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Hybrid enzymatic *graft-through* and *graft-from* polymerization of DNA bottlebrush polymers

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Abstract

Bottlebrush polymers are promising materials with tunable molecular dimensions that give rise to versatile mechanical, optical, and therapeutic properties. However, the precise role of each molecular dimension in a material remains difficult to study; for example, single-molecule experiments have been limited by dispersity in backbone molecular weight, low side-chain molecular weight, and low side-chain density. Here we introduce fully DNA-based bottlebrush polymers with well-defined dimensions enabled by enzymatic polymerization strategies. Monodisperse backbones result from template-defined *graft-through* polymerization, which incorporates dense side chains that are further lengthened using *graft-from* polymerization. Single-molecule atomic force microscopy indicates that dense, high-molecular-weight side chains lead to stiffer backbones that are characteristic of bottlebrush polymers. Our enzymatic polymerization strategy opens a new class of DNA-based bottlebrush polymers that can offer dynamic, molecular-scale insights into polymer material properties, with potential to serve as a new material platform for molecular machines, tunable photonics, and nucleic acid delivery.

21 Main

22 Comb and bottlebrush polymers—consisting of linear backbones with either loosely or densely
23 grafted side chains, respectively—have emerged as tunable soft materials including tissue-like
24 elastomers,¹ therapeutic delivery vehicles,² biomimetic lubricants,³ and printable photonic
25 crystals.⁴ The broad-reaching material properties accessed by comb and bottlebrush polymers
26 stem from versatile underlying molecular architectures.⁵⁻⁷ Key architectural features include
27 backbone and side-chain compositions, molecular weights, and side-chain density and
28 positions. Recent efforts in polymerization chemistry have focused on achieving independent
29 control of each feature to yield a practically endless design space.

30 To efficiently design new materials using comb and bottlebrush polymers, it is crucial to
31 establish molecular-scale insights into the impact of each architectural feature on physical
32 properties. For example, a recent effort decoupled an intrinsic stiffness–extensibility tradeoff for
33 bottlebrush elastomers. Bottlebrush polymers are semiflexible chains, and their integration into
34 elastomeric materials enables low crosslinking densities without introducing entanglements.⁸
35 The resulting elastomers are “super-soft” (low moduli, ~100 Pa), fracture resistant, and
36 chemically versatile.⁹⁻¹¹ However, bottlebrush backbone bonds are susceptible to scission due to
37 steric repulsion between side chains, which limits extensibility. “Foldable” backbones were
38 introduced to reduce steric repulsion by increasing the space between side chains.¹² Backbone
39 folding was promoted by co-polymerizing spacer monomers that were chemically incompatible
40 with side chains. Incompatible spacers folded into backbones that preserved high-density side
41 chains while reducing susceptibility to scission.^{13,14} Foldable bottlebrush elastomers with spacer-
42 to-side-chain ratios from 0 to 1.4 maintained similar moduli, and the largest ratios—the most
43 folded backbones—achieved 20-fold greater breaking strains.¹² This advance was enabled by
44 molecular-scale understanding of bottlebrush polymers, indicating the wealth of insights to be
45 gained by probing sub-molecular features of bottlebrush polymers.

46 Single-molecule investigations of comb and bottlebrush polymers have improved insights into
47 the role of molecular architecture on conformations and dynamics at interfaces and in materials.
48 Single polymers deposited onto surfaces are routinely visualized using atomic force microscopy
49 (AFM),¹⁵⁻¹⁷ which reveals spontaneous extension by bottlebrush polymers. In AFM images,
50 molecular width serves as a proxy for side-chain length at high graft density, as well as
51 individual side-chain lengths and positions at low graft density. Super-resolution imaging is an
52 emerging approach to visualize fluorescently labeled bottlebrush polymers in concentrated

solutions^{18,19} and solid-state thin films.^{20,21} A comparison of bottlebrush polymer flexibility as a function of side-chain molecular weight revealed discrepancies between single-molecule measurements and theoretical predictions of scaling behaviors and conformations in swollen films and bottlebrush melts.¹⁹ The differences appeared to result from a resolution limit for bottlebrush polymers with shorter side chains, thereby highlighting the importance of control over independent architectural features and a need to synthesize bottlebrush polymers with larger dimensions.

DNA-based materials offer improvements in synthetic control and larger dimensions by using biochemical templates to define backbone and side-chain properties. Previously, double-stranded DNA (dsDNA) comb polymers were constructed using enzymatic replication of chemically modified backbones and side chains, which were coupled using *graft-onto* strain-promoted azide–alkyne cycloaddition (SPAAC).²² This method yielded dsDNA comb polymers with well-defined, micron-scale dimensions and multi-color labels to distinguish backbones from side chains using fluorescence optical microscopy.²³ Single-molecule rheology of dsDNA comb polymers revealed the role of side-chain number, position, and molecular weight on dynamic stretching and relaxation processes.^{23–25} However, *graft-onto* approaches are not amenable to synthesizing DNA bottlebrush polymers due to steric hindrance and electrostatic repulsion between multiple DNA components. To date, DNA-containing bottlebrush polymers have included either non-DNA side chains^{26–31} or non-DNA backbones.^{32–34} For side chains, *graft-onto* coupling of low-molecular-weight side chains and *graft-from* polymerization of non-DNA components have limited the ability to resolve side chains from backbones during visualization. For backbones, *graft-through* polymerization of DNA-containing macromonomers resulted in greater polydispersity than enzymatic replication from DNA templates.³⁴

We report the synthesis of fully DNA-based bottlebrush polymers, which comprise template-defined dsDNA backbones and densely grafted single-stranded DNA (ssDNA) side chains. Our hybrid approach combined enzymatic *graft-through* incorporation of chemically modified deoxyribonucleotide triphosphates (dNTPs) into dsDNA backbones, bio-orthogonal *graft-onto* coupling of ssDNA oligonucleotides to dsDNA backbones, and enzyme-catalyzed *graft-from* polymerization of dense, high-molecular-weight ssDNA side chains (**Fig. 1a**). *Graft-through* polymerization was evaluated by successful polymerase chain reaction (PCR) in the presence of modified dNTPs. *Graft-from* polymerization used terminal deoxynucleotidyl transferase (TdT) to demonstrate addition of dNTPs. Single-molecule AFM quantified backbone contour lengths, persistence lengths, and transitions between comb and bottlebrush polymer regimes based on

side-chain lengths and densities. Overall, this modular synthetic approach offers detailed control over the molecular-scale features of DNA bottlebrush polymers.

