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Precisely-defined Aptamer-*b*-Poly(phosphodiester) conjugates prepared by Phosphoramidite Polymer Chemistry

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Abstract

Uniform conjugates combining a DNA aptamer (either anti-MUC1 or ATP aptamer) and a synthetic polymer segment were synthesized by automated phosphoramidite chemistry. This multistep growth polymer chemistry enables the use of both natural (*i.e.* nucleoside phosphoramidites) and non-natural monomers (*e.g.* alkyl- and oligo(ethylene glycol)-phosphoramidites). Thus, in the present work, six different aptamer-polymer conjugates were synthesized and characterized by ion-exchange HPLC, circular dichroism spectroscopy and electrospray mass spectrometry. All these methods evidenced the formation of uniform molecules with precisely-controlled chain-length and monomer sequences. Furthermore, aptamer folding was not affected by polymer bioconjugation. The method described herein is straightforward and allows covalent attachment of homopolymers and copolymers to bio-functional DNA aptamers.

Aptamers are small single-stranded oligonucleotides (ssDNA) that selectively recognize and effectively bind to their targets, including proteins and live cancer cells. Due to their stability and facility of selection and synthesis, DNA aptamers have been extensively applied in biosensing, biotechnology and biomedicine, as promising alternatives to monoclonal antibodies.^{1,2} Although these “chemical antibodies” are discovered using an enzymatic process, like most synthetic oligonucleotides they are readily synthesized using solid-phase phosphoramidite chemistry, in an automated process that has now been practiced for several decades.³ This chemical synthesis allows the site-specific introduction of non-nucleotide linkers such as fluorescent dyes and mobility modifiers such as ethylene glycol and polyethylene glycols (PEG).⁴ Additionally, automated solid-phase synthesis allows for uniform post-synthetic modification of aptamers and site-selective polymer attachment of various functional groups. In particular, it enables the facile incorporation of chemical functionalities (such as primary amines and thiol precursors) either at the 3' or 5' terminals of the aptamers, which can be useful for site-specific conjugation to various synthetic polymers.

Post-modifications of chemically modified aptamers has yielded aptamer-polymer conjugates (APCs) with a variety of natural and synthetic polymers such as biodegradable polymers, graft copolymers, amphiphilic block copolymers and dendritic polymers.⁴⁻⁹ However, in all reported studies, the synthetic segments that have been coupled to aptamers are polydisperse macromolecules synthesized by conventional polymerization methods such as chain-growth or step-growth polymerizations.¹⁰ Thus, APCs usually lack the remarkable programmability, sequence selectivity, monodispersity, and fine structural control that DNA can offer. Moreover, molecular heterogeneity is even more increased if APCs are further modified with different functionalizing domains, such as drug-binding or fluorescent domains.^{11,12} In addition, most of the APCs reported in the literature have been synthesized by coupling of macromolecular reagents. This bioconjugation strategy requires often excess of one reagent and therefore

complicated purifications.¹³ In this context, a particularly attractive goal is therefore the development of new methods for the creation of APCs with a high level of macromolecular precision,¹⁴ providing structural and functional diversity, by uniting the chemical programmability of DNA with the stability and ease of functionalization of synthetic polymers.^{15,16} Here, we report that solid-phase phosphoramidite chemistry can be used as a single tool to prepare APCs. In this approach a synthetic polymer segment is directly grown from the aptamer during the multistep synthesis.

Solid-phase phosphoramidite chemistry is not restricted to the use of phosphoramidite nucleosides (A, T, C, and G) but can also be performed using a wide variety of non-natural monomers.¹⁷⁻²⁰ For example, our group has recently described the synthesis of non-natural informational polymers using this approach.²¹⁻²⁵ In this case, phosphoramidite chemistry is no longer a biochemical method but becomes a polymer chemistry tool. Very interestingly, phosphoramidite chemistry (PPC) can be used to combine biological and non-biological segments. For example, Sleiman and coworkers have used PPC and commercially available monomers to synthesize monodisperse DNA–polymer conjugates.²⁶ The obtained amphiphilic DNA block copolymers could be later used orthogonally in conjunction with DNA nanotechnology to create higher order 3D-nanostructures.^{27,28} In recent publications, our group has shown that using a conventional DNA synthesizer, it is possible to synthesize bio-hybrid macromolecules containing a long non-natural segment connected to one or two ssDNA extremities and to assemble them into precise linear or non-linear constructs.²⁹ However, to our knowledge, PPC has never been used to date to synthesize molecularly-defined polymer-aptamer conjugates. In this letter, we report the synthesis and characterization of uniform APCs by automated phosphoramidite chemistry.

