ChemBioChem

Review
doi.org/10.1002/cbic.202200510

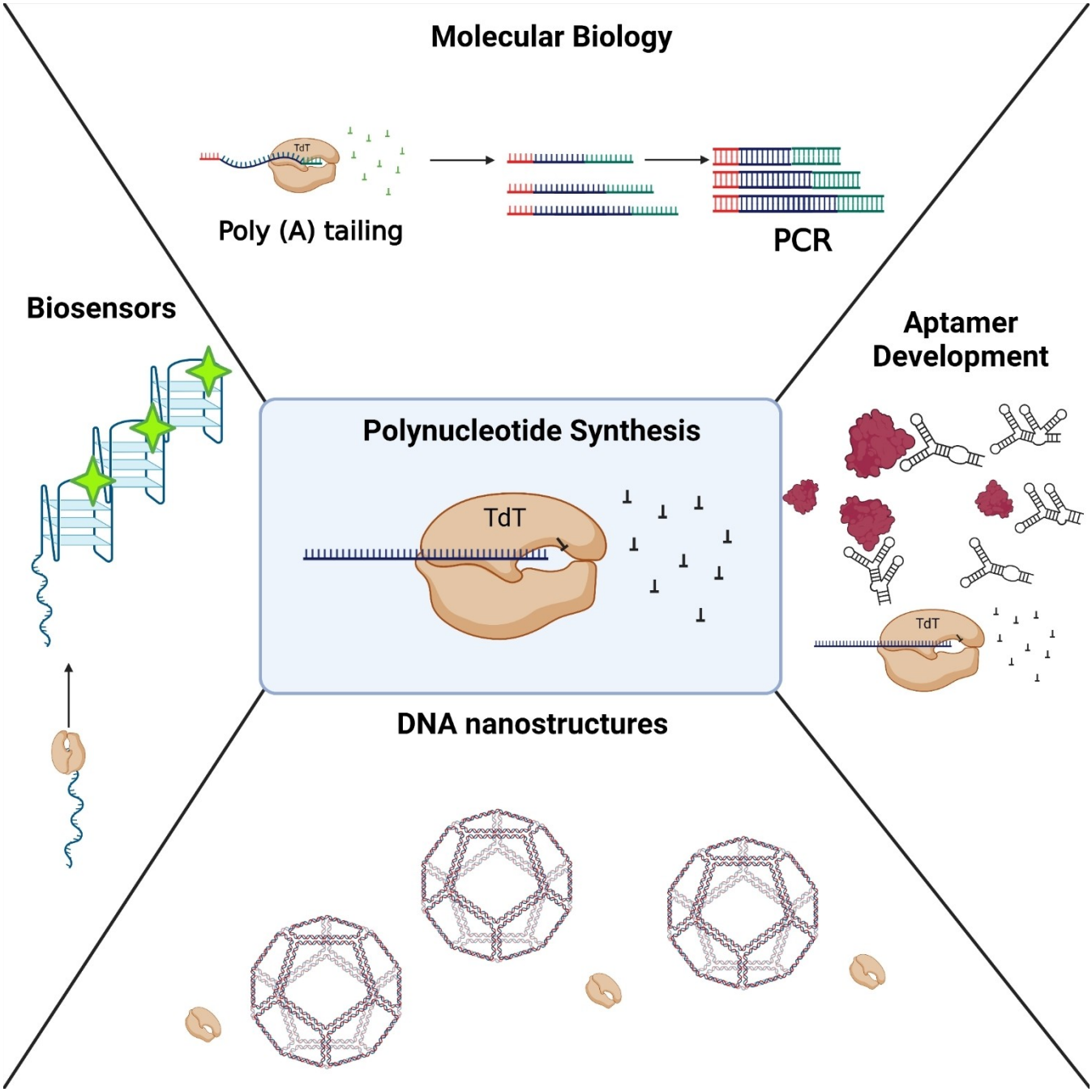
Chemistry Europe
European Chemical Societies Publishing

www.chembiochem.org

VIP *Very Important Paper*

Applications of Terminal Deoxynucleotidyl Transferase Enzyme in Biotechnology

Jon Ashley,*[a] Indiia G. Potts,[a] and Femi J. Olorunniji[a]



The use of polymerase enzymes in biotechnology has allowed us to gain unprecedented control over the manipulation of DNA, opening up new and exciting applications in areas such as biosensing, polynucleotide synthesis, and DNA storage, aptamer development and DNA-nanotechnology. One of the most intriguing enzymes which has gained prominence in the last decade is terminal deoxynucleotidyl transferase (TdT), which is one of the only polymerase enzymes capable of catalysing the template independent stepwise addition of nucleotides onto an oligonucleotide chain. This unique enzyme has seen a significant increase in a variety of different applications. In this review, we give a comprehensive discussion of the unique properties and applications of TdT as a biotechnology tool, and the application in the enzymatic synthesis of poly/oligonucleotides. Finally, we look at the increasing role of TdT enzyme in biosensing, DNA storage, synthesis of DNA nanostructures and aptamer development, and give a future outlook for this technology.

1. Introduction

The unique properties and applications of polymerase enzymes have interested researchers for the last few decades. From the advent of using polymerases to amplify DNA to allow scientists to study the genomics and unravel the human genome, to the use of CRISPR to edit DNA sequences, polymerase enzymes have revolutionized the biomolecular sciences. Of all the polymerase enzymes described in the literature, terminal deoxynucleotidyl transferase (TdT) has stood out as a unique polymerase enzyme owing to the fact that it is one of the few polymerase enzymes which display template-independent activity.[1,2] More recently, researchers have found other polymerases which display template-independent catalytic activity such as primpol polymerase that can synthesize short RNA sequences and polymerase θ that can catalyse the synthesis of short dinucleotide oligonucleotides in the presence of manganese.[3,4] However, the potential applications of both primpol and polymerase θ have yet to be explored. TdT allows for the incorporation of single nucleotides onto elongating oligonucleotide or double-stranded DNA (dsDNA) initiators through a stepwise addition.[5] RNA based sequences have also been demonstrated as initiators but the catalytic efficiency of incorporation of both dNTPs (**1**–**4**) and rNTPs (**10**–**13**) onto RNA based initiators was lower when compared to ssDNA initiators incorporating dNTPs (as seen by the lower observed sizes in gels.[6–9] TdT plays an important role in immunology in the development of lymphocytes. As a key enzyme in V(D)J recombination (the process by which lymphocytes alter and shuffle DNA for increasing the diversity of T-cell receptors and immunoglobulins[10]) where it is responsible for incorporating random nucleotides onto one strand in a 5′ to 3′ direction.[11] The potential biotechnology applications of TdT has expanded in the last two decades due to its unique template-independent properties and this can be seen by the increasing number of scientific papers in the use of TdT in areas such as molecular biology, biosensing, enzymatic DNA synthesis, aptamer development as well as the emerging areas of DNA nanotechnology and DNA based storage systems.

