laboratories for DNA purification. C_{18} ZipTip was purchased from Millipore for DNA desalting before MALDI-TOF MS measurements.

Capture Probe Immobilization. The gold substrates were cleaned in a piranha solution prior to use (*70% H_2SO_4, 30% H_2O_2, hazardous solution, potentially explosive, handle with care!*). The disulfide bonds at the 3′-ends of the oligonucleotide probes, **C** and **NC**, were reduced to generate free thiol groups for surface immobilization.[7,21] Specifically, 3 μL of stock oligonucleotide solution (**C** or **NC**) at 100 μM was mixed with 0.0025 mmol of DTT (0.1 M, 25 μL) and 1 μL of TEA at room temperature for 20 min. After reduction, the excess amount of DTT was removed using a Micro Bio-Spin 30 column. The concentrations of the reduced oligonucleotides were determined by the UV absorbance at 260 nm. Mass spectrometry (MS) measurements were used to confirm successful reduction of disulfide bonds. Freshly reduced capture probes at 1 μM in a KH_2PO_4 buffer (1 M, pH 4.4) were spotted onto Au substrates at room temperature and incubated in a humid chamber for 16–20 h. The surfaces were then incubated in a 1 mM MCH aqueous solution for 1 h, followed by copious rinsing with DI water and dried under N_2.[22,23]

Detection Probe Conjugation (CTA-D). A solution of CMP (0.10 M) in dichloromethane (DCM) was cooled in an ice bath. A mixture of NHS (0.10 M) and EDC (0.10 M) in dioxane was added dropwise. Upon completion, the reaction mixture was stirred under room temperature for 1 h, followed by filtration to remove any precipitates. The solution was then washed with saturated $NaHCO_3$, followed by washing with water, and dried over $MgSO_4$. Evaporation in vacuo resulted in a crude yellow solid product, with the expected structure confirmed by NMR. ^{1}H NMR (CD_3Cl, 300 MHz): δ 1.6 (s, 12H), 2.8 (s, 4H), 10.3 (s, 1H). ^{13}C NMR (CD_3Cl, 300 MHz): 25.4, 25.8, 53.8, 55.5, 169.6, 173.0, 174.7, 219.4. This crude NHS active ester was used in subsequent experiments without further purification. Oligonucleotide **D** (0.3 nmol) and 5 μL of conjugation buffer (1.0 M $NaHCO_3/Na_2CO_3$, pH 9.0) were added into a centrifuge tube, followed by addition of the freshly prepared CTA ester solution (10 mg/mL in DMF). After a 30-min reaction at room temperature, unreacted NHS ester was removed by gel filtration. MS was again used to confirm the coupling and monitor the coupling efficiency by measuring the amount of oligonucleotides before and after the coupling reaction.

DNA Duplex Formation. Probes **C** and **NC**-attached Au surfaces were incubated with a mixture of target oligonucleotides **C′D′** (3 μL, concentration specified in the text) and detection probes **CTA-D** or **D** (3 μL, 1 μM), with or without RAFT reagent modification, respectively, in a humidity chamber at room temperature. The 1 M NaCl in Tris-EDTA (TE) was used as the hybridization buffer. After 1-h hybridization, stringent washes with a NaCl/phosphate buffer were performed to selectively denature the noncomplementary duplexes. After hybridization, the surface was reacted with 20 μL of a solution containing 4 units of T4 DNA ligase and 30% PEG 8000 in a 10 × T4 DNA ligation buffer without DTT. The reaction was allowed to proceed for 1 h at room temperature.

RAFT Polymerization for DNA Detection. In a typical surface-initiated RAFT polymerization, a solution of OEGMA (5 mL, 13.2 mmol), DI H_2O (5 mL) and AIBN (0.004 mmol) was purged with N_2 for 30 min in an ice bath to reduce the amount of O_2 in the reaction system. The DNA-immobilized Au substrate was placed in a glass container and also degassed by purging N_2

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for 30 min. The OEGMA mixture was then injected into the container where the Au substrate was located, and the container under N_2 protection was then transferred to a water bath of 30 °C to allow polymerization to occur. Polymerization was stopped by quenching the reaction container in an ice bath, and the substrate was removed from the reaction mixture. The substrates were thoroughly rinsed and bathed in methanol overnight to remove nonspecifically adsorbed monomers. The polymer films formed on the substrates were characterized using ellipsometry and attenuated total reflectance Fourier transform infrared spectroscopy (ATR-FT-IR).

