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Role of helicity in the nonenzymatic template-directed primer extension of DNA

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Complexing a DNA primer with an RNA template showed improved nonenzymatic template-directed primer extension, attributed to a shift in the DNA helicity from a B-type towards an A-type helix. A 2-fold (deoxyadenosine) and 4.5-fold (deoxycytidine) increase in conversion from initial DNA primer to a primer + 1 nucleotide product was observed.

Nonenzymatic template-directed primer extension offers an alternate method of ribonucleic acid (RNA) or deoxyribonucleic acid (DNA) synthesis, in a one-pot reaction. With the growing applications of DNA, the need to produce DNA in a scalable and cost-effective manner has increased.[1] Current methods of DNA synthesis are based on solid phase synthesis[2] or polymerase chain reaction (PCR)[3]. These have several drawbacks, including requiring large amounts of solvents, costly enzymes and complex multi-step procedures.[2-4] Templated nonenzymatic synthesis is an attractive alternative as it uses chemically activated nucleotides in an isothermal one-pot reaction for DNA synthesis.[5] The aqueous reaction system is free from enzymes or protected nucleotides and is thus more versatile than traditional methods of DNA or RNA production. The generally accepted mechanism of nonenzymatic template-directed primer extension is the extension of a primer with an activated nucleotide by a nucleophilic substitution reaction on a template.[6] To promote the primer extension reaction, activation of the 5′-phosphate of an incoming nucleotide is required, achieved in this work with 2-aminoimidazole, which has previously been shown to provide good rates and yields for the RNA extension reaction.[5]

To date, nonenzymatic primer extension reactions have mainly been conducted using RNA-based systems rather than DNA-based systems due to DNA primers being significantly slower to extend than RNA primers.[6, 7] It is unclear whether this difference in reactivity is due to an intrinsic chemical difference between the ribose sugar in RNA and the deoxyribose sugar in DNA, or to a macromolecular effect in the DNA- or RNA-based systems. Leu et al.[8] have suggested that the reactivity difference arises from the overall conformation adopted by the DNA or RNA strands used in the nonenzymatic primer extension reactions. RNA traditionally forms an A-type helix and DNA mainly forms a B-type helix.[9, 10] In the nonenzymatic primer extension reaction, an A-type helix conformation allows the primer 3′-hydroxyl (3′-OH) and nucleotide 5′-phosphate substituents to be positioned in close proximity, thus favouring RNA extension.[11, 12] The DNA B-type helix conformation results in a less favourable positioning of the primer 3′-OH relative to the nucleotide 5′-phosphate, which may prevent DNA extension. Importantly, a hybrid DNA-RNA double stranded complex adopts a structure closer to an A-type helix conformation, induced by a strong steric preference of an RNA template for an A-type helix.[13]

Nucleotides are more flexible than a oligonucleotide template or primer and exist in a ring-flip equilibrium in solution between the C3′-endo and C2′-endo conformations.[14] The position of this equilibrium is dependent on the nucleotide identity, with cytidine existing in an even distribution between the C3′-endo and C2′-endo conformations, whilst the other nucleotides favour the C2′-endo conformation in solution.[15] The position of this equilibrium can be shifted by template binding, towards the nucleotide conformation compatible with the overall helix of the double-strand system.[16, 17]

In this work we determine whether the template and nucleotide conformation play a role in improving the conversion of a DNA primer to a DNA primer + 1 nucleotide product.

Figure 1 shows the overall nonenzymatic template-directed primer extension reaction. The primer and helper strand are annealed to a template strand (forming a template-primer-helper complex with a dinucleotide reaction site), with complementary base pairing (Fig. 1a).[18] 2-aminoimidazole activated nucleotides are then added for extension of the primer, which have been shown to dimerise in solution (Fig. 1b), with the dimer being much more reactive.[6, 19] The primer extension reaction proceeds via nucleophilic attack of the primer 3′-OH group on the nucleotide 5′-phosphate to give a primer + 1 nucleotide extension product (Fig. 1c).