In this review, we will discuss the current state-of-the-art in the usage of TdT enzyme in several biotechnology applications. First, we will give a brief overview of the properties of TdT as well as describing the routine use of TdT in molecular biology. We will discuss the use of TdT in oligonucleotide synthesis which is now likely to overtake the classical chemical-based synthesis approaches in the future. We will explore the use of TdT in emerging biotechnologies as a tool for generating polynucleotides in DNA based storage systems and DNA nanostructures which have started to see wider focus in drug delivery applications. We will also explore the increasing number of applications of TdT in biosensor platforms and discuss the recent new application of TdT in aptamer screening. Finally, we will discuss the future direction and give an outlook for this unique enzyme. The use of TdT in synthesis using nucleotide analogues was reviewed recently by Hollenstein *et al.*[12]

2. Properties of TdT Enzyme

TdT is a 58 kDa member of the X family of DNA polymerase enzymes and functions alongside other polymerases such as polymerase λ and polymerase μ in DNA repair.[13,14] TdT has played a pivotal role in the evolution of the vertebrate immune system by introducing diversity in T-cell receptors. The first reported crystal structure of TdT was reported by Delarue *et al.* in 2002 who showed that TdT contained a typical DNA polymerase β-like fold locked into a closed form and that the enzyme forms two binary complexes with the initiator sequence and the incoming nucleotide.[15] Like all polymerases, TdT requires the presence of a divalent metal ion to catalyse the phosphoryl transfer reaction which is needed for nucleotide incorporation.[16,17] However, TdT can utilize a multitude of different divalent metal ions such as Co^{2+}, Mn^{2+}, Zn^{2+}, and Mg^{2+}. Previous studies into the kinetic properties of TdT showed that in general, for a homopolymer sequence, the incorporation of dATP showed a specificity order of $Mg^{2+} > Zn^{2+} > Co^{2+} > Mn^{2+}$ and reporting a K_M and V_{max} values of between 5 and 50, respectively.[18] Variation in kinetics can also be seen from each type of divalent metal ion. Co^{2+} shows a catalytic preference for the pyrimidines dCTP and dTTP while Mg^{2+} shows a preference for the incorporation of dATP and dGTP. Mn^{2+} generally shows a lower catalytic efficiency for

[a] *J. Ashley, I. G. Potts, F. J. Olorunniji*
School of Pharmaceutical and
Biomolecular Sciences, Liverpool John Moores University
James Parsons Building, 3 Byrom St, Liverpool L3 3AF (UK)
E-mail: j.ashley@ljmu.ac.uk

incorporation of dNTPs while Zn^{2+} has the opposite effect of incorporating dNTPs.[19] In terms of the kinetic mechanism, previous reports have suggested that TdT-catalysed synthesis of polynucleotides proceeds via a living chain growth condensation based polymerization mechanism (Figure 1), meaning that the resultant products of the synthesis are homopolymers with narrow size distributions.[20] They demonstrated that the ratio of nucleotide concentration to initiator concentration can affect the size distribution of the products. It is worth noting that upon introducing a mix of dNTPs, the size distributions of the resultant polynucleotides broaden significantly, observed as smears in native gels.[21,22]

3. Applications of TdT Enzyme

3.1. The application of TdT in molecular biology

The first use of recombinant TdT enzyme was demonstrated in rapid amplification of complimentary ends assay (RACE) in the 80s whereby nucleic acid sequences are amplified from a mRNA sequence.[23] The first stage of the assay involves reverse transcription of the mRNA into cDNA followed by PCR amplification. To facilitate the PCR amplification, TdT is utilized to add a homopolymeric tailing region to allow for the hybridization of the reverse primer containing a complimentary homopolymeric region. This has allowed researchers to study map transcripts and perform next generation sequencing on RNA samples. Another classical application of TdT in molecular biology is the terminal end labelling of DNA at the 3′ end.[24] This has allowed for the functionalization of DNA with different nucleotide analogues (Scheme 1 and Scheme 2) such as fluorescent tagged nucleotides (**6**,**14**) as chemical reporters and chemically modified nucleotides to increase nuclease resistance.[12] Researchers have demonstrated the incorporation of redox reporters onto both DNA and RNA oligonucleotides at the 3′ end.[25,26] Both fluorescence modified nucleotides and more hydrophobic (**8**) nucleotides have also been demonstrated as substrates for end labelling.[27,28] This ability to end label oligonucleotides has allowed researchers to impart additional functionality onto DNA through a simple and convenient method to allow for further applications of nucleic acids. For instance, in 2012 and 2015, Winz *et al.* demonstrated the use of TdT in the end labelling of DNA and RNA with different click chemistries (**7**) to allow for further conjugation of different functional groups (biotin, fluorescent dyes etc) through copper catalysed reactions of azides with an alkynes.[29–31] In 2017 researchers demonstrated the end labelling of DNA with a his tag mimic (**9**) for application in DNA immobilization.[32]