Instrumentation. An Applied Biosystems Voyager DE-STR MALDI-MS was used to monitor the coupling reaction between amino-labeled DNA and the RAFT agent and the reduction of disulfide bonds on DNA. The spectra were collected by averaging 100 laser shots in a linear positive detection mode using a 20-Hz N_2 laser. The optimal MS performance was achieved at an accelerating voltage of 25 000 V and 300 ns delay time. A solution of HPA (35 mg/mL), diammonium citrate (7 mg/mL), and 10% acetonitrile in water was used as the MALDI matrix. DNA solutions were desalted using C_{18} ZipTips.

^{1}H and ^{13}C NMR spectra were collected on a Mercury 300 MHz spectrometer. In ^{1}H NMR spectra, the monomer vinyl signals of OEGMA were detected at 5.5 and 6.0 ppm. As POEGMA formation proceeded, the relative intensity of proton signals in the methacrylate backbone at 0.6–1.0 ppm increased, suggesting the formation of polymer. $CDCl_3$ was used as the solvent.

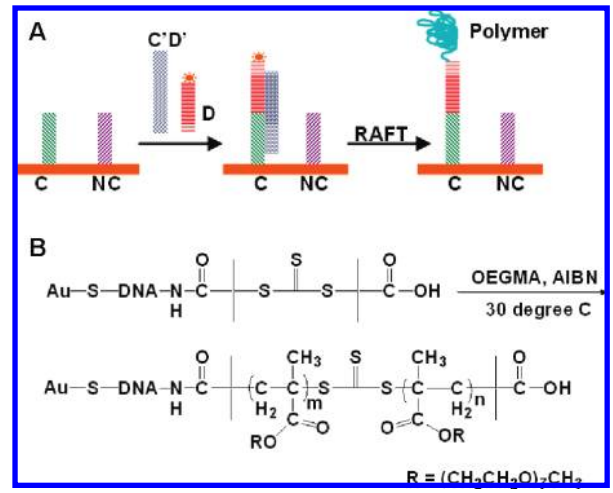
The film thickness was measured using an AutoEL-III Automatic ellipsometer (Rudolph Research). The instrument irradiated the substrates at a 70-deg incident angle. The reflective index of 1.51 was used for the analysis of the polymer films. All surface measurements were conducted with dried samples. Each substrate was measured before and after the RAFT reaction, and the difference in film thickness was calculated and attributed to film growth.

Gel permeation chromatography (GPC) was performed on a PolymerLab GPC 50 Plus instrument equipped with a refractive index detector. Separation was conducted using a Pore column (MW operating range 200–2 000 000, nominal particle size 5 μm, PolymerLab). THF was used as the mobile phase at a flow rate of 0.5 mL/min at 30 °C. The migration time was calibrated using polystyrene standards (MW range 580–377 400, PolymerLab). Polystyrene (EasiCal PS-2) from PolymerLab was used as the GPC standard, and HPLC grade tetrahydrofuran (THF) from Fisher Scientific was used as the GPC eluent. Molecular weights and poyldispersity indexes (PDI) of the resulting polymers were estimated using the PolymerLab software.

Attenuated total reflectance Fourier transform infrared spectra were collected using a Bio-Rad FTS-6000 FT-IR single-beam spectrometer equipped with a Miracle AG single reflection horizontal ATR accessory (Pike Technologies, Inc.). Each spectrum was recorded at 4-cm^{-1} resolution with 100 accumulated scans and was normalized to the blank of 100 scans collected from an empty ATR cell. All spectra were corrected for differences in refractive index using Bio-Rad Win-IR Pro software.

Surface topography was measured by tapping mode AFM using a Digital Instruments Nanoscope IIIa microscope. Images were captured at a scan resolution of 512 points/line, scan rate of 0.5 Hz, scan area of 40 μm × 40 μm, and z-range of 200 nm. The drive frequency was set approximately to the resonant frequency of the cantilever being used. All cantilevers used had a resonant frequency of approximately 72 kHz.