To investigate the conformation of the template-primer-helper complexes formed, eight systems with variants of a primer and helper (either DNA or RNA) bound to either a DNA or RNA template were formed (Fig. 2a, **1** – **8**). In the absence of activated nucleotides, each system (**1** – **8**, Fig. 2a) was analysed

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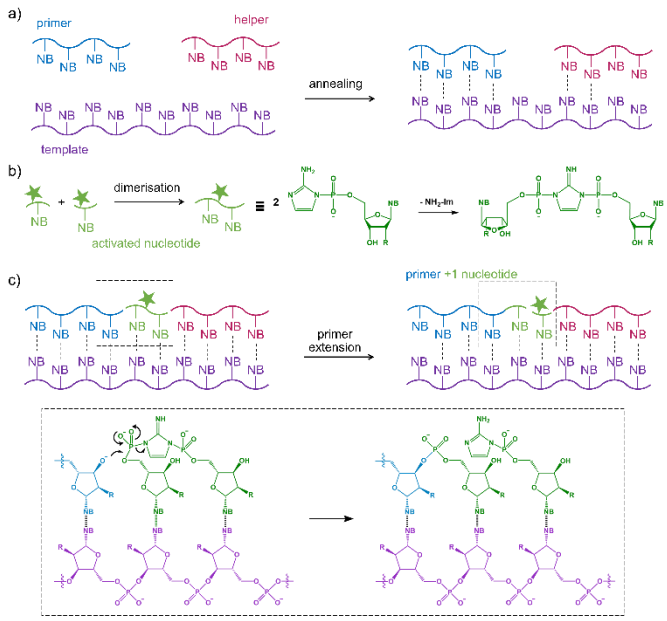


Figure 1. Reaction mechanism of nonenzymatic template-directed primer extension. (a) Annealing of template-primer-helper complex. The primer and helper strands bind to the template by complementary base pairing creating a dinucleotide reaction site. (b) Dimerisation of 2-aminoimidzole activated nucleotides.[16] (c) Primer extension occurs through a nucleophilic substitution reaction where nucleophilic attack of the 3′-hydroxyl (3′-OH) of the deoxyribose/ribose sugar on a primer strand with the 5′-phosphate of the incoming 2-aminoimidazole activated nucleotide, eliminating the activating group (leaving group), forms the primer + 1 nucleotide product.[6]

using CD spectroscopy to identify the helicity (conformation) (Fig. 2b)[10, 20, 21] (see ESI,† Section S1.3). The difference in helicity between DNA and RNA was clear, with a characteristic maximum in ellipticity at 280 nm and a minimum at 255 nm for the B-type pure DNA system (**1**) and a maximum at 260 nm observed for the A-type pure RNA system (**8**).

To determine whether a change in helicity was induced by RNA, each core component of the system was changed from DNA to RNA to provide increasing RNA content (%) (Fig. 2a).

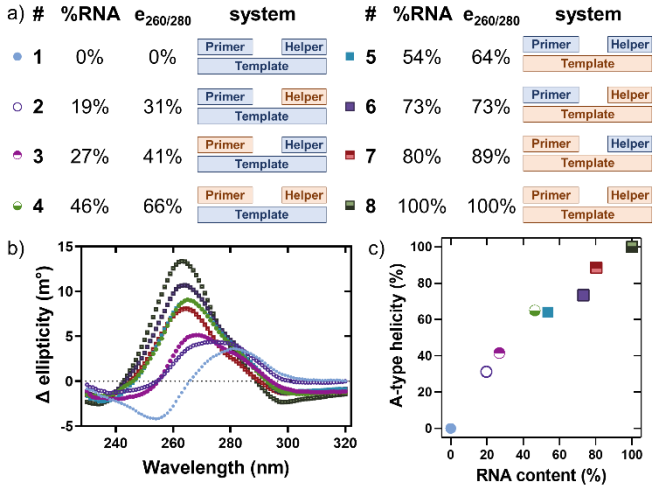


Figure 2. (a) Schematic of the eight systems (**1 – 8**) investigated. Each system contains a template (lower bar), primer (upper left bar), and helper strand (upper right bar) with DNA-based in blue and RNA-based in orange. (b) A-type helicity (%) of the DNA-RNA systems (**1 – 8**) measured by CD at equimolar concentration, in triplicate, with smoothed average. (c) Normalised A-type helicity (%) calculated from the e_{260}/e_{280} ratio for systems (**1 – 8**) against the normalised RNA content (%) from base pair composition.

