Solid-Phase ATRP

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Solid-Phase Incorporation of an ATRP Initiator for Polymer–DNA Biohybrids**

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Abstract: *The combination of polymers with nucleic acids leads to materials with significantly advanced properties. To obviate the necessity and complexity of conjugating two macromolecules, a polymer initiator is described that can be directly covalently linked to DNA during solid-phase synthesis. Polymer can then be grown from the DNA bound initiator, both in solution after the DNA-initiator is released from the solid support as well as directly on the solid support, simplifying purification. The resulting polymer-DNA hybrids were examined by chromatography and fluorescence methods that attested to the integrity of hybrids and the DNA. The ability to use DNA-based supports expands the range of readily available molecules that can be used with the initiator, as exemplified by direct synthesis of a biotin polymer hybrid on solid-support. This method expands the accessibility and range of advanced polymer biohybrid materials.*

Combining polymers with biologically related molecules has spawned a diverse range of tremendously enhanced polymer biohybrid materials.[1–6] Among these, DNA block copolymers (DNABCps) are a novel class of bioconjugates that have a DNA segment covalently attached to an organic polymer segment.[7–9] By tuning the properties of the DNA and the organic polymer, the properties of the DNABCp can be tailored. Such control has led to DNABCps that form reversible micelles and nanoscale assemblies, including some that are responsive to DNA-based recognition events.[10–16] These DNABCps have been used in sensitive biological sensors for single-nucleotide polymorphism detection and as scaffolds for organic reactions.[17–19] Potent drug-delivery vehicles based on DNABCps have also been used with antiviral DNA and towards anticancer therapeutics.[20–23] We previously used a DNABCp-based linker to prepare a non-covalent protein–polymer hybrid.[24]

The primary method to obtain DNABCps involves separate syntheses of the polymer and the DNA segments, followed by a conjugation reaction to covalently attach them.[7,8] This strategy, known as "blocking-to" (b-t), offers the advantage that the composition of the polymer segment is well known prior to conjugation to the DNA segment. The DNA segment, available through modern automated solid-phase synthesis methods, can include highly specific sequences that incorporate various modifications, such as fluorescent dyes or biotin as well as reactive groups for conjugation to the polymer segment. The polymer segment of the DNABCp may be readily prepared by reversible deactivation radical polymerization (RDRP) methods that allow the preparation of well-defined polymers with precise control of chain-end functionality, molecular weight (MW), and MW distribution (MWD).[3,8,17,25–28] One of the most common RDRP methods is atom-transfer radical polymerization (ATRP), which is tolerant to a broad range of functional groups and chain ends.[29–33] Therefore, ATRP has enjoyed wide use in the preparation of a diverse spectrum of biological conjugates, such as protein polymer hybrids, polysaccharide polymer conjugates, and DNABCp.[13,18,24,25,30,31,34–37]

An alternate strategy for the synthesis of DNABCps would be "blocking-from" (b-f), wherein an initiator is covalently attached to the DNA and the polymer is subsequently synthesized using chain extension reactions.[38] There are some advantages to this b-f strategy over the b-t approach. First, b-f allows for simpler purification of the low MW monomers and catalyst from the polymer bioconjugates. Second, in the b-f strategy the reactions are monomer addition reactions, whereas in b-t the conjugation reaction is between two incompatible large molecules (the polymer and the DNA), which is sterically more challenging.

There have been a few recent reports of the b-f strategy for preparing DNABCp using either ATRP or reversible addition–fragmentation chain transfer polymerization.[18,39–41] In these reports, the DNA was functionalized with an amine that could be reacted with an activated ester of the initiator. These DNAs were immobilized on different surfaces (for example, a gold surface, gold nanoparticles) followed by polymer growth. As the polymers were grown from surfaces via the attached DNA, this precluded any direct analysis and characterization of the DNABCps. We therefore sought

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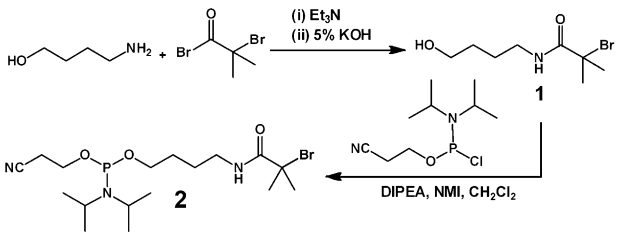
a more direct and facile method to prepare DNA bound initiators and to characterize the polymer biohybrids to assess the degree of control over the b-f copolymerization.