Scheme 2. (A) Schematic Illustration of RAFT-Based DNA Detection and (B) the Chemical Reaction of RAFT Polymerization Grafting on DNA-Immobilized Surfaces



RESULTS AND DISCUSSION

Scheme 2A illustrates the detailed experimental procedure in RAFT-based DNA detection: a **c**omplementary capture oligonucleotide probe, **C** (5′-pTAA CAA TAA TCC CTC A_{20}-$(CH_2)_3$-SH), was immobilized on a Au surface at room temperature. A **n**on**c**omplementary oligonucleotide (5′-pGGC AGC TCG TGG TGA A_{20}-$(CH_2)_3$-SH) was immobilized on a separate substrate as the control (**NC**). To ensure that polymers grow only at the specific location where DNA hybridization occurs (*spatially distinguishable*), the RAFT CTAs were directly coupled to the DNA detection probes.[24,25] Specifically, CMP as the CTA reagent used throughout our studies was converted to a NHS ester, confirmed by NMR: δ 1.6 (s, 12H, the protons of the methyl groups), δ 2.8 (s, 4H, the protons of the succinimide ring) in ^{1}H NMR; δ 219.4 (the carbon in thiocarbonyl) and δ 169.6 (the carbons of amide) in ^{13}C NMR. This NHS ester was then coupled to the amino groups at the 5′-end of the DNA **d**etection probe, **D** (5′-NH_2-$(CH_2)_6$-A_{20} ATC CTT ATC AAT ATT) to form the probes **CTA-D**. The coupling reaction was completed within 1 h with a ~100% conversion efficiency, as monitored by mass spectrometry ($\Delta m/z$ = 264, Supporting Information Figure 1). Note that the coupling reaction was carried out at a stoichiometric 1:1 ratio. No dual coupling product was observed at m/z = 11,472 (i.e., no formation of DNA-CMP-DNA). During the sensing experiments, the target oligonucleotide, **C′D′** with its sequence complementary to **C** and **CTA-D** probes (5′-GAG GGA TTA TTG TTA AAT ATT GAT AAG GAT), was hybridized to both probes that brought the RAFT CTA reagent close to the surface. To permanently affix the RAFT agent at the location where the probe **C** was immobilized, a T4 ligase was used to connect the nicks between the probes **C** and **CTA-D**.

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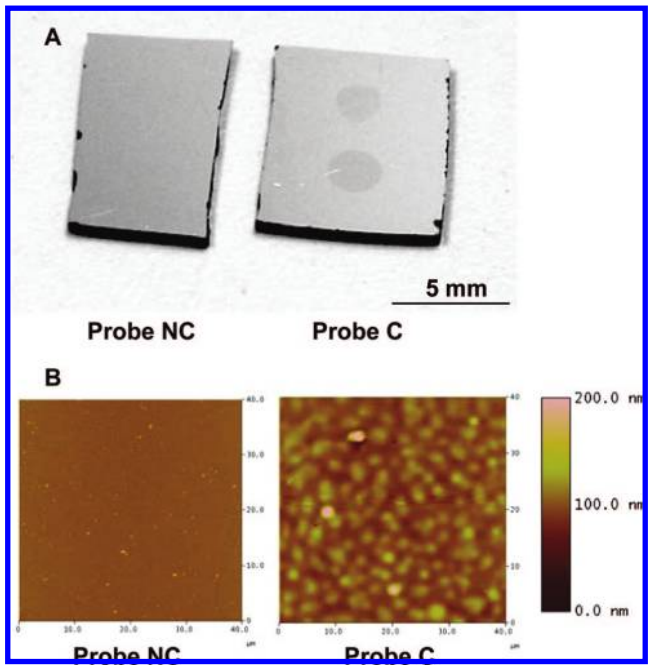

Figure 1. (A) A photographic picture of the Au substrates immobilized with the DNA capture probes **NC** (left) or **C** (right) after DNA **C′D′** hybridization and 2-h RAFT polymerization. Two spots in replication were clearly visible on the substrate immobilized with the probes **C**. (B) AFM images of POEGMA film growth on DNA-coated Au substrates after 5-h RAFT polymerization. The images are 40 μM $\times$ 40 μM. The scale bars showing surface roughness are placed on the side of the images. Experimental conditions: [**C′D′**] = 1 μM, [**CTA-D**] = 1 μM, hybridization volume = 3 μL, 1 h hybridization, OEGMA: H_2O = 1:1 (v/v), AIBN = 0.004 mmol, T = 30 °C.