The RNA content was calculated from the number of RNA bases present relative to the total length of the reaction system (56 bases), given the identity of the template (30 bases), primer (15 bases) and helper (11 bases) used. Figure 2b shows a decrease in the ellipticity maxima from 280 nm (e_{280}) to 260 nm (e_{260}) with an increase in the RNA content of the system. For each system (**1 – 8**), the ratio e_{260}/e_{280} was taken, with the value normalised between pure DNA (**1**) (as 0%) and pure RNA (**8**) (as 100%) (Fig. 2c). There is a clear linear increase in the normalised ellipticity ratio (e_{260}/e_{280}) with increasing percentage RNA content (Fig. 2b), indicating a shift from B-type to A-type helicity upon complexing increasing amounts of RNA within each reaction system.

The primer extension reactions were then performed on each of the systems (**1 – 8**) with 2-aminoimidazole activated nucleotides to explore the effect of helix conformation on reactivity. To compare the effect of nucleotide ring flip equilibrium on primer extension, all eight systems were studied with either adenosine-based nucleotides; deoxyadenosine (dA-2AI) or adenosine (rA-2AI) or cytidine-based nucleotides; deoxycytidine (dC-2AI or cytidine (rC-2AI) (see ESI,† Section S1.4). Each reaction was performed in triplicate and monitored over 144 h with aliquots taken at various timepoints (see ESI,† Section S1.5). The aliquots were quenched, then separated via urea-PAGE to monitor the extent of primer extension, as shown for exemplar systems in Figure 3a-c. The fluorescence intensity of the primer (labelled with carboxyfluorescein (FAM)) and the FAM-labelled primer + 1 nucleotide product bands were determined, with the intensity profiles fitted via Gaussian deconvolution. To determine the conversion, the ratio of reacted primer (primer + 1 band) to the total amount initially in the system (primer and primer + 1 bands) was determined for each timepoint and the conversion versus time plot was fitted to a one-phase association curve with an initial starting point of zero.

Figures 4a and 4b shows the maximum conversion (primer extension) reached at the end of 144 h for each reaction system (**1 – 8**) reacted with dA-2AI (Fig. 4a) or rA-2AI (Fig. 4b) versus the A-type helicity of each system (determined by the e_{260}/e_{280} ratio (Fig. 2c)).

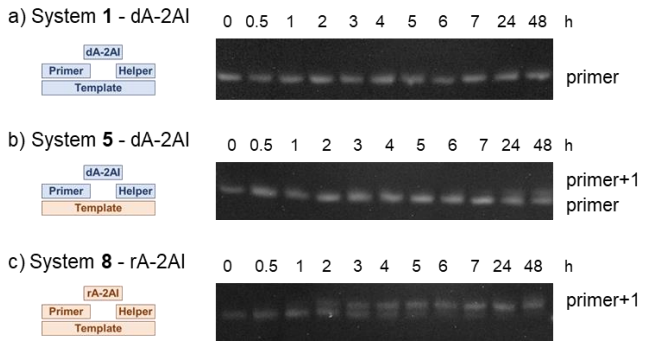
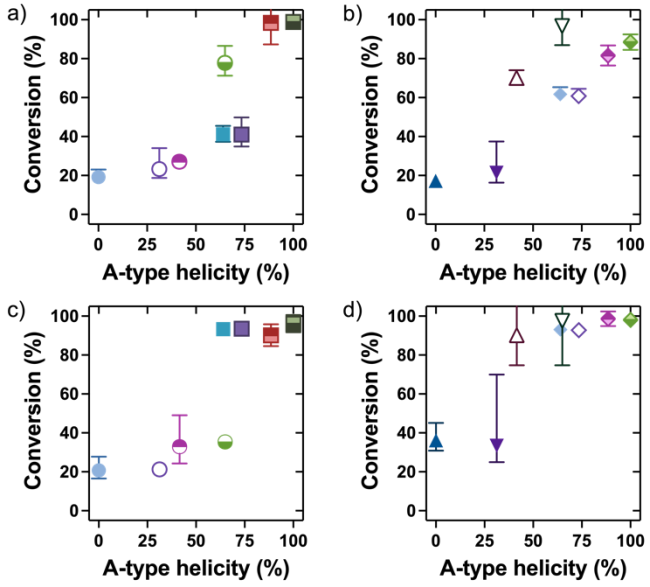