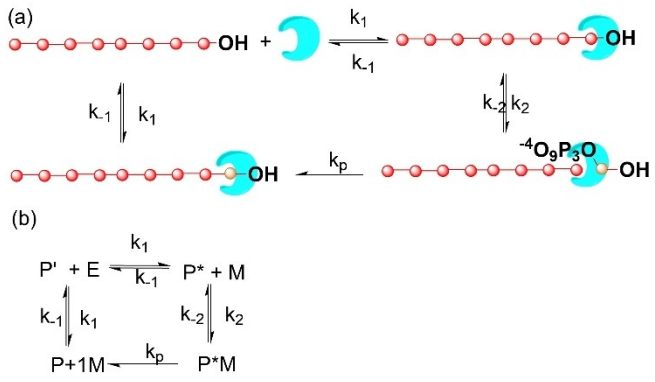


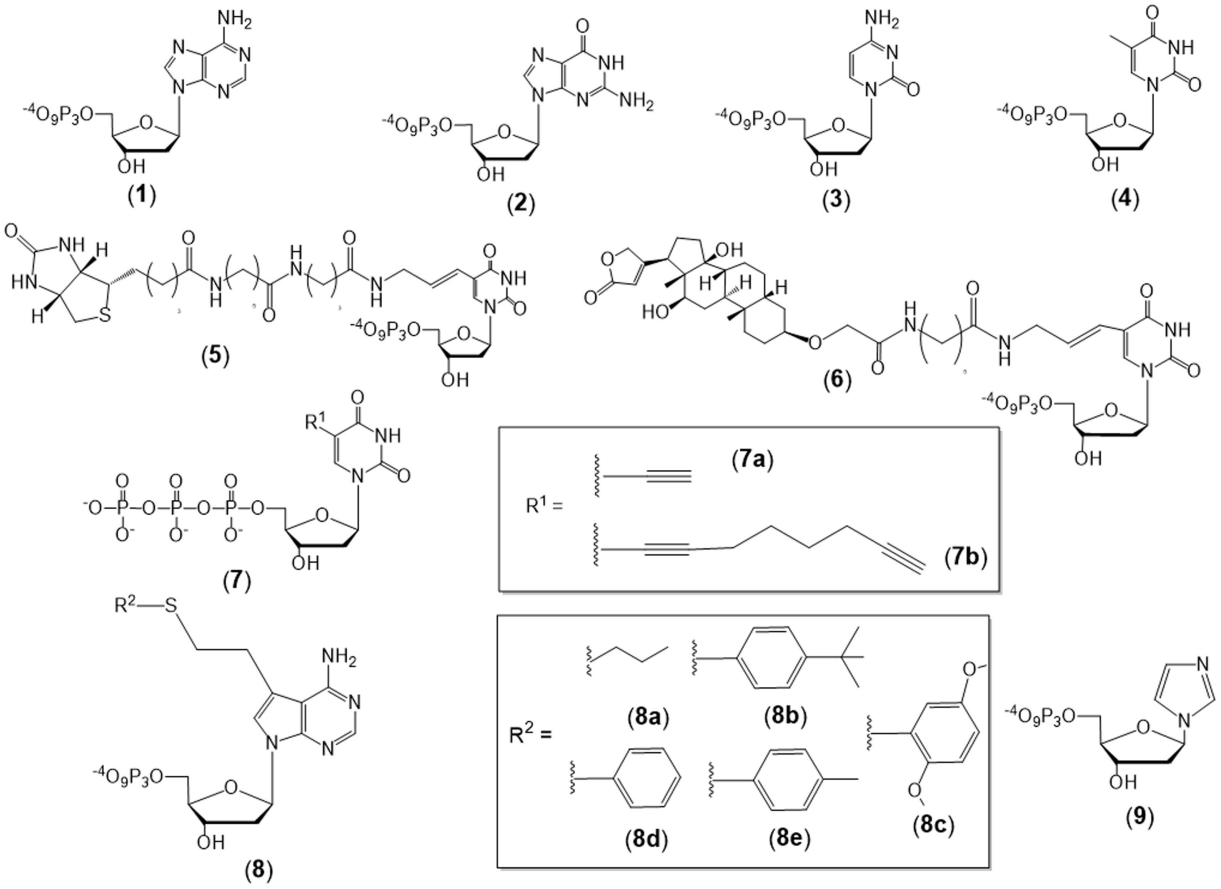
Figure 1. Mechanism of TdT-catalysed polymerisation reactions (a) TdT binds to the initiator followed by the dNTP to form the complex. The chain propagation occurs when a new phosphodiester bond is formed between the 3′-OH and α-phosphate on the dNTP. (b) Chemical mechanism of the living polymerisation reaction in the synthesis of polymers.

As mentioned earlier, TdT has established its role as a workhorse enzyme in routine biomolecular assays such as RACE.[33] Another example of a biomolecular assay that utilizes TdT in the TUNEL assay which has allowed researchers to detect the fragmentation of DNA during apoptosis (cell death).[34] TdT allows for the attachment of fluorophores to the 3′ OH end of dsDNA to highlight where breaks in the DNA occur allowing for the quantification of cells undergoing apoptosis.

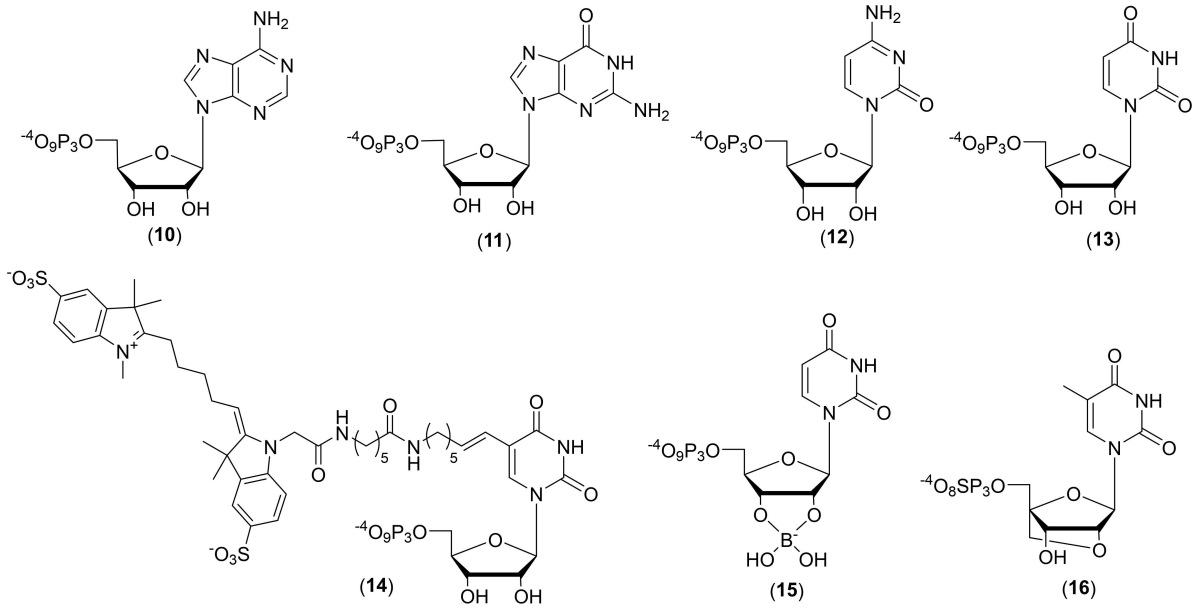
3.2. The application of TdT in polynucleotide synthesis