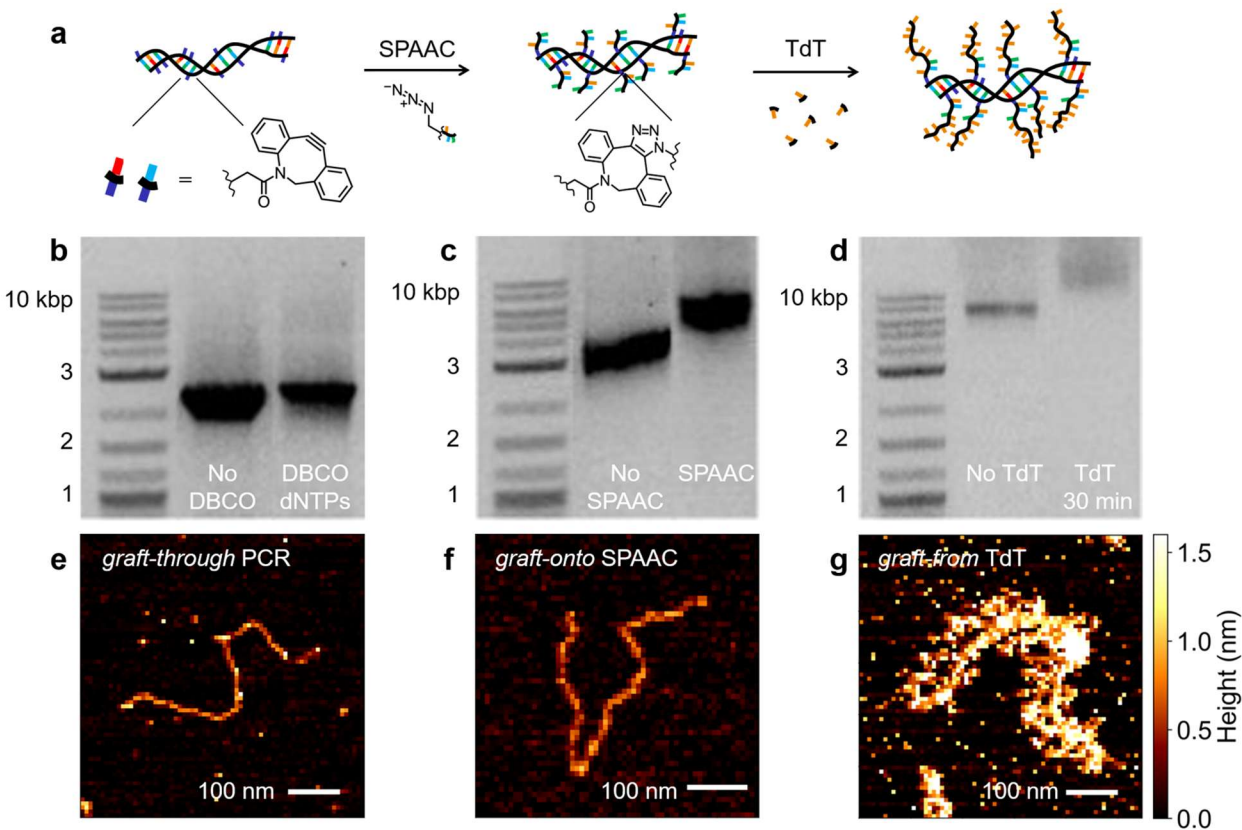


Fig. 1. Hybrid enzymatic synthesis, validation, and single-molecule visualization of DNA bottlebrush polymers. **a**, PCR amplification of linear dsDNA with 5-DBCO-PEG₄-dNTPs enables *graft-through* integration of reactive sites and subsequent *graft-onto* addition of 5'-azido-oligonucleotide ssDNA initiators via strain-promoted azide-alkyne cycloaddition (SPAAC). Then, *graft-from* reactions extend ssDNA initiators via terminal deoxynucleotidyl transferase (TdT)-catalyzed addition of dNTPs. Agarose gel electrophoresis indicates hindered electrophoretic migration of representative **b**, *graft-through* products (PCR with 25% replacement of dTTP and dCTP with 5-DBCO-PEG₄-dUTP and 5-DBCO-PEG₄-dCTP, respectively); **c**, *graft-onto* intermediates after SPAAC; and **d**, *graft-from* products after TdT-catalyzed dNTP addition (30 min reaction with dTTP). Hindered migration is consistent with larger effective molecular weights after each synthetic step. Tapping-mode AFM visualizes the molecular conformations of representative DNA intermediates and products, specifically **e**, *graft-through* products (PCR with 25% replacement of dTTP and dCTP); **f**, *graft-onto* intermediates after SPAAC; and **g**, DNA-based bottlebrush polymers from TdT-catalyzed *graft-from* reactions (30 min reaction with dTTP). AFM scale bars are 100 nm.

Results and Discussion

Enzymes mediate *graft-through* and *graft-from* polymerization of dense side chains

The synthesis of DNA bottlebrush polymers began with PCR amplification, which served as a *graft-through* approach to incorporate reactive dibenzylcyclooctyne (DBCO) throughout dsDNA backbones. Each Watson–Crick base pair (bp) was subject to potential chemical modification by replacement of natural pyrimidines dTTP and dCTP with 5-DBCO-PEG₄-dUTP and 5-DBCO-PEG₄-dCTP, respectively. PEG₄ linkers improve the steric accessibility of DBCO during post-amplification coupling, and pyrimidine modifications at the C5 position are tolerated by several native and engineered DNA polymerases.^{23,35,36} *Taq* polymerase tolerated over 50% molar replacement of dTTP and dCTP during amplification of 2.2 kilobase pair (kbp) templates from λ DNA (**Fig. S1**), corresponding to a theoretical modification of up to half of all bp. *Graft-through* products with 25% replacement of dTTP and dCTP exhibited slower electrophoretic migration than unmodified 2.2 kbp amplicons (**Fig. 1b**), suggesting larger average sizes of modified products. Slower migration and larger sizes are attributed to larger molar masses of DBCO-PEG₄-dNTPs than natural dNTPs, as well as chemical and physical interactions between DBCO-PEG₄ and agarose gel matrices.

Graft-from polymerization of side chains used TdT to catalyze the addition of dNTPs to 3' termini of DNA. TdT is a template-independent DNA polymerase that adds any natural dNTP to any 3' terminus, with routine addition reactions exceeding 300 dNTPs.³⁷ TdT has enabled DNA growth from patterned surfaces,^{38,39} *de novo* oligo synthesis as an alternative to solid-phase synthesis,^{40,41} and iterative homopolymer addition for data storage.⁴² To enable dNTP addition to 3' termini, 5'-azido-oligonucleotide “initiators” were coupled to DBCO-PEG₄-modified dsDNA using SPAAC. 5'-azide modifications enabled the display of 3' termini—the preferred substrates for TdT. Initiators comprised small, flexible ssDNA oligomers to reduce steric hindrance and improve coupling efficiency compared to prior syntheses of comb polymers using larger, semiflexible dsDNA side chains.^{23,24} SPAAC products migrated between 5.0–8.0 kbp dsDNA, consistent with at least 200% increases in effective sizes (**Fig. 1c**). Finally, initiators were extended using TdT-catalyzed addition of dTTP, which led to significantly hindered electrophoretic migration (**Fig. 1d**). TdT-extended products migrated more slowly than 10.0 kbp dsDNA, indicating over 5-fold effective size increases from the starting 2.2 kbp dsDNA.