Figure 3. Exemplar denaturing urea polyacrylamide gel electrophoresis (urea-PAGE) of 0-48 h of three key systems investigated, imaged via carboxyfluorescein (FAM) fluorescence of the FAM-labelled primer. Systems are shown as per Figure 2 with DNA-based in blue and RNA-based in orange, with nucleotide identity included (upper bar). Bands are labelled, with (a) System **1** showing no change from primer band. (b) System **5** showing a low level of conversion from primer to primer + 1 product. (c) System **8** showing complete conversion from primer to the primer + 1 product.



e)

#		dA-2AI	dC-2AI		rA-2AI	rC-2AI	system	A-type
		Conversion						Helicity
1	●	19%	21%	▲	17%	36%	Primer Helper Template	0%
2	○	23%	21%	▼	21%	33%	Primer Helper Template	31%
3	◒	27%	33%	△	70%	90%	Primer Helper Template	41%
4	◒	78%	35%	▽	97%	98%	Primer Helper Template	66%
5	■	41%	93%	◆	62%	93%	Primer Helper Template	64%
6	■	41%	94%	◇	61%	93%	Primer Helper Template	73%
7	■	98%	90%	◆	82%	99%	Primer Helper Template	89%
8	■	99%	96%	◆	89%	98%	Primer Helper Template	100%

Figure 4. Maximum conversion from one-phase association of the incoming 2-aminoimidazole activated nucleotides in systems (1 – 8) versus the A-type helicity (%) calculated from the e_{260}/e_{280} of each system based on (a) dA-2AI, (b) rA-2AI, (c) dC-2AI, or (d) rC-2AI. Error bars show 95% confidence intervals for the maximum conversion when fitted to the one-phase association curve. (e) Legend showing system number, symbol, maximum conversion (%) for each nucleotide identity (DNA = d, RNA = r), schematic representation of system components with DNA (blue) and RNA (orange), and A-type helicity (%) of each system.

The addition of an activated DNA nucleotide to the pure DNA system (**1** + dA-2AI, 0% A-type helicity (Fig. 4a)) showed a low conversion of 19%, as expected, due to the system's unfavourable B-type helix conformation. There was little difference observed when using an RNA nucleotide (**1** + rA-2AI) with a conversion of 17% (Fig. 4b). Due to the B-type helix of the DNA components, the nucleotides are both in the C2'-endo conformation.[14] When changing from a DNA helper to an RNA helper (system **2**), despite an increase in A-type helicity (31%), no increase in conversion was observed with either the addition of dA-2AI (23%) or rA-2AI (21%). Even though the RNA helper could induce an A-type helix in the DNA template, this shift in helicity did not appear to translate upstream to the primer and reaction site, resulting in the low conversion level observed. Little difference is seen between the DNA or RNA nucleotide conversions due to both being in the C2'-endo conformation.[14] System **3** consists of an RNA primer, DNA template and DNA helper with an A-type helicity of 41%. In system **3** the primer is now in the more favourable A-type conformation, with conversion of the dA-2AI measured at 27%. With rA-2AI, a larger increase in conversion is observed at 70%. Here, we propose that the RNA primer favours conversion through an increase in the A-type helicity of this system around the reaction site, compared to the B-type helicity of systems **1** and **2**, with a DNA primer. The much higher 70% conversion with rA-2AI reflects the stronger preference for RNA nucleotides to adopt the C3'-endo conformation compared to the preference for the C2'-endo conformation of the DNA nucleotide which resulted in the lower 27% conversion with dA-2AI. System **4** consists of an RNA primer, DNA template and RNA helper with an A-type helicity of 66%. Here, the RNA primer and helper are expected to promote an A-type helix along the entire length of the DNA template. Much higher conversions are now observed with both dA-2AI (78%) and rA-2AI (97%). An A-type system would promote both nucleotide variants to adopt the more reactive C3'-endo conformation.