A series of six different APCs was prepared in this work using standard phosphoramidite chemistry on a DNA synthesizer (Table 1). Two different model aptamers were considered

herein as proof-of-principles. The first one is a 24-mer version of the anti-MUC1 aptamer (**A1**), which has been identified by Missailidis and coworkers as a bioreceptor for breast cancer biomarker mucin 1 (MUC1) protein.^{30,31} The MUC1_{apt} folds into a stable structure with three binding thymine residues in the terminal loop.³² The second model aptamer is the well-known and widely used DNA aptamer for ATP or adenosine (ATP_{apt}) that was first described by Huizenga and Szostak in 1995.³³ This aptamer (**A2**) is composed of 27 nucleotides and folds into a secondary structure which consists of an internal loop region and two stems flanking the loop.³⁴ The nucleotide sequences of **A1** and **A2** are listed in Table 1. Commercially available nucleoside phosphoramidite with conventional protecting groups were used as building blocks for the synthesis of the aptamer segments (Chart 1).³⁵ For the synthesis of the non-natural segments, two different phosphoramidite monomers were considered (Chart 1), containing a butyl (**M1**) and a tetraethylene glycol spacer (**M2**). As shown in Table S1, each model aptamer was conjugated to homopolymers of either **M1** (**APC1**, **APC2**) or **M2** (**APC3**, **APC4**). In addition, two triblock copolymers containing a first block (15-mer) of **M2**, a second block (12-Mer) of **M1** and a terminal aptamer were synthesized (**APC5**, **APC6**). In order to assess the influence of polymer conjugation on aptamer properties, aptamers **A1-A2** (Figures S1-S2) and synthetic segments **P1-P3** (Figures S3-S5) were also prepared (Table 1). The six APCs, the two aptamers and the three synthetic segments were all synthesized on a DNA synthesizer using conventional phosphoramidite protocols.²² However, it is important to mention that previously-reported cleavage conditions²² led herein to APCs with partially-deprotected nucleobases (data not shown) and therefore an optimized cleavage procedure employing mild heating was used in this work.

Chart 1. Molecular structure of the phosphoramidite monomers used in this work.