After each synthetic step, AFM revealed changes to molecular structures (**Fig. 1e-g**). DNA intermediates and products were purified using spin columns, deposited on freshly cleaved

136 mica, and imaged using tapping-mode AFM.⁴³ DBCO-PEG₄-modified dsDNA adopted flexible
137 conformations, consistent with unmodified dsDNA⁴⁴⁻⁴⁶ (**Fig. 1e**). SPAAC products also adopted
138 flexible conformations (**Fig. 1f**), but thicker backbone contours, regions of increased stiffness,
139 and sharper bending angles were attributed to the presence of oligonucleotide initiators. TdT-
140 catalyzed *graft-from* polymerization with dTTP yielded bottlebrush-shaped DNA (**Fig. 1g**).
141 Dense side chains protruded more than 10 nm from the backbone, and the bottlebrush polymer
142 showed greater variation in molecular height than both precursors. Following the successful
143 synthesis and visualization of fully DNA-based bottlebrush polymers, we investigated the range
144 of molecular features that could be accessed by the hybrid polymerization approach.

145 TdT-catalyzed polymerization depends on 3' terminus and added dNTP compositions

146 Each synthesis step controls different molecular features. During *graft-through* PCR, DNA
147 template sequences and 5-DBCO-PEG₄-dNTP concentrations determine the backbone
148 molecular weight and side-chain density, respectively. This study focuses on a 2.2 kbp template,
149 which tolerates over 50% molar replacement of dTTP and dCTP (**Fig. S1**) and produces
150 backbone contour lengths that are amenable to AFM imaging (**Fig. 1e-g**). During *graft-onto*
151 SPAAC, initiator-to-backbone stoichiometry influences side-chain density. To maximize side-
152 chain density, excess initiators were added to backbones (800:1 molar ratio). During *graft-from*
153 TdT-catalyzed polymerization, dNTP and initiator composition influence TdT reactivity and
154 subsequent side-chain molecular weights. For bottlebrush polymers, homopolynucleotide side
155 chains are preferred to reduce secondary structure formation.⁴⁷⁻⁴⁹ Initiators with as few as 2 or 3
156 nucleotides can initiate TdT addition; however, longer initiators improve addition efficiency.^{37,50}

157 To evaluate the multiplexed parameter space of initiator sequences and dNTP compositions,
158 TdT-catalyzed polymerization was screened in solutions of ssDNA oligonucleotides prior to
159 testing a subset of conditions using *graft-onto* intermediates. A series of initiators varied the
160 oligonucleotide length, 3' terminal dNTP, and GC content (**Table 1**). Sequences were derived
161 from initiator "34C", which is a common PCR primer for amplicons from λ DNA including the 2.2
162 kbp backbone.^{23,24,51} Initiators were synthesized without 3'-phosphate groups to avoid inhibiting
163 TdT reactions. Solution-based screening does not replicate sterically hindered configurations
164 that arise at the beginning of *graft-from* polymerization; however, as polymerization proceeds, 3'
165 termini continually extend away from the backbone into less hindered aqueous environments.
166 Screening products were analyzed using polyacrylamide gel electrophoresis, which resolves
167 DNA shorter than 1.0 kbp more effectively than agarose (**Fig. 2a**). Effective ssDNA sizes were

estimated in nucleotides (nt) using densitometry and comparison to dsDNA standards (**Fig. 2b**), despite potentially different electrophoretic mobilities of ssDNA and dsDNA.

Table 1. Oligonucleotide initiators for solution-based screening reactions with TdT. Sequences are aligned to show overlap with 34C.

Name	Sequence	%GC
34A	5'-CTG ATG AGT TCG TGT CCG TAC AAC TGG CGT AAT A -3'	47.1
34C	5'-CTG ATG AGT TCG TGT CCG TAC AAC TGG CGT AAT C -3'	50.0
34G	5'-CTG ATG AGT TCG TGT CCG TAC AAC TGG CGT AAT G -3'	50.0
34T	5'-CTG ATG AGT TCG TGT CCG TAC AAC TGG CGT AAT T -3'	47.1
25T	5'-CTG ATG AGT TCG TGT CCG TAC AAC T-3'	48.0
15T	5'-TCG TGT CCG TAC AAC T-3'	53.3
9T	5'-CG TAC AAC T-3'	44.4
11C1	5'-CTG ATG AGT TC-3'	45.4
11C2	5'-CG TGT CCG TAC-3'	63.6
11C3	5'-C TGG CGT AAT C-3'	54.5

To identify a robust dNTP and initiator pairing for *graft-from* polymerization, combinatorial screening reactions added either dATP, dCTP, dGTP, or dTTP to initiators with different 3' termini (**Fig. 2c**, 34A, 34C, 34G, 34T, 30 min reaction). All combinations resulted in successful oligonucleotide extension, and dNTP and 3' termini compositions both influenced effective ssDNA sizes. Initiators 34A and 34T yielded the longest polymerizations with most dNTPs, with average ssDNA sizes over 100 nt. The combination of 34C and dATP produced ssDNA of 200 ± 50 nt, but 34C with dCTP, dGTP, and dTTP did not exceed 80 nt. Initiator 34G yielded the shortest polymerizations with all dNTPs.

Initiators 9T, 15T, 25T, and 34T explored the influence of initiator length with the same termini and similar GC content. Only 34T successfully added all dNTPs (**Fig. 2d**, 30 min reaction). dATP addition proceeded with all four initiators, whereas dCTP, dGTP, and dTTP addition required different minimum initiator lengths. Surprisingly, initiator length had a negligible influence on final effective size, suggesting TdT requires a threshold length to catalyze dNTP addition. Once addition was initiated, added dNTP composition and reaction time determined the effective size. Initiators 11C1, 11C2, and 11C3 explored the influence of GC content with the same termini and length (**Fig. S2**, 30 min reaction). These initiators yielded consistent dTTP addition and suggested a negligible role of GC content. Interestingly, the 3' terminal dNTP may influence the threshold length required to catalyze dNTP addition.