The subsequent RAFT reaction is illustrated in Scheme 2B: monomethoxy-capped OEGMA was used as the monomer for its good water solubility. The reaction mixture of OEGMA and H_2O at a 1:1 volume ratio with 0.004 mmol of AIBN as the RAFT initiator was kept on an ice bath and purged with N_2 to remove residual O_2 in the system. The solution was then introduced to a reaction vial containing the DNA-coated substrate, followed by heating the solution mixture to 30 °C. This low reaction temperature was used to ensure chemical stability of the Au−thiol interaction in DNA immobilization and to allow slow release of free radicals from AIBN.[26] The radicals from AIBN attacked the CTA reagents at the distal points of DNA molecules to yield CTA-derived radicals, followed by chain growth on the surface in a combined "graft-to" and "graft-from" fashion. The reaction was stopped in 2 h by removing the substrate from the reaction mixture, followed by rinsing with methanol overnight to remove nonspecifically adsorbed polymers and monomers. Similar to the previous ATRP-based DNA sensing experiment, direct surface inspection showed formation of thick polymer films at the specific spots where the probes **C** were immobilized as the optical clarity changed under room light (Figure 1A). Visually no film was discernible at the spot **NC**. Quantitative measurements using an ellipsometer indicated that an average of 78-nm-thick POEGMA film was formed at the spots **C** after the 2-h reaction, whereas for the control spots, **NC**, an average film thickness of 1.9 ± 0.3 nm was measured, confirming the feasibility of using RAFT in

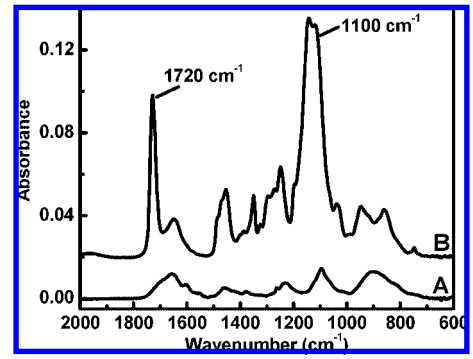

Figure 2. ATR-FTIR spectra of CTA-coupled DNA molecules immobilized on a Au surface before (A) and after (B) the formation of POEGMA. Experimental conditions: [**C′D′**] =1 μM, [**CTA-D**] = 1 μM, hybridization volume = 3 μL, 1-h hybridization, OEGMA:H_2O = 1:1 (v/v), AIBN = 0.004 mmol, T = 30 °C, 2-h RAFT.

polymerization-based DNA sensing. It is important to note that the ellipsometric measurements only provide a qualitative readout on positive polymer film growth. AFM images of a set of CTA-DNA-coated substrates after a 5-h RAFT reaction showed a very rough surface with polymer islands distributed across it (Figure 1B). The intensity scale on the side shows an average of 150+ nm in the height difference for the island features. These islands cause severe light scattering, and subsequently allow direct visualization of spots **C**. The control spot showed relatively smaller and fewer surface features, and macroscopically no discernible spots visible. In the previously reported ATRP-based experiments, a film thickness of less than 15 nm was measured after a 5-h reaction.[21] A conservative estimate here shows that more than an order of magnitude of improvement in polymer growth efficiency was realized. Meanwhile, the background signal measured after RAFT was less than what was observed from the ATRP assays, probably due to the elimination of electrostatic attraction of metal ions on the surface.[7]

ATR-FTIR was used to chemically characterize the sensor surface. The infrared spectrum of the surface immobilized with CTA-coupled DNA molecules showed a characteristic DNA peak at 1670 cm^{-1} from NH-bending in the amide bonds (Figure 2A). Additional peaks at 1460 cm^{-1} from the purine and pyrimidine rings and 1110 cm^{-1} from the DNA phosphodiester backbones were also observed.[17,27] Formation of POEGMA on the surface was evident from the large increase in the peak intensity at 1720 cm^{-1} (C=O) (Figure 2B). The increase in the peak intensity at 1110 cm^{-1} corresponded to the expected C−O stretching in POEGMA side chains. Polymer growth in solution can also be monitored using ^1H NMR. Specifically, the chemical shifts at 5.5 and 6.0 ppm corresponding to the vinyl groups in OEGMA were monitored by removing a portion of the reaction mixture during RAFT. As polymerization proceeded, the intensities of these two shifts decreased. Meanwhile, a broad band corresponding to the proton signals of the methacrylate backbone at 0.6–1.0 ppm appeared as the polymer reaction proceeded. Similar band formation was observed for the ethylene oxide proton signals at 4.0 ppm, confirming the formation of POEGMA (Supporting Information Figure 2).[17,28,29]