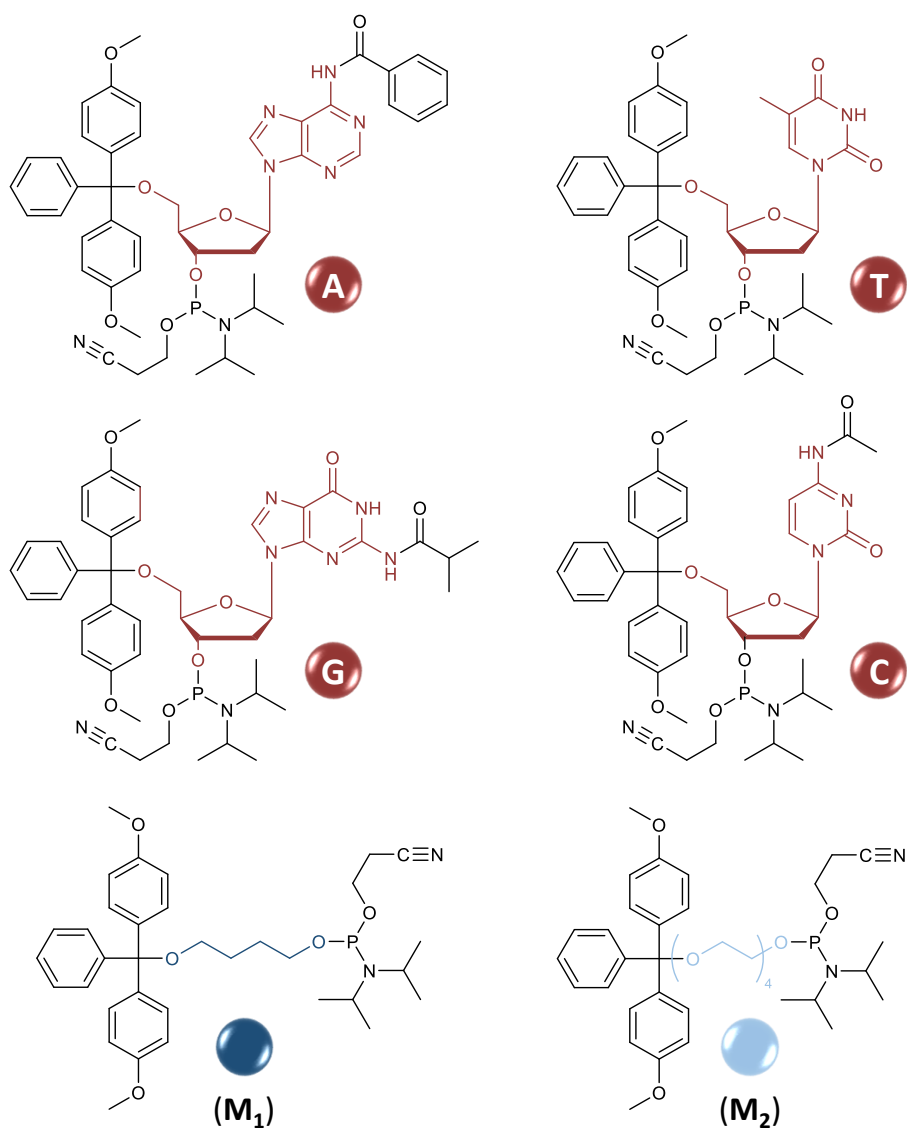


Figure 1 shows the characterization of the conjugate **APC1**, which combines **A1** and **P1** (*i.e.* homopolymer of **M1**). The formed conjugate was characterized by ion-exchange HPLC, circular dichroism (CD) spectroscopy and electrospray mass spectrometry (ESI-MS). Due to the pronounced difference in chemical nature of the natural and non-natural segments, the HPLC retention times of **A1** (21.04 min) and **P1** (13.59 min) are markedly different (Figure 1b). For the corresponding conjugate **APC1**, a retention time in between these two extremes (19.91 min) was found. Furthermore, the HPLC signal of **APC1** was found to be near

monodisperse with minor impurities. Monodispersity was also confirmed by ESI-MS measurement (Figure 1c) which shows formation of a dominating peak series of expected molar mass. Small HPLC and MS impurities correspond to truncated sequences missing one monomer unit, which are classical defects in such syntheses.²² All these results account for the formation of a uniform conjugate with precisely-controlled chain-length and monomer sequence. Perhaps more importantly, Figure 1d compares the CD spectra measured for **APC1**, **A1** and **P1**. While the non-natural segment **P1** exhibits a flat signal, the individual aptamer **A1** and the conjugate **APC1** show comparable signals, thus indicating that **P1** conjugation does not prevent aptamer folding.³⁶ Comparable results were obtained for **APC2**, which combines segments **A2** and **P1** (Figure S6) and were confirmed by UV melting measurements (Figure S7).. The conjugation of **P2** (*i.e.* homopolymer of **M2**) to **A1** and **A2** also led to successful results (Figures S8-S9). In HPLC, the oligo(ethylene glycol)-based segment **P2** (9.29 min) (Figure S4) has a strikingly different elution time than **A1** and **A2** (22.04 min) (Figure S2). Still, the covalent attachment of **P2** to **A1** and **A2** did not lead to any significant change in CD spectroscopy (Figures S8b-S9b), thus evidencing that PPC ‘PEGylation’ does not modify aptamer properties.³⁷ Yet, synthetic segments **P1** and **P2** lead to APCs with different solution properties. For instance, the different chemical nature of **P1** and **P2** is reflected by electrophoresis measurements (Figure S10), in which **P2**-based APCs have notably different migration rates than their **P1**-based analogues. Furthermore, APCs containing blocky synthetic segments (**APC5**, **APC6**) were also successfully synthesized and characterized (Figures S10-S12).

Table 1. Characterization of the aptamers, polymers and APCs synthesized in this work.

	<i>Sequence</i>	<i>Mass_{th}^a</i>	<i>Mass_{exp}^b</i>	<i>Yield</i>
		[Da]	[Da]	[%]
A1	5'-GCA GTT GAT CCT TTG GAT ACC CTG-3'	7436.193 ^c	7436.400 ^c	70
A2	5'-ACC TGG GGG AGT ATT GCG GAG GAA GGT-3'	8484.450	8484.286	70
P1	5'-(M1) ₂₇ -3'	4347.738	4347.921	83
P2	5'-(M2) ₂₇ -3'	7159.022	7158.955	55
P3	5'-(M2) ₁₅ -(M1) ₁₂ -3'	6211.582 ^d	6211.593 ^d	47
APC1	5'-(M1) ₂₇ -GCA GTT GAT CCT TTG GAT ACC CTG-3'	11440.894	11440.772	46
APC2	5'-(M1) ₂₇ -ACC TGG GGG AGT ATT GCG GAG GAA GGT-3'	12591.100	12591.079	43
APC3	5'-(M2) ₂₇ -GCA GTT GAT CCT TTG GAT ACC CTG-3'	14251.176	14251.145	31
APC4	5'-(M2) ₂₇ -ACC TGG GGG AGT ATT GCG GAG GAA GGT-3'	15402.384	15402.322	21
APC5	5'-(M2) ₁₅ -(M1) ₁₂ -GCA GTT GAT CCT TTG GAT ACC CTG-3'	13304.738 ^d	13304.515 ^d	22
APC6	5'-(M2) ₁₅ -(M1) ₁₂ -ACC TGG GGG AGT ATT GCG GAG GAA GGT-3'	14454.944 ^d	14454.729 ^d	20

^a Calculated at isotopic maximum. ^b Estimated from negative mode ESI mass spectra. ^c

Detected as [M-H+Na]. ^d The detected molecules still contained DMT terminal groups.