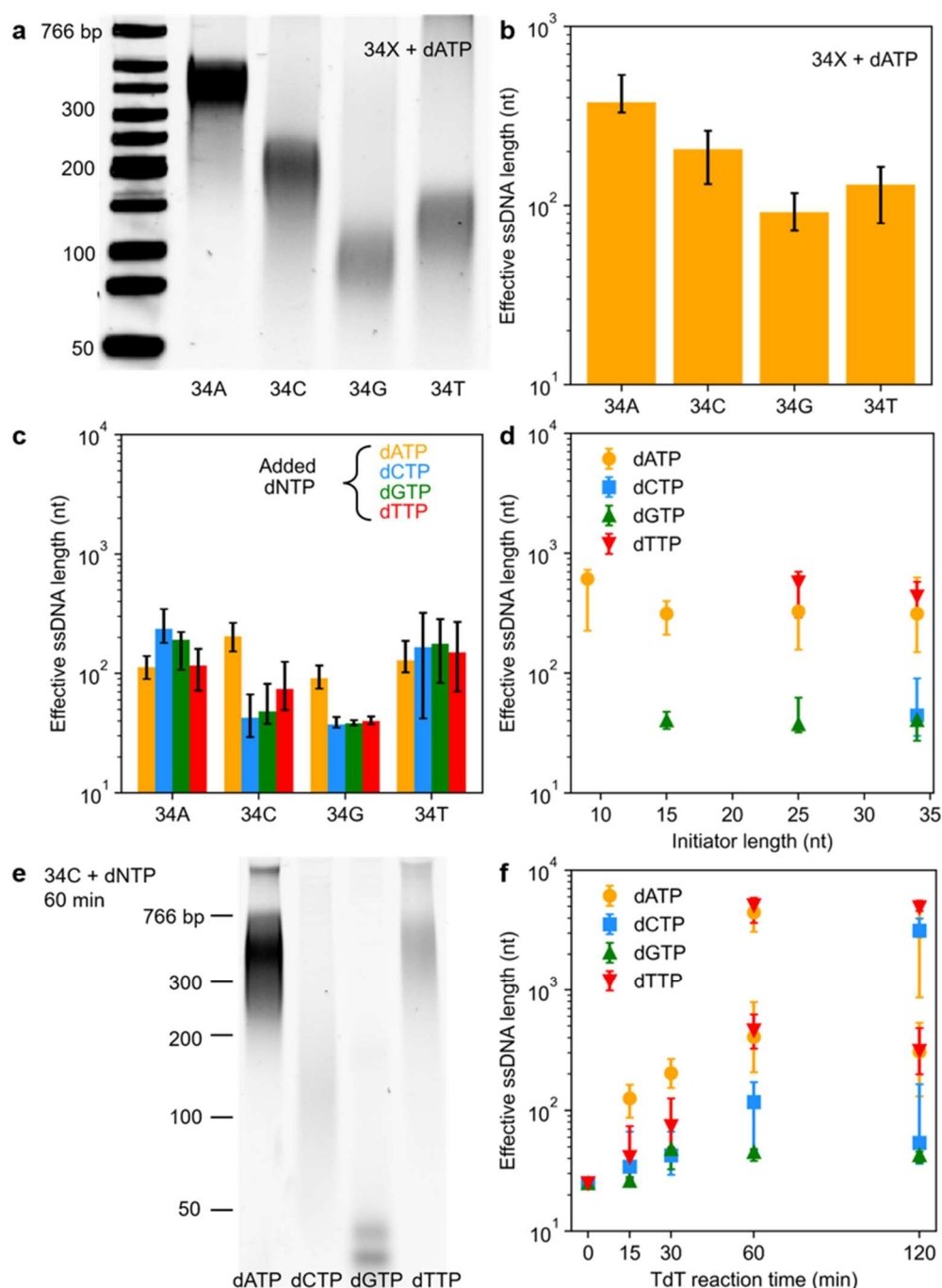


Fig. 2. Screening initiator composition, dNTP composition, and reaction time on TdT-catalyzed addition in solution. **a**, dATP addition reactions using initiators with different 3' termini are quantified using polyacrylamide gel electrophoresis. **b**, Effective ssDNA sizes are estimated by assuming similar electrophoretic migration of 100 bp dsDNA and 100 nt ssDNA. Bar charts reflect product distributions quantified using densitometry for each band, specifically indicating the maximum intensity and full width at half maximum. **c**, Combinatorial screening of dNTP and 3' termini compositions using 34-nt initiators. **d**, Initiator length screening suggests threshold initiator lengths are required to catalyze addition of each dNTP. **e**, Representative broad and bimodal TdT product distributions after 60 min. The full-size gel with DNA ladders is shown in **Fig. S3**. **f**, Longer TdT reactions yield larger effective ssDNA sizes and broader product distributions.

Finally, initiator 34C was used to evaluate dNTP addition as a function of reaction time (**Fig. 2e,f**). 34C was selected due to the range of ssDNA sizes reached after 30 min reactions with different dNTPs. Longer reactions yielded larger ssDNA, consistent with a living chain-growth polycondensation mechanism.⁵² In some cases, 60 min or longer reactions led to polydisperse or bimodal product distributions (**Fig. 2e, Fig. S3**). Broad product distributions may result from varying TdT activity over longer reactions, preferential extension of longer substrates, or hindered migration of spontaneous secondary structures. Ultimately, dTTP addition to initiator 34C was selected for *graft-from* polymerization of bottlebrush polymers. This combination produced ssDNA with low polydispersity during shorter reactions. Moreover, poly(dT) is less prone to spontaneous secondary structure formation than other homopolynucleotides,^{48,52} and poly(dT) provides future chemical versatility via facile addition of chemically modified dUTPs.

Independent *graft-through* and *graft-from* polymerization reactions tune the molecular dimensions of DNA bottlebrush polymers

The tunability of DNA comb and bottlebrush polymers was evaluated by varying *graft-through* and *graft-from* polymerization conditions. Side-chain density was tuned by dNTP substitution ratios during *graft-through* PCR. dTTP and dCTP were substituted with 5-DBCO-PEG₄-dNTPs at molar ratios of 1:8, 1:4, 1:2, or 1:1, such that reactions with 1:8 ratios contained 3.1% 5-DBCO-PEG₄-dUTP, 21.9% dTTP, 3.1% 5-DBCO-PEG₄-dCTP, 21.9% dCTP, 25% dATP, and 25% dGTP. Similarly, reactions with 1:1 ratios contained 12.5% 5-DBCO-PEG₄-dUTP, 12.5% dTTP, 12.5% 5-DBCO-PEG₄-dCTP, 12.5% dCTP, 25% dATP, and 25% dGTP. Side-chain length was tuned by performing *graft-from* polymerization for 15, 30, or 60 min.