Recombinant DNA synthesis has allowed researchers and industry to produce oligonucleotides with specific sequences and randomized sequences on demand. The classical methods for synthesizing DNA involve the covalent attachment of an initial nucleoside phosphonamidite to a solid phase material which is then followed by the gradual stepwise addition of nucleotide residues through cycles of deblocking, coupling, capping and oxidation. This has allowed for the routine synthesis of oligonucleotides of up to 100 nt. Longer sequences of up to 200 nt can be synthesized if required but this comes at the cost of poorer yields and sequence errors. These errors increase in frequency as the polynucleotide length grows and as GC rich sequences are required. Companies such as IDT (Coralville, USA) offer a synthesis service to produce custom sequences known as Ultramers up to 200 nt in length. Polynucleotides over 200 nt becomes prohibitively expensive to synthesize and display a higher degree of error accumulation along the sequence making the synthesis of these sequences impractical. To add to this, the reagents and solvents used in the synthesis of oligonucleotides are hazardous to the environment. Another approach is to use gene-based synthesis whereby genetic cassettes are utilized to synthesize sequences of DNA down to single nucleotide accuracy but this approach is often error prone due to the use of PCR.[35] Researchers have looked at using polymerase enzymes such as TdT to overcome this size limitation and to provide green chemistry methodologies for industry. The race to develop the first enzymatic method for the synthesis of polynucleotides has intensified in recent years.[36]

In 2018, Palluk *et al.* demonstrated the sequence-specific synthesis of 10–20 nt oligonucleotides by using polymerase nucleotide conjugates that facilitated the incorporation of each nucleotide in a stepwise addition.[37] This meant that each TdT polymerase molecule was conjugated with an NTP tether which



Scheme 1. Summary of natural dNTPs: dATP (**1**); dGTP (**2**); dCTP (**3**); dTTP (**4**); and base modified nucleotides including biotin-dTTP (**5**); fluorescent dig-11-dUTP (**6**); click chemistry functional groups alkyne-dUTP (**7a-b**) and hydrophobic functional group containing-dATP analogues (**8a–e**) and his-tag mimic (**9**).



Scheme 2. Summary of natural rNTPs: rATP (**10**); rGTP (**11**); rCTP (**12**); rUTP (**13**) and chemically modified rNTPs; cy-UTP (**14**); borate-UTP complex (**15**) and α-thio-LNA-TTP (**16**).

upon extension would form a TdT-DNA complex. The DNA fragment is released from this complex through reversible cleavage. Mathews et al. demonstrated the synthesis, characterization, and application of 3′-O modified 2′-deoxyribonucleoside triphosphates (dNTPs) which were end capped with photolabile protecting groups. These protecting groups can be selectively removed to allow single step addition of each nucleotide and control the sequence specific synthesis of oligonucleotides.[38] Research has focused on the synthesis of new nucleotide analogues containing unique protecting groups and the assessment of these analogues as tolerated substrates for TdT to improve the sequence controlled TdT catalysed enzymatic synthesis of larger polynucleotides. Flamme *et al.* demonstrated the synthesis of nucleotides with benzoyl and pivaloyl protecting groups to allow for the enzymatic sequence-controlled synthesis of both DNA and XNA oligonucleotides.[39,40]

In another example, with nucleotide analogues containing borate (**15**) based chemistries as reversible protecting groups were demonstrated, which can be tolerated as substrates for TdT for the controllable synthesis of DNA.[24]

In addition, there has been a focus on the protein engineering of TdT variants to improve the efficiency of incorporation of nucleotide analogues containing protecting groups. Lu *et al.* recently demonstrated a strategy for improving the catalytic stepwise addition of 3′-ONH_2-modified nucleotides using an engineered terminal deoxynucleotidyl transferase from *Zonotrichia albicollis* (ZaTdT), which demonstrated a three times increase in catalytic activity compared to mammalian TdT and allowing for increase tolerance of nucleotides modified with protecting groups.[40]

Researchers have also looked new approaches at using TdT to introduce chemically modified nucleotides to polynucleotides during synthesis to boost their functionality in terms of changing the chemical and physical properties (e.g., boosting the hydrophobic character of the base group) or increasing *in vivo* performance of the DNA by increasing resistance towards nuclease enzymes and increasing the *in vivo* circulation times. In classical recombinant synthesis of DNA, researchers can include nucleotide analogues into the synthesis, but the costs, yields and complexity of the synthesis associated with these methods have limited the large-scale synthesis of chemically modified DNA and degree of modification in oligonucleotides.[41] Therefore, the emergence of enzymatic based methods capable of synthesizing chemically modified oligonucleotides is a key focus of current research efforts. Nucleotides containing chemically modified regions such as those made to the phosphate backbone (e.g., thiolates), stereoisomers of DNA (e.g., L-DNA), modifications to the sugar base (e.g., locked nucleic acid (LNA)) and base group modifications could all be used as potential substrates in the TdT catalysed synthesis of DNA. This could potentially lead to an increase in the degree of incorporation throughout a synthesized poly/oligonucleotide and thereby increase their functional properties and *in vivo* performance, while lowing costs and improving yields.

Schaudy *et al.* investigated TdT's ability for sequence preference and initiator promiscuity by attaching initiator sequences to glass slides using photolithographic in situ synthesis as a potential solid phase synthesis approach for chemically modified oligonucleotides.[42] They found that the initiator sequence with the 3′ termini of 2′OMe-RNA (3′-OH), L-DNA (5′-OH), and hexaethylene glycol have all acted as substrates for TdT enzyme, although the efficiency of incorporation was lower than for the natural 3′OH terminal group.[41] In 2021, Flamme *et al.* demonstrated the use of TdT to synthesize chemically modified locked nucleic acid (LNA) oligonucleotides by using the nucleotides α-thio-dTTP, LNA-TTP and α-thio-LNA-TTP as substrates (**16**).[43] They found that while the analogue α-thio-LNA-TTP was not well tolerated as a substrate for TdT synthesis while both LNA-TTP and α-thio-dTTP showed increased tolerance for incorporation by TdT enzyme onto the initiator.

Researchers have also taken advantage of TdT's ability to incorporate base-modified nucleotides to synthesize polynucleotides with tunable *in vivo* stability to address some of the current limitations in oligonucleotide-based therapeutics.[44] They demonstrated that increased sizes of polynucleotide and type of nucleotide used could be essentially tuned to avoid degradation from nuclease enzymes. The synthesis of larger polynucleotides could also potentially prevent renal clearance owing to their sizes which can go beyond the renal size limit of 8 nm.[45]

Overall, the use of TdT in enzymatic synthesis of polynucleotides is emerging as the most important synthesis approach for polynucleotides in the next few years which will cut the costs of large size DNA products and remove the current size bottleneck. With the emerging reports on the increased tolerance of TdT to incorporate chemically modified nucleotides, we should see a growth in the applications of chemically modified polynucleotides for introducing increased functionality, new types of protecting groups for stepwise synthesis and increase in nuclease resistance for future oligonucleotide therapeutics.