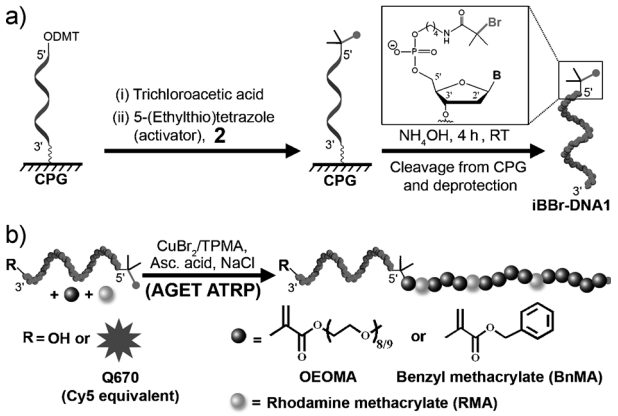
Herein, we report a straightforward method to incorporate an ATRP initiator onto DNA that is compatible with automated DNA synthesis on solid support. Following the coupling of the initiator, polymer synthesis from the DNA initiator can be performed through activators generated by electron transfer (AGET) ATRP[42–44] in solution or even directly on the solid support prior to deprotection and release of the DNA sequence. We analyzed the resultant DNABCps using gel permeation chromatography (GPC) and fluorescence spectroscopy, which confirmed that the DNA remained intact under the polymer synthesis conditions, and we obtained DNABCp with narrow MWD and control over the desired MW using different monomers. We show that the DNABCps have DNA functionally preserved through hybridization experiments. Our approach also permits ready and rapid functionalization of polymers with the diverse small molecules that are available for use in solid-phase synthesis of nucleic acids. To exemplify this, we synthesized a biotin-modified polymer on solid support using the same ATRP initiator phosphoramidite. Overall, we present a strategy that allows direct access to DNABCps and other functionalized polymers.

For DNA synthesis on a solid support, the most common and direct method for attaching functional groups or small molecules is phosphoramidite coupling chemistry.[45] However, the incorporated functional group or small molecule needs to be stable under the nucleic acid deprotection conditions. Thereby, we first prepared an ATRP initiator phosphoramidite capable of surviving typical DNA deprotection conditions (concentrated ammonia at room temperature for 4 h). An amide linked initiator, **1**, was prepared from 4-aminobutanol and α-bromoisobutyryl bromide (Scheme 1).



Scheme 1. Synthesis of the ATRP initiator phosphoramidite.

Washing the product with 5% KOH ensured that no ester linked initiator, which might also form through reaction of the hydroxy group of 4-aminobutanol, remained as a contaminant. Following extraction, the intermediate **1** was reacted with 2-cyanoethyl-*N,N*-diisopropylchlorophosphoramidite to yield the ATRP-initiator phosphoramidite **2**. This ATRP initiator phosphoramidite could then be readily coupled to the 5′-hydroxy group of a DNA sequence on a solid support, obtained after acid deprotection of the terminal dimethoxytrityl (DMT) group (Scheme 2). The coupling of **2** was performed in 6 min using standard reagents (see the Supporting Information, Table S1, for detailed conditions) in near quantitative yields. As a test case, we performed the initiator coupling to 23-mer DNA (DNA1) on a 1 μmol-scale controlled pore glass (CPG) support. Following the coupling and incorporation of the initiator, ammonium hydroxide treatment for 4 h at room temperature cleaved the DNA from the CPG support and removed the nucleobase and cyanoethyl protecting groups, yielding the initiator-linked DNA, iBBr-DNA1 (see the Supporting Information, Table S2, for the sequence). The integrity of the initiator on the DNA was confirmed using mass spectrometry (MALDI-TOF; Supporting Information, Figure S2). Thus, with this phosphoramidite coupling approach, an ATRP initiator could be directly incorporated into the DNA sequence during standard solid-phase synthesis and afforded concomitant purification of DNA with initiator. Compared to previously reported activated ester couplings that are performed following DNA synthesis, deprotection, and purification,[18,39,40] this method offers both a simplified access and greater yield of the DNA with initiator.