To monitor changes to molecular structures at each step, AFM was used to compare unmodified dsDNA, *graft-through* PCR products, *graft-onto* SPAAC products, and *graft-through* TdT products (**Fig. 3**). Representative images of *graft-through* PCR products show that most dNTP substitution ratios produce similar molecular heights and flexibility to unmodified dsDNA. A notable exception is the lowest ratio (1:8), which produced pearls-on-a-string morphologies that persisted through several reaction steps. This morphology is attributed to attractive hydrophobic interactions between DBCO-PEG₄-dNTPs, which may form local clusters when sparsely distributed along a DNA backbone.

Graft-onto SPAAC products appear taller and thicker than *graft-through* products, indicating successful coupling of azido-oligonucleotide initiators to DBCO-PEG₄-modified backbones. The architectural transition from comb to bottlebrush polymer is defined by the dNTP substitution

ratio. The 1:8 ratio yielded pearls-on-a-string morphologies, suggesting comb polymers with sparse grafting. The 1:4 ratio produced molecules with taller regions that were not noticeably thicker in the x-y raster plane. These features suggest comb polymers with moderate grafting, wherein initiators have enough space along the backbone to adopt coiled conformations. For ssDNA, the radius of gyration is between 2–3 nm depending on ionic strength, which varies between reaction steps and imaging; the contour length is approximately 20 nm.⁵³ Molecular contours with nanometer-scale heights are more consistent with coiled conformations than extended conformations. 1:2 and 1:1 ratios led to thicker molecular contours, with line widths exceeding 20 nm. Thicker molecules suggest densely grafted initiators that adopt extended conformations, which are characteristic of bottlebrush polymers.

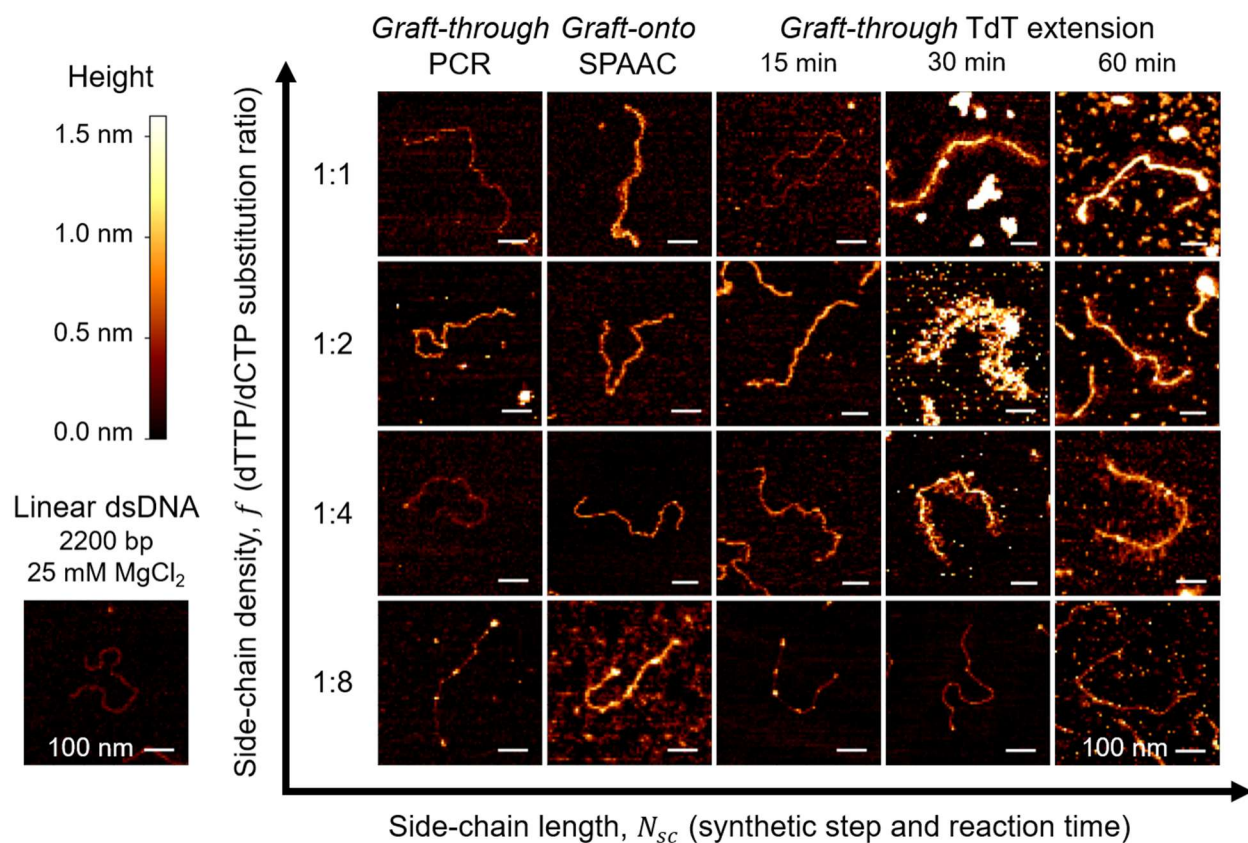


Fig. 3. Single-molecule visualization at each synthetic step reveals architectural transitions from comb polymers to bottlebrush polymers upon increasing side-chain density and length. Representative molecules for each reaction condition were selected from larger ensembles ($N = 6$ to 56 molecules). Scale bars are 100 nm.

As *graft-from* reactions proceeded for longer times, longer side chains enhanced the comb and bottlebrush architectures defined by side-chain density. For the 1:8 dNTP substitution ratio, longer polymerization yielded several prominent side chains and disrupted pearls-on-a-string morphologies. For other substitution ratios, elongated side chains emerged after only 15 min

and grew prominently after 30 and 60 min. After polymerization for 30 or 60 min, images of deposited samples revealed smaller fragments in the background. Deposited fragments are attributed to either polymerization of residual, ungrafted initiators or chain scission during solution transfer and deposition. DNA solutions were gently handled with wide-bore pipette tips to reduce flow-induced fragmentation;^{54,55} however, bottlebrush polymers are prone to spontaneous scission upon adsorption to a substrate.⁵⁶⁻⁵⁸ Despite the risk of scission, some molecules reached very large dimensions—for example, the 1:2 ratio after 30 min—but such features were not observed across the entire ensemble from a given reaction condition.

Molecular dimensions were quantified by extracting contour lengths L_c and persistence lengths ℓ_p from ensembles of deposited molecules (**Fig. 4**). Individual molecules were identified and traced using TopoStats⁵⁹ (**Fig. 4a**). For unmodified dsDNA ($N = 43$ molecules), L_c ranged from 230 nm to 780 nm, with a mean of 590 ± 30 nm and median of 655 nm. These lengths are consistent with the theoretical L_c of 750 nm, predicted using 0.34 nm per bp⁶⁰ (**Fig. 4b**). Shorter contours are attributed to either 3D structures that are not captured by the tracing algorithm or chain scission during deposition. To determine ℓ_p , tangent vector correlation functions were fit to equation (1):

$$\langle \cos \theta \rangle = \exp(-L/2\ell_p) \quad (1)$$

where $\langle \cos \theta \rangle$ is the mean cosine of the angle θ between two segments separated by length L (**Fig. 4c**). For linear dsDNA, ℓ_p of 57.4 ± 0.2 nm agrees with previous reports,^{44,46,61} and the error represents the square root of the variance of the fit.