3.3. The application of TdT in DNA data storage

Although the use of DNA as a potential information storage medium has been around for the last few decades, it is only in the last several years that innovations in the area have made this future technology a more likely prospect.[46] DNA based storage systems have significant advantages over traditional types of data storage such as increased longevity and increase in storage capacity.

Researchers have utilized TdT in DNA as information storage medium.[47–49] In 2018, researchers from Harvard Medical school demonstrated a *de novo* enzymatic synthesis strategy to utilize DNA in data storage.[50] They used TdT to catalyse the synthesis of oligonucleotides strands with short homopolymeric extensions. To limit the degree of polymerization, they added apyrase (AP) enzyme to the reactions to convert the nucleoside triphosphates into their TdT-inactive diphosphate and monophosphate precursors. In 2020, Lee *et al.* described a multiplexed enzymatic DNA synthesis which utilizes a Co^{2+} cage as the mechanism to perform maskless lithography.[51] Upon uncaging of the Co^{2+} divalent metal ions, the enzymatic

reaction occurs. The researchers demonstrated the encoding of 12 unique DNA sequences using this approach, giving a turn on signal. More recently, Bhan *et al.* demonstrated a simplified system employing a single enzyme, TdT, to transduce environmental signals into DNA.[52] Essentially, researchers took advantage of an optimized TdT-based untemplated recording of temporal local environmental signals (TURTLES) to enable high-resolution spatial and temporal recordings of physiological signalling molecules that fluctuate on the time scale in less than an hour. This goes some way to addressing the current bottleneck of converting the biological signal of the DNA code to an output electrical signal. Overall, TdT clearly has a role in DNA data storage which could help allow for the replacement of traditional data storage systems.

3.4. The application of TdT in DNA nanotechnology

With increasing interest in the development of novel biomaterials for several biomedical applications, DNA has proven to be versatile building block for supramolecular structures such as DNA origami (DNA nanostructures) and DNA based hydrogels.[53–55] The use of enzymes such as a TdT in the synthesis of DNA nanostructures has become increasingly important to help produce more elaborate and elegant shapes, increase their functionalities and aid their precise engineering.[55] Researchers recently demonstrated the self-assembly of DNA hydrogels using the enzymatically polymerized DNA building blocks to reduce the number of DNA building block motifs required for their formation.[56] Researchers utilized a X-shaped DNA motif as well as terminal deoxynucleotidyl transferase (TdT) to elongate the DNA building blocks, and to allow hybridization between dual building blocks via their complementary TdT-polymerized DNA tails and led to gel formation. In 2021, Yang *et al.* demonstrated the site-specific functionalization of DNA origami structure to produce a series of polynucleotide-based brushes which could be utilized in future 3D cell cultivation and 3D bioprinting applications.[57] As an emerging area, TdT will continue to aid researchers in the design of elaborate DNA-nanostructures.

3.5. The application of TdT in biosensing

The unique ability of TdT to randomly add multiple deoxyribonucleoside triphosphates (dNTPs) to the 3′ OH end of ssDNA in a template-independent reaction has been exploited as a gateway for several biosensing applications and have been demonstrated on several biosensing platforms (Table 1).

The examples described reflect a sample of applications that highlight the enzyme's versatility and potential in developing novel biosensors or in enhancing the performance of existing ones. Two general strategies for the use of TdT to amplify the signal output of a biosensor are shown in Figure 2. TdT-based technologies for the sensitive detection of clinically relevant enzymes are an emerging aspects of biosensor toolkits available for clinical diagnosis. TdT-generated poly dA tails have been used for the sensitive detection of uracil-DNA glycosylase (UDG) activity,[58,59] DAM methylase,[60] and polynucleotide kinase.[61] MicroRNAs have emerged as important biomarkers for several disease conditions and there is significant interest in detection methods that are both sensitive and easy to use. New approaches for sensitive detection of *miRNA* have been developed using systems coupled with TdT-dependent amplification of target signals.[62] A label-free strategy incorporating strand displacement and TdT-assisted amplification was used to optimize the detection of miRNA-21, a key biomarker for several malignancies.[63] Another approach incorporating TdT with copper nanoclusters was used for quantitative detection of miRNA-21.[64] A multiple amplification strategy that utilizes TdT

Table 1. Summary of TdT assisted biosensors.

Analyte	Output signal	Detection limit (LOD)	Reference
Enzyme based analytes			
uracil-DNA glycosylase (UDG)	Fluorophore/quencher	5×10^{-6} U/mL and 0.090 U/mL	[58,59]
polynucleotide kinase (T4 PNK)	Fluorophore/quencher	1×10^{-4} U/mL	[58]
DAM methylase	Fluorophore/quencher	0.002 U/mL	[60]
microRNA based analytes			
miRNA-21	Fluorescence	18.7 pM	[64]
microRNA-162a	Photoelectrochemical	0.18 fM	[65]
Pathogens and exosomes analytes			
E. coli	Plasmonic/Raman	2 CFU/mL	[69]
Salmonella	Colorimetric	6 cfu/mL	[70]
hepatitis C virus	Electrochemical	32 fg/mL	[71]
Covid-19	Electrochemical	45 fM	[72,73]
microcystin	Electrochemical	15 pM	[81]
cancer cells exosomes	Colorimetric/enzyme-linked aptamer-sorbent assay	6.7×103 particles/μL	[82]
Small molecule and proteins			
ampicillin	Photoelectrochemical (PEC)	4.97 fM	[78]
kanamycin	Colorimetric	9 pM	[83]
thrombin	Fluorescence Electrochemical, fluorescence, (respectively)	2.0 fM, 0.31 pM, 0.1 nM, (respectively)	[84,85,86]
potassium ions	Fluorescence	0.4 μM	[86]

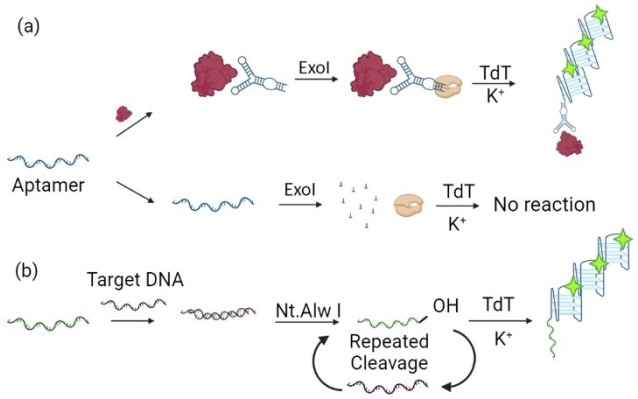


Figure 2. Overview of some general strategies for the use of TdT in the amplification of signal for the detection of (a) proteins and small molecules using aptamers (b) target DNA/mRNA using hybridisation probes.