Scheme 2. Solution-phase synthesis of DNABCp using AGET ATRP. a) After removal of the 5′-ODMT from the DNA (in the CPG bead), the ATRP initiator phosphoramidite was conjugated to 5′-OH. Cleavage from the solid support and removal of the base and cyanoethyl protecting groups yielded the DNA with the ATRP initiator (iBBr-DNA1). b) Direct synthesis of the DNA-polymer hybrid in solution by AGET ATRP using the initiator-modified DNA. Two different initiator modified DNAs (with either 3′-OH or 3′-Quasar670 dye) were used to synthesize polymers with OEOMA or benzyl methacrylate and rhodamine methacrylate as the monomers.

Recently, procedures have been developed that allow ATRP under biologically relevant conditions using AGET ATRP.[29,31,38,42,46] There are several advantages to this over traditional ATRP, including addition of the oxidatively stable copper/ligand complexes to the reaction and the reaction rate adjustment by controlling the feed rate of the reducing agent to diminish the total copper concentration.[29,47] To develop conditions suitable for block copolymer growth from iBBr-DNA1, the initiator concentration and reducing agent feed rate were kept constant, while varying several key reaction parameters, such as reaction time, catalyst, monomer, and salt concentration (Supporting Information, Table S3). The monomer chosen for the polymer synthesis was oligo(ethylene oxide) methacrylate (OEOMA, $M_n = 475$), owing to its biocompatibility and suitability in various biomedical appli-

cations (Scheme 2b).[48–50] A small percentage of rhodamine methacrylate (RMA) was also incorporated into the reaction mixture to facilitate visualization and characterization of the resultant DNABCp (Scheme 2b). With the iBBr-DNA1 concentration set to 1 mM, a 5 mM solution of ascorbic acid reducing agent was added to the reaction mixture by a syringe pump at a feed rate of 500 nL min^{-1} (2.5 nmol min^{-1}). One of the variables in the reaction was the NaCl concentration. The addition of NaCl to the reaction enhances the concentration of the deactivator leading to better control over the polymerization.[47] Here we varied NaCl concentration from 50 to 300 mM. Another parameter was the concentration of the catalyst, $CuBr_2$/TPMA (1:8), which was varied from about 1% to about 5% (by mol to monomer). The targeted degrees of polymerization were from about 100 to about 500, and the total reaction times varied from 0.5 to 2 h.

To characterize the resulting block copolymers, DNA1-*b*-POEOMA-*co*-RMA, the reaction mixtures were diluted in dimethylformamide (DMF) with 0.5% diphenyl ether as internal standard and injected into a DMF GPC, calibrated using poly(ethylene oxide). Well-defined polymers were grown from iBBr-DNA1 with good control over targeted MW and narrow MWD (Figure 1a; Supporting Information, Figure S3). To determine if the polymerization affected the DNA structure, a second DNA-initiator sequence with a 3′-terminal fluorescent dye, Quasar670 (Q670; a Cy5 equivalent) was prepared (Scheme 2b) using similar conditions (Supporting Information, Table S3, Entry 8). If the polymerization caused cleavage of the DNA, there would be a loss of Q670 dye after purification of the reaction mixture. Conversely, if the polymerization did not lead to DNA scission, there would be Förster resonance energy transfer (FRET) from the rhodamine dye in the grafted polymer to the Q670 dye at the 3′-end of the DNA. When characterizing the hybrids for DNA integrity using fluorescence spectroscopy (Figure 1), the products were purified using dialysis. The fluorescence spectra of the different hybrids show that FRET from the rhodamine to Q670 only in the case of the Q670 terminated DNABCp (Figure 1b, solid trace) but not when free dye was mixed with the DNA1-b-POEOMA-co-RMA. This indicated the DNA was not degraded during the polymerization.