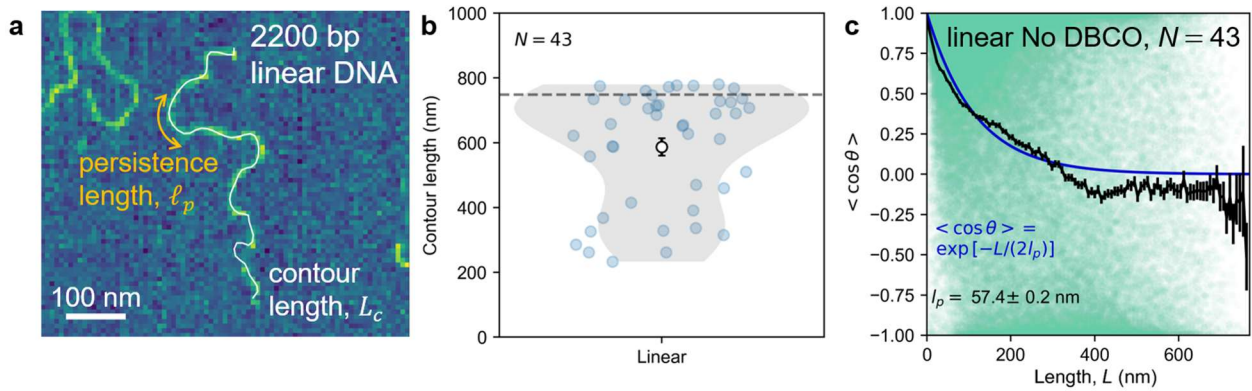


Fig. 4. Quantification of contour lengths L_c and persistence lengths ℓ_p . **a**, Representative contour tracing (white) of linear dsDNA (green) deposited on mica (blue). Chain flexibility is quantified from ℓ_p (orange). **b**, A violin plot displays the L_c distribution for linear dsDNA (2.2 kbp, $N = 43$ molecules). The dashed line indicates the theoretical L_c of 750 nm. **c**, ℓ_p are fit from tangent vector correlation functions. The black line represents the ensemble-average tangent

vector correlation function, and the blue line shows the fit of all data points (green) to an exponential decay function.

Graft-through, *graft-onto*, and *graft-from* reactions produced nonmonotonic changes in L_c that reflect changes in side-chain chemistry (**Fig. 5, Fig. S4**). First, *graft-through* PCR consistently led to shorter L_c . The 1:8 substitution ratio produced the shortest molecules, averaging 350 ± 40 nm. Shorter L_c are consistent with pearls-on-a-string morphologies (**Fig. 3**) and attributed to intramolecular associations between sparse, hydrophobic DBCO groups. For 1:4 and 1:2 ratios, L_c increased to 510 ± 30 nm and 540 ± 10 nm, respectively. The trend towards L_c for unmodified dsDNA suggests a balance between intramolecular associations and steric crowding upon greater incorporation of 5-DBCO-PEG₄-dNTPs. Next, *graft-onto* SPAAC generally increased L_c compared to *graft-through* PCR ensembles. For 1:8 and 1:4 ratios, longer L_c are consistent with enhanced steric crowding by initiators. For the 1:2 ratio, shorter L_c are attributed to the limited number of molecules observed after deposition ($N = 6$). In all cases, L_c did not exceed its theoretical limit, indicating that initiators did not contribute excess torsion or overstretching of dsDNA backbones.

Graft-from polymerization led to longer, more broadly distributed L_c than the starting *graft-onto* ensembles. For the 1:8 substitution ratio, longer reactions led to L_c with comparable averages to unmodified dsDNA, suggesting that ssDNA side chains disrupt intramolecular associations between hydrophobic DBCO groups. For 1:4 and 1:2 ratios, longer reactions led to broader L_c distributions. A few shorter bottlebrush molecules were attributed to deposition-induced chain scission. In these cases, fragments of bottlebrush-like structures were occasionally observed in the vicinity of a larger, deposited bottlebrush polymer. Larger bottlebrush molecules extended to contours exceeding the theoretical L_c for unmodified dsDNA. Excess contours likely result from the TopoStats algorithm, which could not discern side chains from backbones—especially near the ends of molecules. It is also possible for TdT to add dNTPs to the 3' ends of dsDNA backbones. Overall, most molecules remained constrained by L_c of the dsDNA backbone.