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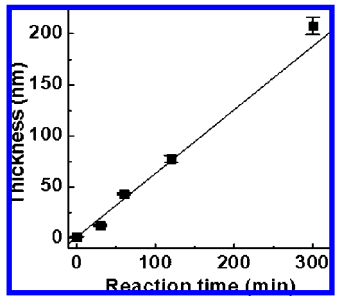

Figure 3. A plot of the measured POEGMA film thicknesses on the DNA-coated surfaces as a function of polymerization time. A linear fitting was used to guide the eye. The error bars were calculated experimental standard deviations from four replicates. Experimental conditions: [**C′D′**] = 1 μM, [**CTA-D**] = 1 μM, hybridization volume = 3 μL, 1-h hybridization, OEGMA:H_2O = 1:1 (v/v), AIBN = 0.004 mmol, T = 30 °C.

To achieve maximal detection sensitivity, preserving the "living" characteristics of RAFT would be preferred to allow "infinite" polymer growth, i.e. amplification, when needed. Figure 3 shows a linear growth of polymer films as a function of the reaction time, suggesting the existence of a controlled/"living" polymerization reaction on the surface. Gel permeation chromatographic (GPC) is another technique that is routinely used in polymer characterization. The oscillating property of the symmetric CTA reagents used in this study allowed polymer growth on the surface and in solution simultaneously. Given the difficulty in producing sufficient amount of polymer on a surface for GPC characterization (chain density < 10^{12} molecules/cm^2),[21] the polymers collected from the reaction mixture in the same reaction container were used in GPC, though certain differences in polymer growth kinetics and final M_n were suspected.[24,25] One of the representative GPC elution chromatograms collected from the solution after 300 min RAFT polymerization showed a Gaussian-shaped elution band at 14.03 min with an estimated M_n of 31.6K (Supporting Information Figure 3). Note that this calculated M_n was only a rough estimation due to the use of polystyrene (PS) as the standards. The comblike structure of POEGMA was drastically different from that of PS; thus a difference in the relationship between hydrodynamic volume and molecular weight was suspected.[30] Nevertheless, PS standards are routinely used in GPC characterization of POEGMA in which the relative values of M_n are valid parameters in the characterization of polymers.[31] Reaction conversion was calculated by rationing the height of the polymer band to that of the monomer, eluted at 18.28 min. A linear relationship was observed when reaction conversion was plotted against the reaction time (Figure 4A). In the control experiment where incubation with noncomplementary target DNA molecules resulted in the absence of CTA on the surface the reaction conversion reached the plateau in an hour, indicating early termination of free radical polymerization. Another linear plot was obtained by plotting the calculated M_n as a function of conversion, directly confirming the "living" nature of polymer growth in