target amplification was used for the detection of plant microRNA-162a.Click or tap here to enter text. Exosomes are nanoscale vesicles secreted by cells and carry biomolecules such as proteins, nucleic acids, or sugars. Their characteristics could provide information on the nature and properties of cells from which they originate. Exosomes are becoming increasingly important as biomarkers for several clinical conditions.[66] Recent studies have demonstrated the use of TdT-based technologies for the detection of exosomes. Huang *et al.* developed a modular TdT biosensor that incorporates enzyme-linked aptamer-sorbent assay for the detection of exosomes associated with colorectal cancer.[67] Another approach incorporates TdT to an exonuclease-based assay for detecting colorectal cancer exosomes.[68]

TdT have been coupled with other technologies either to detect cancer cells, microbes, and viruses. For such applications, TdT has been used for direct detection of pathogens in food, as well as infectious bacteria and viruses. This approach has been used in the detection of pathogenic *E. coli*,[69] Salmonella,[70] hepatitis C virus,[71] SARS-CoV-2,[72,73] and lipopolysaccharides.[74] TdT has also been used along with other sensing cascades for the detection of tumour cells[75] or circulating tumour DNA.[76]

One significant aspect of the versatility of TdT is that it can be used as a secondary tool to enhance the performance of other sensing technologies. For example, the sensitivity of catalytic G-quadruplex/hemin DNAzymes have been improved using TdT-assisted signal amplification.[77–79] Liu *et al.* improved the sensitivity of alkaline phosphatase by incorporating TdT-catalysed extension at 3′-OH end generated by the phosphatase.[80] The extension is hybridized with a G-rich capture DNA, and in the presence of hemin, a chromogenic hemin/G-quadruplex reaction is triggered. Using a similar approach, Abnous *et al.* coupled TdT with an aptamer-based biosensor for ultrasensitive detection of microcystin.[81] A similar TdT/aptamer approach was used for the detection of metastatic colorectal cancer cells.[82] Similar systems in which TdT was used to achieve signal amplification have been used for the detection of kanamycin,[83] thrombin,[84,85] potassium ions.[86] In combination with a CRISPR-Cas/Aptamer system, the detection of cocaine was also demonstrated.[87]

The application of TdT enzyme in biosensing has emerged as a new strategy for the detection of several small molecules and biomolecules and shows great potential in the realization of *in vivo* sensing currently held back by the lack of viable platforms available.

3.6. The use of TdT in aptamer development

We recently applied the use of TdT as a means for screening aptamers.[21] Libraries with variable sizes were generated using different ratios of nucleotides. Initiator concentrations and time of reaction allowed us to effectively tune the aptamers to increase their binding towards human thrombin and human lactoferrin model proteins. Termed as enzyme generated aptamers (EGA), this method allowed us to successfully find EGAs with low nanomolar binding within a single round. This also meant that we could screen EGAs based on both their size and sequence and allows for the screening of larger polynucleotides for binding towards the target. As an expansion to this method, and inspired by the methods used to generate molecularly imprinted polymers (MIPs),[88] we also demonstrated that the protein target could be utilized as a template during the synthesis of EGAs using TdT resulting in EGAs which could distinguish between human and bovine lactoferrin by a factor of 10.[89] This new development marks an exciting application in aptamer development and could reduce the complexity, cost, and time of classical SELEX methods.[90] This method removes the need for repeated rounds of selection, the degree of PCR bias is reduced and does not rely on random screening but rather rational design. The methodology could also be used as a simple method for the development of chemically modified aptamers. As discussed earlier, the list of chemically modified nucleotides that are tolerated by TdT make this a feasible approach. However, it remains to be seen how applicable this method is for developing aptamers against more challenging targets such as small molecules and whole cells.

4. Future Outlook and Conclusion

The use of TdT enzyme as a biotechnology tool has grown substantially in the last decade. The use of TdT in labelling of oligonucleotides at the 3′ end and in other molecular biology techniques such as RACE and tunnel assays is now considered a routine technique. In this review we discussed how TdT holds the key to opening up the sequence specific enzymatic synthesis of polynucleotides >200 nt that is currently the bottleneck in the development of new oligonucleotide drugs, drug delivery systems and DNA based information systems where long polynucleotides are needed. However, there are some hurdles that need to be overcome regarding the replacement of classical solid-phase synthesis. The flexibility of TdT to incorporate some non-natural nucleotides varies widely from substrate to substrate and there is a need to determine the

degree of tolerance of new nucleotide analogues as substrates for TdT and to engineer TdT variants with improved catalytic activity. This is especially critical if we are to translate enzyme-based synthesis to the largescale industrial synthesis. TdT has also shown to be a very promising tool for the development of nanostructures in terms of functionalizing DNA and generating homopolymers, and a promising strategy for the development of aptamers, which could simplify their development. In biosensing, TdT has being used as a basis by which we can detect analytes of interest by generating G-rich quadruplexes and specific fluorescence dyes as well as being utilized in electrochemical based sensors.[91]

We envisage that TdT-based developments in aptamer technology will improve other approaches aimed at enhancing sensitivity and versatility of biosensing applications using G-Quadruplex or DNAzymes.[91] Overall, the use of TdT enzyme has shown great potential in a wide range of applications making it a truly versatile strategy and biotechnology tool which should see an increase in applications over the next few years.

Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

Keywords: aptamers · biosensors · DNA nanotechnology · enzymes · terminal deoxynucleotidyl transferase