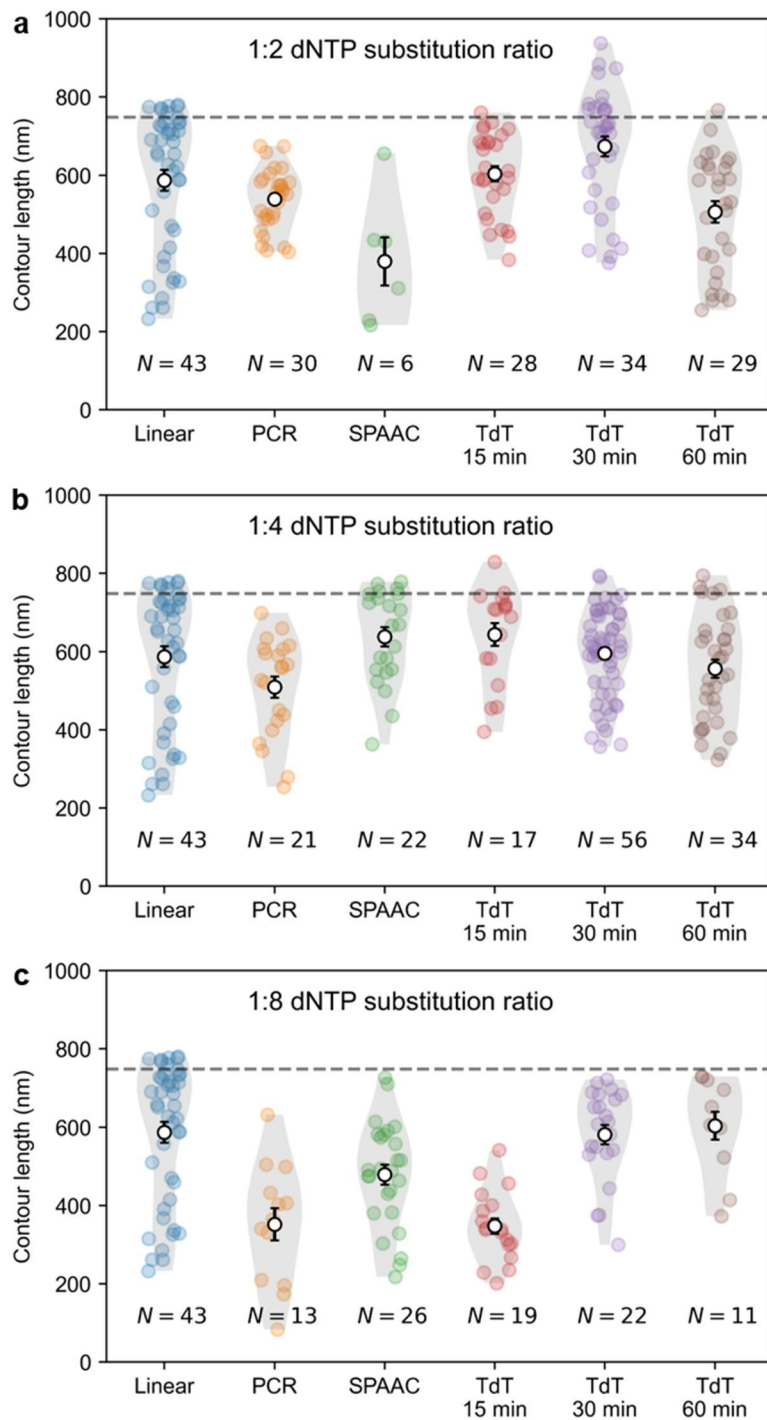


Fig. 5. Contour length distributions at each synthetic step suggest a balance between side-chain interactions and steric crowding on L_c . Distributions are shown for **a**, 1:2, **b**, 1:4, and **c**, 1:8 dNTP substitution ratios. For the 1:1 substitution ratio, molecular deposition onto mica was more challenging and resulted in limited ensemble sizes ($N < 15$) as shown in **Fig. S4**.

Graft-from polymerization products were classified as either comb or bottlebrush polymers by determining ℓ_p (**Fig. 4c**, **Fig. 6**). 15 min reactions led to ℓ_p within 10% of the value for dsDNA, indicating no significant changes in chain flexibility and comb polymer architectures (**Fig. 6a**). Interestingly, the 1:8 substitution ratio produced the stiffest molecules in this comparison set ($\ell_p = 62.2 \pm 0.3$ nm), likely due to stiffer “strings” connecting aggregated “pearls.” The 1:8 substitution ratio produced modest, nonmonotonic changes in ℓ_p after 30 min and 60 min reactions (49.9 ± 0.2 nm and 64.5 ± 0.4 nm, respectively). In contrast, higher substitution ratios of 1:4, 1:2, and 1:1 consistently produced stiffer ensembles after 30 min and 60 min reactions, with ℓ_p ranging from 66.0 ± 0.2 nm to 98.9 ± 0.5 nm (**Fig. 6b-d**). Progressive stiffening as *graft-from* polymerization proceeds is consistent with the tunable synthesis of DNA bottlebrush polymers.

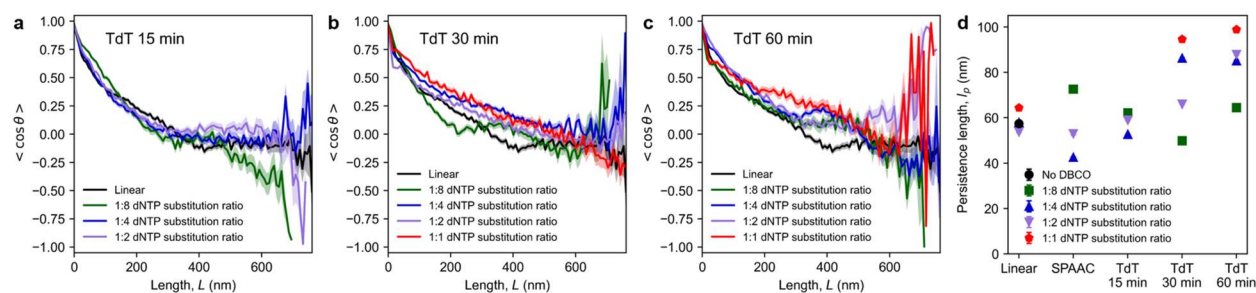


Fig. 6. Ensemble-average tangent vector correlation decay functions show stiffening of bottlebrush polymers with higher dNTP substitution ratios after longer *graft-from* reactions. **a**, 15 min reactions produced minimal changes in stiffness for all dNTP substitution ratios. As dNTP substitution ratios increase, progressive stiffening after **b**, 30 min and **c**, 60 min reactions indicated the successful polymerization of bottlebrush DNA polymers. **d**, Persistence lengths quantify stiffness of DNA comb and bottlebrush polymers. Error bars represent the square root of the variance of the fit and are smaller than the symbols.

Conclusions

We developed an enzymatic polymerization strategy to produce DNA comb and bottlebrush polymers with tunable architectural features. This strategy provided control over backbone molecular weight and side-chain density, composition, and molecular weight. *Graft-through* PCR tolerated high molar substitution of pyrimidines with chemically modified analogs, enabling the incorporation of dense side chains throughout dsDNA backbones. Solution-based screening of TdT-catalyzed polymerization identified initiators for *graft-onto* coupling to dsDNA backbones and controlled conditions for *graft-from* polymerization of high-molecular-weight side chains.

DNA-based polymers in this work were designed with appropriate dimensions to characterize size and flexibility using single-molecule AFM, which identified conditions to generate either

comb or bottlebrush polymers. Generally, comb-to-bottlebrush transitions were driven by increasing the side-chain density or molecular weight. Hybrid *graft-through* and *graft-from* polymerization is well-suited for future syntheses of DNA bottlebrush polymers with well-defined, high-molecular-weight backbones to investigate single-polymer dynamics using fluorescence optical microscopy. This approach would provide unprecedented insights into molecular-scale features and mechanisms underlying the design of bottlebrush polymers for adhesion, lubrication, fracture resistance, self-assembly, and more. Broadly, the enzymatic polymerization strategy is a promising platform for advances in nucleic-acid-based delivery, molecular machines, and tunable photonics.

Methods

Materials

A detailed list of materials is included in **Table S1**.