To assess the compatibility of this method with a wider range of monomers and polymerization conditions, we used iBBr-DNA1 in AGET ATRP to prepare hydrophobic polymers using benzyl methacrylate (BnMA) and RMA (see the Supporting Information, Table S3, entry 9 for conditions). Owing to its charged phosphate backbone and water solubility, iBBr-DNA1 can act as a reactive surfactant in dispersion polymerization to form DNA1-*b*-PBnMA-*co*-RMA. The hydrophobicity of the polymer component results in aggregation of DNA1-*b*-PBnMA-*co*-RMA into large DNA-latex (DTEX) particles by dialysis of the DNA1-*b*-PBnMA-*co*-RMA into acetone and then into ultrapure water (Figure 2). The DTEX-DNA1 particles were characterized by dynamic light scattering and zeta potential analysis, which showed well defined particles with a diameter of 1.3 ± 0.09 μm (Figure 2b, inset) and a zeta potential of negative 25.8 ± 1 mV.

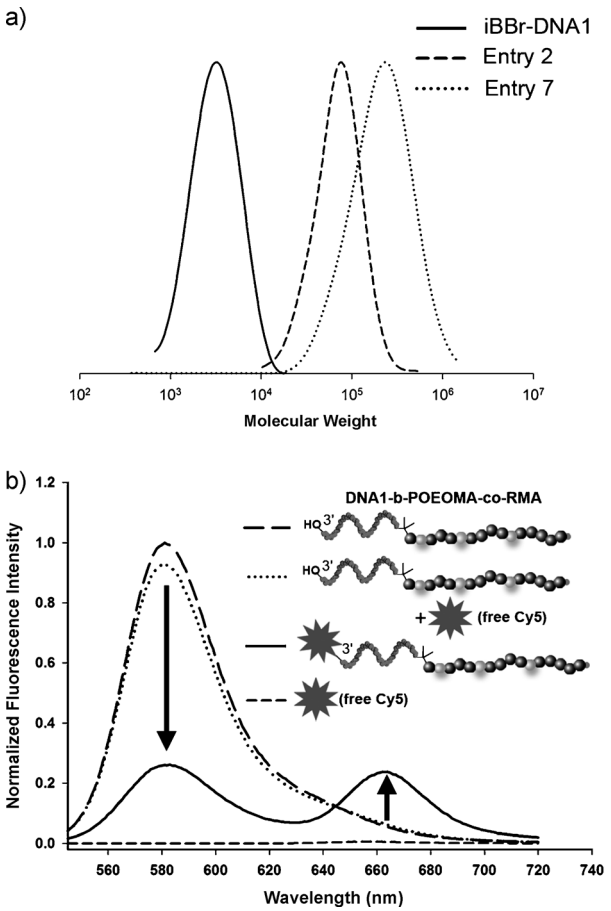


Figure 1. Characterization of the DNABCp synthesized using AGET ATRP in solution phase. a) GPC traces of the iBBR-DNA1 (solid trace) and the polymer–DNA conjugates (dashes, entry 2 and dotted, entry 7 in the Supporting Information, Table S3). The GPC traces show a significant shift of MW after polymerization, with no residual initiator iBBr-DNA1. The GPC characterization of all polymers are in the Supporting Information, Figure S3. b) Fluorescence spectra (with excitation at 530 nm) of the different DNA polymer hybrids show that DNA is directly conjugated to the rhodamine containing polymer. The DNA-polymer hybrid with terminal 3′-OH alone (long dashes) or together with a free dye (dotted), shows no energy transfer. However, the DNA-polymer hybrid with Q670 dye at 3′-terminus shows significant energy transfer (solid trace, arrows) indicating that the Q670 is in a close proximity to the rhodamine in the polymer chain and confirms the integrity of the DNA during polymerization using AGET ATRP.