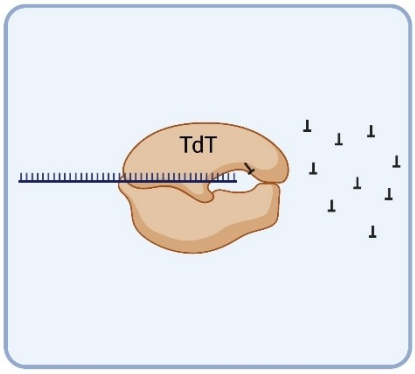
[1] E. A. Motea, A. J. Berdis, *Biochim. Biophys. Acta Proteins Proteomics* **2010**, *1804*, 1151–1166.
[2] F. J. Bollum, *J. Biol. Chem.* **1960**, *235*, 2399–2403.
[3] S. G. Rudd, J. Bianchi, A. J. Doherty, *Mol. Cell Oncol.* **2014**, *1*, 1, e960754.
[4] T. Kent, P. A. Mateos-Gomez, A. Sfeir, R. T. Pomerantz, *eLife* **2016**, *5*, 1–25.
[5] A. A. Kuznetsova, T. E. Tyugashev, I. v. Alekseeva, N. A. Timofeyeva, O. S. Fedorova, N. A. Kuznetsov, *SSRN Electronic J.* **2022**, *5*, 1–16.
[6] H. Han, J. Cui, D. Zhou, D. Hua, W. Peng, M. Lin, Y. Zhang, F. Li, X. Gong, J. Zhang, *Chin. Chem. Lett.* **2022**, https://doi.org/10.1016/j.cclet.2022.05.047.
[7] T. Baladi, J. R. Nilsson, A. Gallud, E. Celauro, C. Gasse, F. Levi-Acobas, I. Sarac, M. R. Hollenstein, A. Dahlén, E. K. Esbjörner, L. M. Wilhelmsson, *J. Am. Chem. Soc.* **2021**, *143*, 5413–5424.
[8] B. Zhao, Z. Gong, Z. Ma, D. Wang, Y. Jin, *Acta Biochim. Biophys. Sin.* **2012**, *44*, 129–135.
[9] I. D. Vilfan, W. Kamping, M. van den Hout, A. Candelli, S. Hage, N. H. Dekker, *Nucleic Acids Res.* **2007**, *35*, 6625–6639.
[10] A. A. Kuznetsova, T. E. Tyugashev, I. v. Alekseeva, N. A. Timofeyeva, O. S. Fedorova, N. A. Kuznetsov, *Life Sci. Alliance* **2022**, *5*, 1–16.
[11] E. Market, F. N. Papavasiliou, *PLoS Biol.* **2003**, *1*, 24–27.
[12] I. Sarac, M. Hollenstein, *ChemBioChem* **2019**, *20*, 860–871.
[13] F. J. BOLLUM, *J. Biol. Chem.* **1960**, *235*, 2399–2403.
[14] K. Bebenek, L. C. Pedersen, T. A. Kunkel, *Biochemistry* **2014**, *53*, 2781–2792.
[15] M. Delarue, J. B. Boulé, J. Lescar, N. Expert-Bezançon, N. Jourdan, N. Sukumar, F. Rougeon, C. Papanicolaou, *EMBO J.* **2002**, *21*, 427–439.
[16] M. Yoneda, F. J. Bollum, *J. Biol. Chem.* **1965**, *240*, 3385–3391.
[17] L. M. Chang, F. J. Bollum, *J. Biol. Chem.* **1971**, *246*, 909–916.
[18] M. R. Deibel, M. S. Coleman, *J. Biol. Chem.* **1980**, *255*, 4206–4212.
[19] L. M. S. Chang, F. J. Bollum, *J. Biol. Chem.* **1990**, *265*, 17436–17440.
[20] L. Tang, A. N. Jr, A. Chilkoti, S. Zauscher, *Angew. Chem. Int. Ed.* **2017**, *56*, 6778–6782; *Angew. Chem.* **2017**, *129*, 6882–6886.
[21] J. Ashley, A.-L. Schaap-Johansen, M. Mohammadniaei, M. Naseri, P. Marcatili, M. Prado, Y. Sun, *Nucleic Acids Res.* **2021**, *49*, 1065–1074.
[22] K. D. Bhilare, U. Mohan, U. C. Banerjee, *J. DNA RNA Res.* **2017**, *1*, 1–9.
[23] M. A. FROHMAN, *Recomb. DNA Methodol. II* **1995**, *8998*, 655–671.
[24] E. K. Jang, R. G. Son, S. P. Pack, *Nucleic Acids Res.* **2019**, *47*, E102.
[25] A. Simonova, L. Havran, R. Pohl, M. Fojta, M. Hocek, *Org. Biomol. Chem.* **2017**, *15*, 6984–6996.
[26] P. Horáková, H. Macíková-Cahová, H. Pivoková, J. Paek, L. Havran, M. Hocek, M. Fojta, *Org. Biomol. Chem.* **2011**, *9*, 1366–1371.
[27] M. Slavíčková, R. Pohl, M. Hocek, *J. Org. Chem.* **2016**, *81*, 11115–11125.
[28] M. Ondruš, V. Sýkorová, M. Hocek, *Chem. Commun.* **2022**, 11248–11251.
[29] M. L. Winz, E. C. Linder, T. André, J. Becker, A. Jäschke, *Nucleic Acids Res.* **2015**, *43*, 1–10.
[30] M. L. Winz, A. Samanta, D. Benzinger, A. Jäschke, *Nucleic Acids Res.* **2012**, *40*, 1–13.
[31] N. Z. Fantoni, A. H. El-Sagheer, T. Brown, *Chem. Rev.* **2021**, *121*, 7122–7154.
[32] P. Röthlisberger, F. Levi-Acobas, I. Sarac, B. Baron, P. England, P. Marlière, P. Herdewijn, M. Hollenstein, *Chem. Commun.* **2017**, *53*, 13031–13034.
[33] P. G. Adamopoulos, P. Tsiakanikas, I. Stolidi, A. Scorilas, *BMC Genomics* **2022**, *23*, 1–14.
[34] K. Kyrylkova, S. Kyryachenko, M. Leid, C. Kioussi, *Methods Mol. Biol.* **2012**, *887*, 41–47.
[35] M. J. Czar, J. C. Anderson, J. S. Bader, J. Peccoud, *Trends Biotechnol.* **2009**, *27*, 63–72.
[36] J. Perkel, *Nature* **2019**, *566*, 9.
[37] S. Palluk, D. H. Arlow, T. De Rond, S. Barthel, J. S. Kang, R. Bector, H. M. Baghdassarian, A. N. Truong, P. W. Kim, A. K. Singh, N. J. Hillson, J. D. Keasling, *Nat. Biotechnol.* **2018**, *36*, 645–650.
[38] A. S. Mathews, H. Yang, C. Montemagno, *Org. Biomol. Chem.* **2016**, *14*, 8278–8288.
[39] M. Flamme, D. Katkevica, K. Pajuste, M. Katkevics, N. Sabat, S. Hanlon, I. Marzuoli, K. Püntener, F. Sladojevich, M. Hollenstein, *Asian J. Org. Chem.* **2022**, *11*, e202200384.
[40] M. Flamme, S. Hanlon, H. Iding, K. Puentener, F. Sladojevich, M. Hollenstein, *Bioorg. Med. Chem. Lett.* **2021**, *48*, 128242.
[41] X. Lu, J. Li, C. Li, Q. Lou, K. Peng, B. Cai, Y. Liu, Y. Yao, L. Lu, Z. Tian, H. Ma, W. Wang, J. Cheng, X. Guo, H. Jiang, Y. Ma, *ACS Catal.* **2022**, *12*, 2988–2997.
[42] A. Shivalingam, T. Brown, *Biochem. Soc. Trans.* **2016**, *44*, 709–715.
[43] E. Schaudy, J. Lietard, M. M. Somoza, *ACS Synth. Biol.* **2021**, *10*, 1750–1760.
[44] R. Gu, T. Oweida, Y. G. Yingling, A. Chilkoti, S. Zauscher, *Biomacromolecules* **2018**, *19*, 3525–3535.
[45] C. F. Adhipandito, S. H. Cheung, Y. H. Lin, S. H. Wu, *Int. J. Mol. Sci.* **2021**, *22*, 11182.
[46] D. Panda, K. A. Molla, M. J. Baig, A. Swain, D. Behera, M. Dash, *J. Biotechnol.* **2018**, *8*, 1–9.
[47] Y. Hao, Q. Li, C. Fan, F. Wang, *Small Structures* **2021**, *2*, 2000046.
[48] C. K. Lim, S. Nirantar, W. S. Yew, C. L. Poh, *Trends Biotechnol.* **2021**, *39*, 990–1003.
[49] L. F. Song, Z. H. Deng, Z. Y. Gong, L. L. Li, B. Z. Li, *Front. Bioeng. Biotechnol.* **2021**, *9*.
[50] H. H. Lee, R. Kalhor, N. Goela, J. Bolot, G. M. Church, *Nat. Commun.* **2019**, *10*, 1–12.
[51] H. Lee, D. J. Wiegand, K. Griswold, S. Punthambaker, H. Chun, R. E. Kohman, G. M. Church, *Nat. Commun.* **2020**, *11*, 1–9.
[52] N. Bhan, A. Callisto, J. Strutz, J. Glaser, R. Kalhor, E. S. Boyden, G. Church, K. Kording, K. E. J. Tyo, *J. Am. Chem. Soc.* **2021**, *143*, 16630–16640.
[53] Y. Zhang, L. Zhu, J. Tian, L. Zhu, X. Ma, X. He, K. Huang, F. Ren, W. Xu, *Adv. Sci.* **2021**, *8*, 1–28.
[54] S. H. Um, J. B. Lee, N. Park, S. Y. Kwon, C. C. Umbach, D. Luo, *Nat. Mater.* **2006**, *5*, 797–801.
[55] S. Keller, A. Marx, *Chem. Soc. Rev.* **2011**, *40*, 5690–5697.
[56] B. Xiang, K. He, R. Zhu, Z. Liu, S. Zeng, Y. Huang, Z. Nie, S. Yao, *ACS Appl. Mater. Interfaces* **2016**, *8*, 22801–22807.
[57] Y. Yang, Q. Lu, C. M. Huang, H. Qian, Y. Zhang, S. Deshpande, G. Arya, Y. Ke, S. Zauscher, *Angew. Chem. Int. Ed.* **2021**, *60*, 23241–23247.