System **5** consists of a DNA primer, RNA template and DNA helper with an A-type helicity of 64%. This is an important system for DNA primer extension as a DNA primer is used. A 2-fold increase in conversion was observed, with dA-2AI at 41% compared to the pure DNA system (**1**) at 19% conversion. For rA-2AI a high conversion was also observed, at 62%. In system **5** the DNA primer and nucleotide are both shifted to an A-type conformation by the RNA template and so higher conversion rates are seen than with a DNA template (systems **1** and **2**). System **6** contains an RNA template, DNA primer and RNA helper. The use of an RNA primer compared to system **5** increased the A-type helicity to 73%. However, like system **2**, this further increase in A-type helicity is located to the template-helper region, and therefore does not lead to an increase in conversion relative to system **5**, with dA-2AI at 41% and rA-2AI at 61% conversion. System **7** contains RNA primer, RNA template and DNA helper and has a high A-type helicity (89%) and correspondingly higher conversions for dA-2AI (98%) and rA-2AI (82%). The increased A-type helicity is within the primer reaction site and therefore results in enhanced reactivity with both dA-2AI and rA-2AI, with both adopting the more reactive C3'-endo conformation. The pure RNA system (**8**) has 100% A-type helicity and showed a high conversion for dA-2AI (99%) and rA-2AI (89%), as expected. Here, the slightly lower conversion of rA-2AI is presumed to be due to its lower stability and its propensity for hydrolysis during the reaction.[12] Overall, a higher conversion of a DNA primer with a DNA nucleotide was with an RNA template. Templating an A-type helix conformation within the primer-reaction region provided the greatest impact on conversion. Minimal effects were observed upon changing

from a DNA to RNA helper. To investigate the more reactive C3'-endo conformation for improved conversion, cytidine-based nucleotides were then examined.

Figure 4c and 4d shows the maximum conversion reached at the end of 144 h for each reaction system (**1** – **8**) containing dC-2AI (Fig. 4c) or rC-2AI (Fig. 4d). As expected, the maximum conversions were higher for the cytidine-based nucleotides than the adenosine-based nucleotides. This is consistent with literature, in which the stronger hydrogen bonding of the C – G pair compared to the A – T or A – U pairs results in a faster and higher yielding reaction.[15] In addition, cytidine nucleotides (rC) have an innate preference for the C3'-endo conformation, suggested to contribute to faster reaction rates.[15]