Graft-through polymerase chain reaction (PCR)

PCR was used to amplify dsDNA backbones and incorporate 5-DBCO-PEG₄-dNTPs as reactive sites for *graft-onto* SPAAC. Prior to handling PCR reagents, working surfaces were cleaned with ethanol and Formula 409® disinfectant spray. After cleaning, stock solutions of dNTPs, primers, λ-DNA templates, *Taq* DNA Polymerase, and Thermopol® reaction buffer were thawed from –20 °C on ice. To isolate dNTPs, primers, and λ-DNA templates from *Taq* DNA Polymerase prior to thermal cycling, two separate master mixes were prepared on ice. Aliquots from each master mix were combined into thin-walled PCR tubes, mixed by briefly pipetting, and immediately loaded onto a preheated thermocycler for enzymatic amplification (BioRad C1000). Detailed compositions and thermal cycling protocols are included in **Table S2**. After PCR amplification, samples were held at 4 °C prior to purification and additional reactions. All PCR products were handled with gentle pipetting and wide-bore pipette tips to prevent flow-induced fragmentation.

Graft-onto strain-promoted azide–alkyne cycloaddition (SPAAC)

SPAAC was used to couple ssDNA oligonucleotide initiators with 5'-azide modifications to 5-DBCO-PEG₄-dNTPs incorporated along DNA backbones. SPAAC reactions used an 800:1 molar ratio of oligonucleotide initiators to backbones. Assuming equivalent incorporation of 5-DBCO-PEG₄-dNTPs and unmodified dNTPs, 2.2 kbp dsDNA backbones would contain either 138, 275, 550, or 1,100 DBCO modifications for 1:8, 1:4, 1:2, or 1:1 dTTP/dCTP substitution

ratios, respectively. SPAAC reactions were conducted in 250 mM Tris (pH 8.0), 10 mM EDTA, and 1.0 M NaCl at 70 °C for 18 hr.²³ High concentrations of Tris provided excess buffering capacity at elevated temperature, EDTA inhibited potential nuclease activity, and NaCl screened electrostatic interactions to promote coupling of negatively charged DNA. Completed reactions were cooled to 4 °C for safe handling during purification and additional reactions.

Graft-from terminal deoxynucleotidyl transferase (TdT)-catalyzed polymerization

TdT was used to perform *graft-from* polymerization of ssDNA side chains from oligonucleotide initiators in solution or after SPAAC coupling to dsDNA backbones. TdT reactions used a 5000:1 molar ratio of dNTPs to 3' ends and were carried out in 50 µL reaction volumes containing 1× TdT reaction buffer (50 mM potassium acetate, 20 mM Tris acetate, 10 mM magnesium acetate, pH 7.9 at 25 °C), 0.25 mM CoCl₂, 10 units TdT, and 100 ng to 400 ng purified SPAAC products. TdT reactions were incubated at 37 °C for the specified duration, followed by heating to 70 °C for 10 min to quench the reaction by denaturation of TdT. Completed reactions were cooled to 4 °C for safe handling during purification.

DNA purification

DNA products were purified following each synthetic step. PCR and SPAAC products were purified and concentrated using QIAquick PCR Purification Kits (Qiagen) following manufacturer instructions. DNA products from TdT reactions were purified and concentrated using Vivacon® 2 Centrifugal Concentrator (100,000 MWCO, Sartorius) spin columns, with 3 wash cycles into water. The concentrations of purified DNA samples were determined by measuring absorbance at 260 nm (A₂₆₀) using a NanoDrop One UV-Vis spectrophotometer (Thermo Scientific).

Gel electrophoresis

Agarose gel electrophoresis was used to validate PCR, SPAAC, and TdT reaction products. 1.0 wt% agarose gels were prepared in 1× TAE buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA, pH 8.3). DNA samples were mixed with 1× SYBR Gold nucleic acid stain and loading buffer prior to loading into agarose gels, which were run in 1× TAE buffer at 120 V for 30 min to resolve product bands. Agarose gels were directly imaged using a GelDoc Go UV imaging system.

Polyacrylamide gel electrophoresis was used to validate ssDNA reaction products from TdT-catalyzed polymerization in solution. 12.0 wt% polyacrylamide gels were cast in 1× TBE buffer (100 mM Tris, 100 mM boric acid, 2 mM EDTA, pH 8.3) by crosslinking an acrylamide/bis

solution (29:1) with ammonium persulfate and TEMED. ssDNA samples were mixed with loading buffer prior to loading in polyacrylamide gels, which were run in 1× TBE buffer at 120 V for 60 min to resolve product bands. Polyacrylamide gels were stained by submersion in 1× SYBR Gold nucleic acid stain in 1× TBE and gentle rocking for 30 min prior to UV imaging.

Atomic force microscopy (AFM)

DNA samples were deposited onto freshly cleaved mica to conduct AFM imaging on atomically flat surfaces. Grade V4 muscovite mica discs (Electron Microscopy Sciences) were cleaved by scoring the edge with a razor blade and adhering Scotch® Double-Sided Tape to the top surface. Atomically flat, cleaved mica sheets were generated by peeling off the tape and adhering it to a microscope slide with the mica facing upward.

Prior to deposition, solutions containing 1–2 ng/μL DNA were incubated in 5–25 mM MgCl₂ at 40 °C for 2 hr. Mg²⁺ was used to bridge the negative charge of the DNA phosphate backbone and the negative surface charge of mica, and DNA and MgCl₂ concentrations were adjusted to ensure good surface coverage. For example, 1 ng/μL DNA in 5.0 mM MgCl₂ led to good surface coverage with 2.2 kbp linear dsDNA but poor surface coverage with PCR products containing 5-DBCO-PEG₄-dNTPs. Generally, higher concentrations of MgCl₂ were required for successful deposition of DNA bottlebrush polymers. 20 μL DNA solutions were deposited onto exposed, freshly cleaved mica surfaces. Droplets were equilibrated for 5 min in a covered petri dish before rinsing with 2 mL MilliQ water. Rinsed mica surfaces were dried with house nitrogen prior to AFM imaging.

Deposited DNA samples were visualized using a JPK NanoWizard V5 BioAFM in PeakForce Tapping Mode. SCANASYST AIR-HR (Bruker) tips were used for all imaging experiments ($k = 0.4$ N/m, $f_0 = 130$ kHz). Images were collected using a 1 nN force setpoint, 800 Hz oscillation frequency, 54 nm oscillation amplitude, and 0.7 Hz line scan rate. AFM images were analyzed using TopoStats,⁵⁹ an open-source Python package for molecular identification and contour tracing. Persistence lengths were calculated from contours traced by TopoStats.

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Contributions

Conceptualization: M.C.B., D.J.M.; methodology: M.C.B., L.N., L.X.W.; analysis: M.C.B., L.X.W., D.J.M.; investigation, M.C.B., L.N.; resources: M.C.B., L.N.; data curation: M.C.B.; writing—original draft: M.C.B., D.J.M.; writing—review and editing, all authors; funding acquisition, M.C.B., D.J.M.; supervision, D.J.M.; project administration, D.J.M.

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Competing interests

The authors declare no competing interests.

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