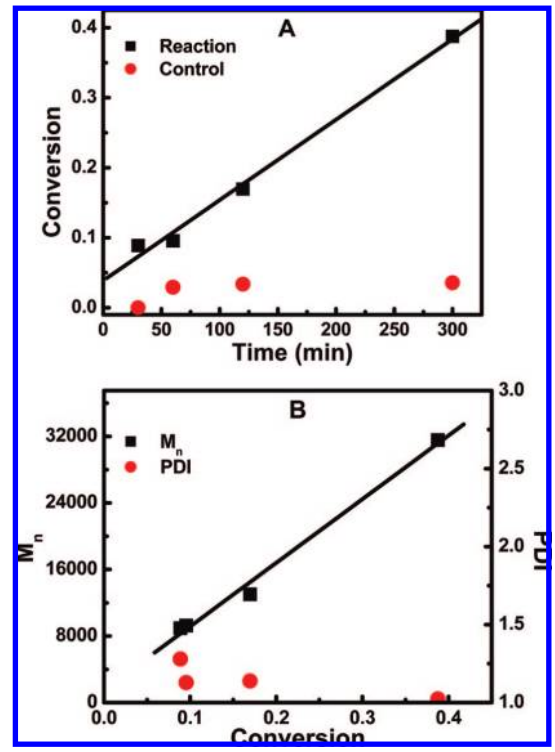

Figure 4. (A) Evolution of OEGMA conversion as a function of reaction time in RAFT-assisted DNA detection and (B) plots of calculated M_n and PDI of the polymer products as a function of reaction conversion. Both linear fittings were added to guide the eye. Experimental conditions: [**C′D′**] =1 μM, [**CTA-D**] = 1 μM, hybridization volume = 3 μL, 1-h hybridization, OEGMA:H_2O = 1:1 (v/v), AIBN = 0.004 mmol, T = 30 °C. Samples were collected at various time intervals from the reaction mixture above the substrates.

solution within the reaction period examined (Figure 4B). The polydispersity index (PDI) of the elution band at 14.03 min was smaller than 1.1, suggesting a narrow size distribution of polymer chains. The calculated PDIs decreased with increasing conversion, which was also consistent with the focusing effect of control/"living" radical polymerizations. Continuous reaction slowly increased solution viscosity. The gelation is suspected to be the result of increased M_n of polymer products as well as the side reactions of POEGMA through hydrogen abstraction or coupling reactions.[32,33]

It is important to note that the polymer chains in solution had a molecular weight of M_n = 31.6K after a 5-h reaction, which was significantly less than what would be expected from a polymer film of 207 nm in thickness (measured by ellipsometry).[34] In addition to the possible errors in film thickness measurements due to surface roughness and GPC measurements due to the use of PS standards, we suspect that the aforementioned side reactions of POEGMA were the main culprit for the much thicker films formed on the surface: the ethylene oxide units in POEGMA are known to be prone to radical attacks and to form new radicals

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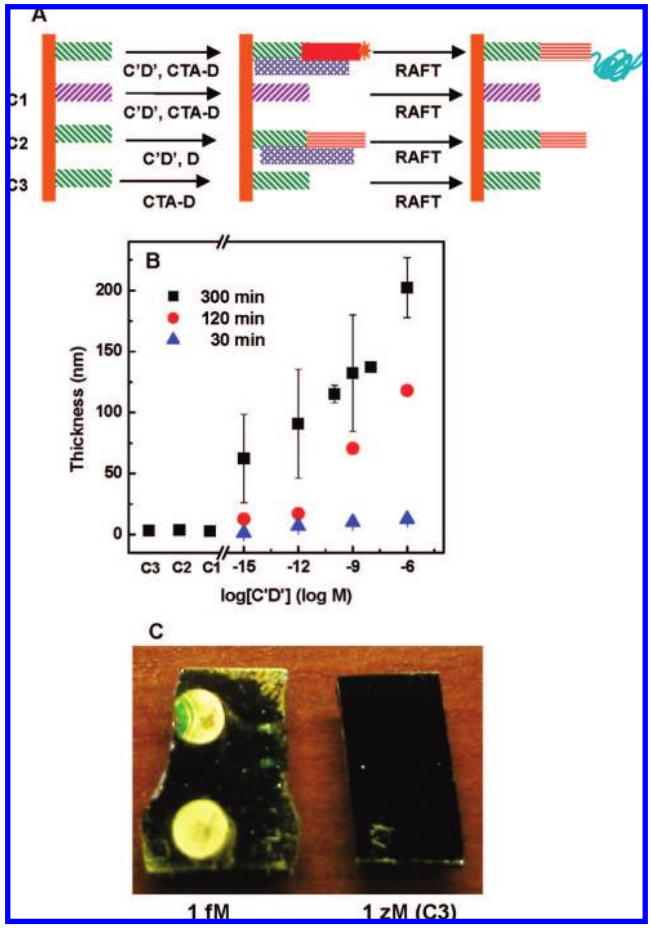