To test the integrity and sequence recognition properties of the DTEX particles imparted by the 23-mer DNA1, the particles were first annealed to a partially complementary DNA strand, DY647-DNA1PC that included a fluorescent dye (Dylight647, a Cy5 equivalent) and 18 matched nucleotides (see the Supporting Information, Table S2, for sequences). Flow cytometry analysis of DTEX-DNA1 (Figure 2b; black solid trace) showed a large increase in the fluorescence in the Cy5 channel when annealed to DY647-DNA1PC (gray solid trace). However, no such increase was observed when a non-complementary sequence Dy647-DNA2 (gray dots) was used, demonstrating the sequence specific recognition by DTEX-DNA1. Furthermore, the partially complementary DY647-DNA1PC strand could be displaced from the particles

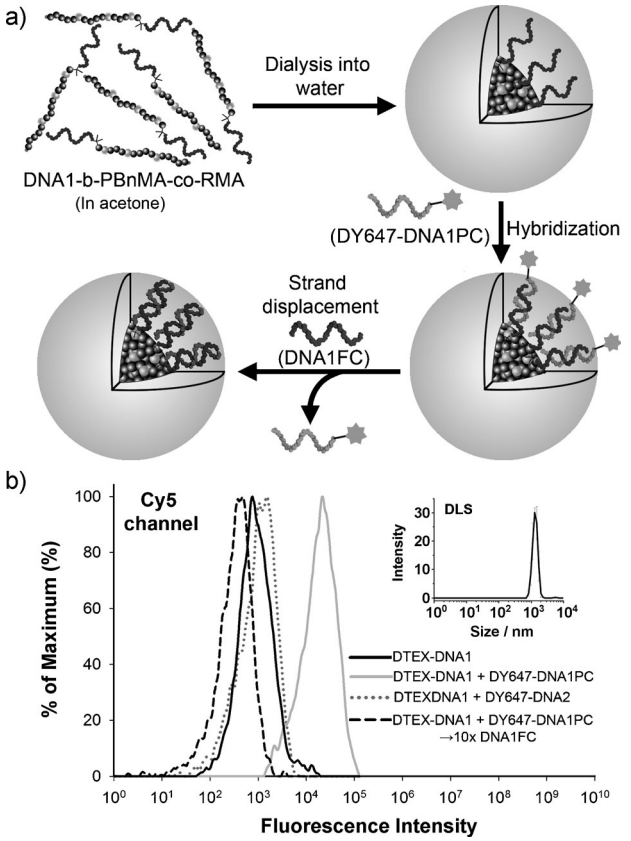


Figure 2. Synthesis and characterization of DNA-latex (DTEX) particles. a) The DNA1-b-PBnMA-co-RMA, obtained by block copolymerization from iBBr-DNA1 using BnMA and RMA monomers, was dialyzed from acetone into water. The hydrophobic polymer chains aggregate to form the core of the particles. The outer surface of the DTEX particles, exposed to water, remains decorated with DNA1 that selectively binds DY647-DNA1PC (with fluorescent dye). This partially complementary strand could be displaced from the DTEX particles using strand DNA1FC that is fully complementary to the DNA1 sequence. b) Flow cytometry with detection in Cy5 channel reveals these sequence specific hybridzations. DTEX-DNA1 (black solid line) shows a large increase in fluorescence when DY647-DNA1PC was hybridized (gray solid line). When a non-complementary strand (DY647-DNA2) was used for hybridization, the Cy5 signal did not increase (gray dots). The DY647-DNA1PC hybridized to the DTEX-DNA1 could be completely displaced by fully complementary strand DNA1FC (with no fluorescent dye) and the Cy5 signal (black dashes) reverted to baseline levels. Inset: volume distribution of the DTEX particles by dynamic light scattering.

with a 10X excess of DNA1FC that is fully complementary (all 23 residues) to the DTEX-bound DNA1. As DNA1FC included no fluorescent dye, the signal in the Cy5 channel of DTEX-DNA1 + DY647-DNA1PC after strand displacement with DNA1FC (black dashes) showed close correspondence to that of the initial DTEX-DNA1 and indicated complete displacement of the DY647-DNA1PC from the DTEX particles. The signal from the rhodamine channel for all particles that contain RMA in the hydrophobic polymer core remained unchanged (Supporting Information, Figure S4), suggesting stability of the polymer core. Overall, these experiments demonstrate that the DNA in these DNABCps retains the ability of sequence specific strand recognition following the synthesis of the polymer chain. We envision that such readily accessible DTEX particles, with their capacity for versatility in the polymer and DNA sequences would be useful towards biosensors and other applications.