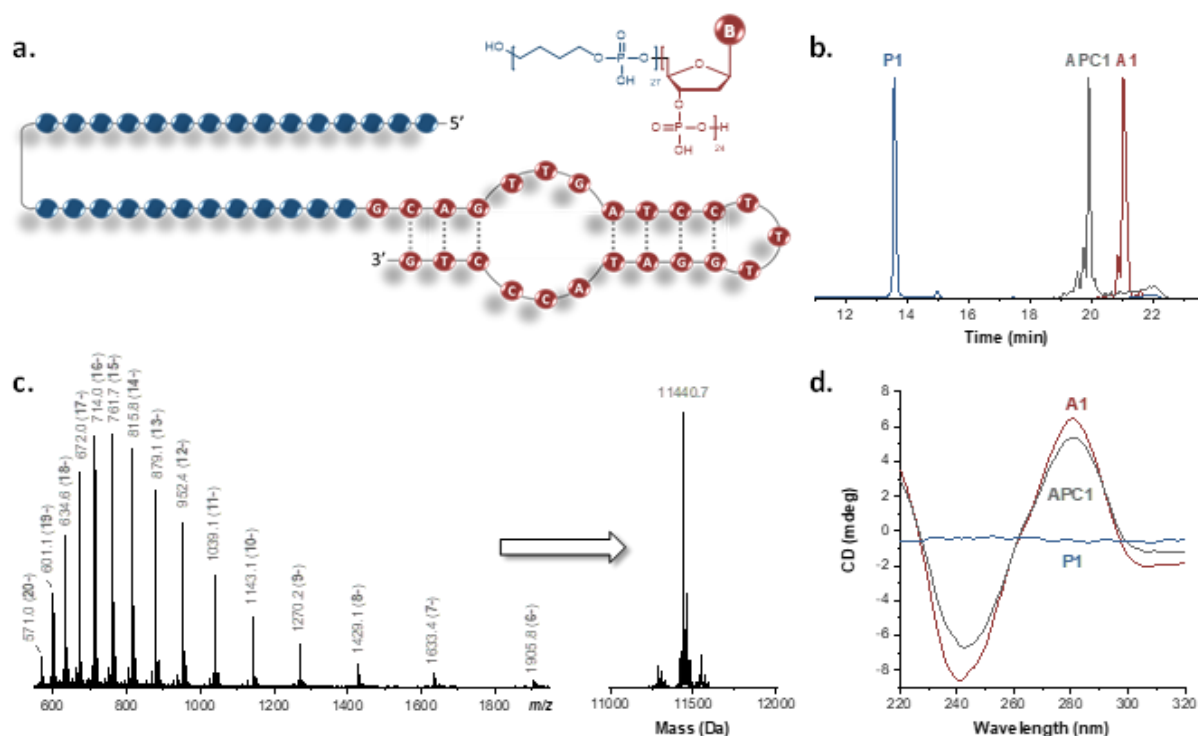


Figure 1. Characterization of the bio-hybrid macromolecule **APC1**. **(a)** General molecular structure and schematic representation of the synthesized conjugate. The letter **B** denotes a nucleobase. The dark blue spheres symbolize **M1**. The red spheres symbolize nucleotides. **(b)** HPLC traces recorded for the individual homopolymer **P1** (blue), the individual aptamer **A1** (red) and the corresponding conjugate **APC1** (grey). **(c)** Negative mode ESI-MS analysis of **APC1**. Original spectrum (left) and its charge state deconvolution (right). **(d)** Circular dichroism spectra measured for the individual homopolymer **P1** (blue), the individual aptamer **A1** (red) and the corresponding conjugate **APC1** (grey).

In summary, this letter describes a straightforward approach to prepare APCs with a uniform molecular structure. As demonstrated herein, automated PPC is a versatile method to link homopolymers or copolymers of defined chain length to aptamers. Importantly, for the two different aptamers studied in this work, polymer conjugation did not affect notably DNA folding. Furthermore, in comparison to previously reported macromolecular ligation

approaches, the present method is practical because it does not require removal of excess of macromolecular reagents. It also opens up interesting perspectives for the design of sequence-defined APCs containing functional groups (*e.g.* prodrugs, fluorescent labels) at defined positions.³⁸

Supporting Information. Full experimental part and additional figures. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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