Figure 5. (A) A schematic drawing of three control experiments used in DNA sensing. (B) Measured POEGMA film thicknesses plotted against target DNA **C′D′** concentrations at different RAFT reaction times. The error bars were calculated from four replicates. Three control samples were also included (**C1**−**C3**). (C) A photographic picture of the Au substrates incubated with the target DNA solutions at 1 fM or 1 zM, followed by a 5 h RAFT reaction. Experimental conditions: [**D**] or [**CTA-D**] = 1 μM, hybridization volume = 3 μL, 1-h hybridization, OEGMA:H_2O = 1:1 (v/v), AIBN = 0.004 mmol, T = 30 °C.

after losing hydrogen.[32,33] The newly formed radicals may participate in fresh polymer chain growth or directly couple to other radical-containing polymer chains in solution. Consequently formation of branched or cross-linked polymers atop one single DNA molecule is suspected, which also explains the observation of large polymer islands on the surface (Figure 1B).[35] This speculation is also supported by the slower film growth when hydroxyethyl methacrylate (HEMA) was used as the reaction monomer. Interestingly, these thick films were still found to semiquantitatively correlate to the amount of polymer materials on a surface, which was used to provide a semiquantitative measurement of DNA target molecules.

Taking advantage of the "living" character of RAFT, detection of low concentrations of DNA targets was realized by simply extending polymerization reaction time. Figure 5 shows detection of target DNA (**C′D′**) on a solid support. To monitor nonspecific background induced by nonspecific hybridization or non-CTA-regulated polymer growth on the surface during the detection, three control experiments were carried out in parallel to DNA detection (Figure 5A): in the first experiment, a Au surface (**control C1#**) coated with noncomplementary probes (**NC**) was exposed to the same DNA mixture containing the target DNA (**C′D′**, 1 μM) and the CTA-coupled detection probes **CTA-D** (1 μM); the second control experiment was carried out with the Au substrate (**control C2#**) coated with the complementary probes **C** but was exposed to a mixture of the target DNA (**C′D′**, 1 μM) and the detection probes **D** (1 μM) without CTA reagents attached; and the third control was conducted with a substrate (**control C3#**) of the complementary probes **C** but was exposed to a mixture of the target DNA (**C′D′**, 1 zM, i.e. <1 DNA molecule in the mixture) and the CTA-coupled detection probes **CTA-D** (1 μM). None of these three control substrates exhibited meaningful film growth. Figure 5B shows the semiquantitative measurements of film thicknesses of different substrates undergoing different reaction periods. It is clear that by extending the polymerization reaction time different levels of amplification can be achieved, i.e. "*amplification-at-will*". Under 5-h reaction, positive detection of target DNA at the fM level (62 ± 36 nm) was unambiguously distinguishable from the background (3 ± 1 nm), regardless of the large error bars due to the semiquantitative nature of polymerization reactions. Given that an incubation volume of merely 3 μL was used during DNA hybridization, this result was equivalent to direct visualization of ~1,800 copies of DNA molecules without PCR amplification. Direct visualization of a single copy of DNA without PCR is theoretically feasible but experimentally limited by Langmuir adsorption isotherm. In other words, the ability to reproducibly capture a few molecules in the sample solution during DNA hybridization that allows attachment of the CTA reagents to the surface imposes the ultimate limit to the achievable sensitivity of this polymerization-based sensing platform. A linear dynamic range of 9 orders of magnitude for the logarithmic DNA concentrations was observed, similar to the literature results using different sensing platforms.[36]

CONCLUSION

In summary, we demonstrated here that RAFT polymerization can be used as an effective amplification method in DNA biosensing. The reaction rate was improved without compromising detection sensitivity. Direct visualization of fewer than 2,000 copies of DNA in solution was demonstrated for the first time. However, it is important to point out that in order to achieve such high detection sensitivity an extended polymerization reaction period is needed, which inevitably reduces assay throughput. Faster polymer reactions based on branch or star polymer formation and cross-linking reactions can be explored to address the issue. Overall, this *amplification-at-will* approach provides an inexpensive alternative to current ultrasensitive sensing platforms and can be applied to monitor other biological interactions beyond the field of genomics.

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SUPPORTING INFORMATION AVAILABLE

MALDI-MS characterization of CTA and DNA coupling reaction, NMR spectra of OEGMA and POEGMA, a table of the DNA sequences used in this study, and a representative GPC spectrum of POEGMA. This material is available free of charge via the Internet at http://pubs.acs.org.

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