To further evaluate the power and utility of solid-phase initiator incorporation, we attempted to grow polymer from the DNA initiator still attached to the solid support (Figure 3). We reasoned this method would facilitate not only the rapid preparation, but also the ready purification of functional bioconjugates from the unreacted monomers and

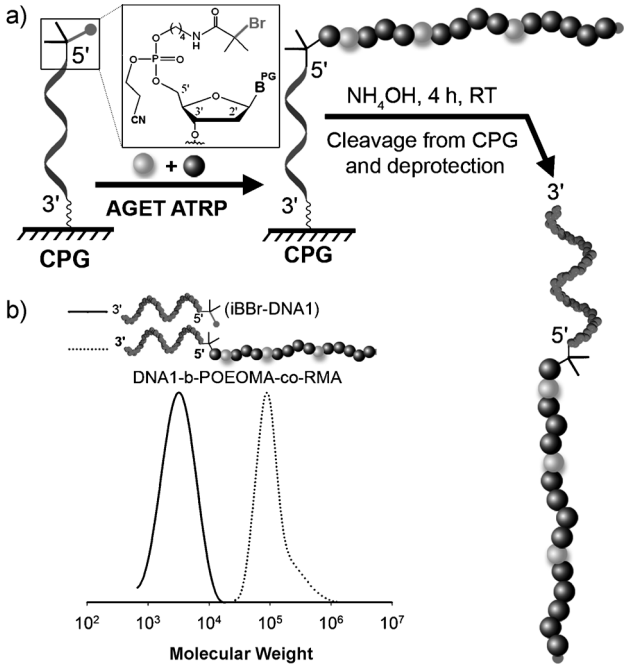


Figure 3. Synthesis and characterization of DNABCp prepared using a solid support. a) After coupling of the initiator phosphoramidite to the 5′-OH of the DNA, the polymer was synthesized using AGET ATRP with the protected DNA still attached to the CPG beads. After ATRP, the DNA-polymer conjugate was cleaved from the CPG bead and the protecting groups from the DNA were removed using standard conditions to form the functional DNABCp. b) GPC traces of the initiator modified DNA (black solid line) and the DNA-polymer conjugate (black dots) synthesized on solid support.

catalyst.[51–55] Additionally, using this strategy, there is an important difference in the DNA strand during polymer synthesis as the protecting groups are still on the nucleobases and the backbone has neutral phosphotriester groups (Figure 3a, inset). The CPG beads with the protected iBBr-DNA1 sequence were suspended in the polymerization medium and a polymerization was conducted in situ using 5% monomers (OEOMA and RMA) and 1.7 mol% Cu (Figure 3a). After extensive washing of the CPG beads with water to remove any unreacted monomer and catalyst, the beads were bright red, suggesting copolymer growth from the DNA initiator on solid support. Cleavage from the CPG beads and concomitant removal of the protecting groups of the DNA bases using standard DNA deprotection conditions yielded the DNABCp. The DNABCp was analyzed using GPC, which

indicated a molar mass of $M_n = 205\,000$ and $M_w/M_n = 1.43$ (Figure 3b) with a slight shoulder at higher MW. This demonstrates the direct preparation of well-defined DNABCps from CPG beads and provides a general approach to the synthesis of biologically related molecules and polymer hybrids through solid phase incorporation of the initiator.