Systems **1** and **2** with DNA template and primer had low conversions of 21% and 21%, with the dC-2AI nucleotide. There was a moderate increase with rC-2AI to 36% (**1**) and 33% (**2**), due to the higher preference of cytidine nucleotides for the C3'-endo conformation. As with the adenosine-based nucleotides, the similarity of conversion between systems **1** and **2** showed that there was minimal difference between the DNA and RNA helper variants. System **3** (RNA primer, DNA template, and DNA helper) showed a moderate increase in conversion with dC-2AI (33%) and a notable increase with rC-2AI (90%). As per the reactions of system **3** with dA-2AI and rA-2AI, the increase in conversion arose from the RNA primer shifting the underlying DNA template to a more A-type helix conformation. The much higher conversion with rC-2AI reflects the strong propensity of rC to adopt the C3'-endo conformation and readily undergo the extension reaction. System **4** (RNA primer, DNA template and RNA helper) shows that by changing the DNA helper to an RNA helper there is a decrease in conversion with dC-2AI (35%) compared to the reaction with dA-2AI (78%). Here, we suggest that the relative influence of the DNA template on the conformation of the nucleotides is greater than that of the RNA primer and helper with dC-2AI and thus is in the less reactive C2'-endo conformation. This is in reverse to results shown with the dA-2AI where the weaker A – T base pairing (compared to C – G) allows the RNA primer and helper to influence the nucleotide conformation to the C3'-endo conformation. Systems **5** – **8** all showed similar trends to the adenosine-based reactions, with high levels of conversion above 90%, due to the higher reactivity of cytidine-based nucleotides. Notably, system **5** (DNA primer, RNA template and DNA helper) showed a final conversion of 93% with dC-2AI, a 4.5-fold increase compared to the pure DNA system (**1**). This reiterated the potential influence of the underlying RNA template in enhancing the reactivity of DNA primer extension. The 4.5-fold increase in conversion with dC-2AI compared to a 2-fold increase in conversion with dA-2AI highlighted the importance of the reaction site conformation in determining reactivity level.

This work suggests that in order to obtain high DNA primer nonenzymatic template-directed extension conversion levels, a C3'-endo conformation is required for both the primer and the incoming nucleotide. This can be achieved by using an RNA template to promote an A-type helicity in the DNA primer and nucleotide. A similar increase in conversion could be gained through use of a locked-DNA (LNA) template to strongly enforce the A-type helicity and would be an interesting future investigation to confirm this hypothesis.[8] The effects of conformation can be further reinforced by a strong intrinsic preference for the C3'-endo conformation of the activated nucleotides, resulting in a higher conversion for cytidine than adenosine activated nucleotides.

There are multiple factors that can influence the level of conversion observed, including monomer binding affinity, however the importance of the reaction site conformation is clear from this work. An increase in conversion was present with an increase in A-type helicity for multiple nucleotide variants (DNA and RNA variants of adenosine and cytidine), showing that the trend of increasing conversion with A-type helical conformation is universal between different nucleotides. Adenosine and cytidine represent partners from the two base-pairs in DNA and RNA, A-T or A-U and C-G. Adenosine has a weaker hydrogen bonding interaction (2 H-bonds) with the template strand than cytidine (3 H-bonds). However, being a purine base, adenosine has a larger π-stacking surface and therefore a stronger interaction with the primer and helper than the pyrimidine base cytidine. In addition, adenosine nucleotides favour the C2′-endo conformation in solution, whilst cytidine nucleotides favour the C3′-endo conformation. These different behaviours may result in differing levels of binding for each nucleotide variant with the primer-template-helper complex. Despite this difference, the effect of the binding site conformation is still dominant, as an increase in conversion can be seen with an increase in A-type helicity, for every nucleotide system. This is shown in the values in Figure 4 and is perhaps clearest in comparing the conversion when moving from system **1** to **5**, for dA-2AI (19% to 41%), dC-2AI (21% to 93%), rA-2AI (17% to 97%), and rC-2AI (36% to 93%).

Furthermore, the electronegativity of the 2'-substituent increasing reactivity[22] is a factor in the higher incorporation of the monomer with an RNA primer compared to a DNA primer. However, this does not explain the increase in the incorporation of a DNA nucleotide with a DNA primer of 19% (system **1**) to 41% (system **5**), when moving from a DNA to an RNA template, as there is no chemical difference in the 2'-substituents on the primer and nucleotide. These results highlight that the increase in conversion is principally due to a templating effect rather than a reactivity difference between reagents.

This work paves the way for improved DNA synthesis through RNA templated nonenzymatic extension, whereby the residual RNA can be removed post reaction using ribonucleases, for example.

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Conflicts of interest

There are no conflicts to declare.

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