A large variety of small-molecule-functionalized solid supports for nucleic acid synthesis are readily available, so the solid-phase ATRP method will allow rapid access to small (bio)molecule functionalized polymers. Any molecule-of-interest bound to CPG can be used to couple the ATRP initiator amidite **2** and AGET ATRP can be conducted on a solid support (Figure 4a). To show this, we used biotin-CPG

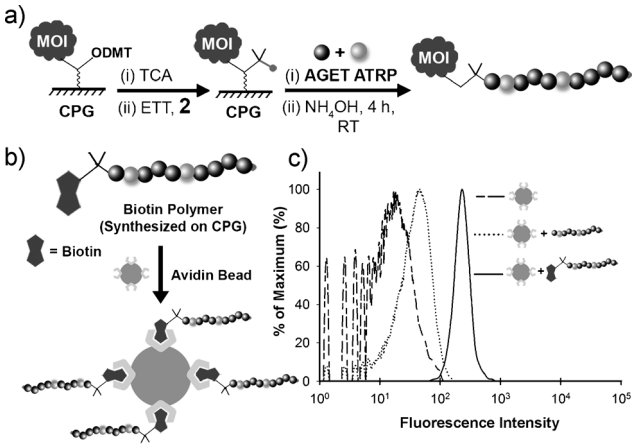


Figure 4. Synthesis of small-molecule polymer conjugates on a solid support. a) A molecule of interest (MOI) attached to solid support can be functionalized with the ATRP initiator. Subsequently, AGET ATRP on solid support can grow the polymer. This method to access polymer conjugates enjoys all the advantages of solid-phase synthesis. b) A biotin–polymer hybrid obtained through the solid-phase method binds to avidin in an assay to characterize the conjugate. c) Flow cytometry indicates the polymer without biotin did not bind to the avidin beads, giving rise to low fluorescence signal (black dot trace). When the biotin–polymer conjugate was used with the avidin beads, a population with significantly higher fluorescence was observed (solid black trace), indicating that the rhodamine polymer is directly conjugated to biotin.

and directly coupled with the ATRP initiator **2** and then conducted a b-f copolymerization of OEOMA and RMA from a solid support (Supporting Information, Figure S5a). After the polymerization, the beads were washed and the biotin functionalized polymer was cleaved from the solid support using ammonium hydroxide. Although there are alternate methods to incorporate biotin into the chain end of a polymer including use of soluble biotin initiators[56,57] and post-synthetic ligation[58,59] our method exemplifies the advantages of solid-phase initiator synthesis coupled with polymer growth. The biotin–polymer hybrid was characterized by GPC; the molar mass of the conjugate determined to be $M_n = 32\,000$ with $M_w/M_n = 1.2$ (Supporting Information, Figure S5b). To confirm that the biotin was intact at the chain end of the polymer, the polymer's ability to bind to avidin microbeads was assessed using flow cytometry (Figure 4c). The avidin microbeads themselves or the avidin microbeads with the OEOMA-RMA copolymer (without biotin) showed little fluorescence in the flow cytometry experiment (Figure 4c, black dash and black dot traces). However, when the biotin terminated polymer was incubated with the avidin microbeads, a significant shift in the peak to higher fluorescence (Figure 3b; solid black trace) indicates binding of the biotin–polymer hybrid with the avidin microbeads.

In conclusion, a robust method is presented for preparing DNABCps compatible with solid-phase nucleic acid synthesis strategies. An ATRP initiator phosphoramidite was prepared using commonly available commercial reagents in a simple two-step procedure. This initiator was incorporated into a 23-mer DNA sequence and the incorporation was confirmed using mass spectrometry. This DNA-initiator could be used to obtain block copolymers with synthetic polymer segments of OEOMA and RMA using AGET ATRP. Optimization of the AGET ATRP conditions provided conjugated polymer segments with well-defined MW and narrow MWD. The use of a Q670 dye-labeled DNA macroinitiator confirmed that the AGET ATRP conditions did not affect the DNA stability, confirming the development of a polymerization procedure that can be readily applied to other DNA sequences for developing functional DNABCps. Successful block copolymerization of BnMA showed not only applicability of an extended monomer range but also preparation of DNA coated latex particles that retained the sequence specific hybridization capabilities of DNA. We have shown that this method can be extended to prepare bioconjugates by directly grafting from CPG beads with attached biomolecules, such as biotin. Our approach has two advantages. First, it enables ready access to a diverse and wide range of functional polymers through molecules that are available on solid support for nucleic acids synthesis. Second, the ability to synthesize the polymers on solid support allows direct and rapid purification of the conjugates. We expect that this ATRP initiator phosphoramidite will enable rapid and ready access to a broad range of functional biomaterials.

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