[58] L. Wang, H. Zhang, Y. Xie, H. Chen, C. Ren, X. Chen, *Talanta* **2019**, *194*, 846–851.
[59] Y. C. Du, S. Y. Wang, Y. X. Wang, J. Y. Ma, D. X. Wang, A. N. Tang, D. M. Kong, *Biosens. Bioelectron.* **2021**, *171*, 112734.
[60] X. Chen, G. Cao, X. Wang, Z. Ji, F. Xu, D. Huo, X. Luo, C. Hou, *Biosens. Bioelectron.* **2020**, *163*, 112271.
[61] Y. C. Du, S. Y. Wang, X. Y. Li, Y. X. Wang, A. N. Tang, D. M. Kong, *Biosens. Bioelectron.* **2019**, *145*, 111700.
[62] J. L. He, T. T. Mei, L. Tang, S. Q. Liao, Z. Cao, *Talanta* **2020**, *219*, 121173.
[63] Y. Yan, H. Zhao, Y. Fang, C. Ma, J. Chen, *Biosensors* **2022**, *12*, 328.
[64] Y. Li, D. Tang, L. Zhu, J. Cai, C. Chu, J. Wang, M. Xia, Z. Cao, H. Zhu, *Anal. Biochem.* **2019**, *585*, 113346.
[65] M. Wang, H. Yin, Y. Zhou, J. Han, T. He, L. Cui, S. Ai, *Microchim. Acta* **2018**, *185*, 257.
[66] L. Wang, Y. Deng, Y. Huang, J. Wei, J. Ma, G. Li, *Chem. Commun.* **2021**, *57*, 8508–8511.
[67] Z. Huang, Q. Lin, X. Ye, B. Yang, R. Zhang, H. Chen, W. Weng, J. Kong, *Talanta* **2020**, *218*, 121089.
[68] L. Wang, Y. Deng, J. Wei, Y. Huang, Z. Wang, G. Li, *Biosens. Bioelectron.* **2021**, *191*, 113465.
[69] Y. Zhou, W. Fang, K. Lai, Y. Zhu, X. Bian, J. Shen, Q. Li, L. Wang, W. Zhang, J. Yan, *Biosens. Bioelectron.* **2019**, *141*, 111419.
[70] Y. Zhang, J. Tian, K. Li, H. Tian, W. Xu, *Anal. Chim. Acta* **2019**, *1075*, 144–151.
[71] Y. Wang, L. Li, Z. Dong, Y. Yu, A. Zhou, X. Zhao, J. Zhang, *Microchim. Acta* **2021**, *188*, 285.
[72] Y. Deng, Y. Peng, L. Wang, M. Wang, T. Zhou, L. Xiang, J. Li, J. Yang, G. Li, *Anal. Chim. Acta* **2022**, *1208*, 339846.
[73] Y. Peng, Y. Pan, Z. Sun, J. Li, Y. Yi, J. Yang, G. Li, *Biosens. Bioelectron.* **2021**, *186*, 113309.
[74] Y. Huang, L. Wang, L. Sha, Y. Wang, X. Duan, G. Li, *Anal. Chim. Acta* **2020**, *1126*, 31–37.
[75] J. Zheng, H. Shi, M. Wang, C. Duan, Y. Huang, C. Li, Y. Xiang, G. Li, *Anal. Chem.* **2020**, *92*, 2194–2200.
[76] H. F. Wang, R. N. Ma, F. Sun, L. P. Jia, W. Zhang, L. Shang, Q. W. Xue, W. L. Jia, H. S. Wang, *Biosens. Bioelectron.* **2018**, *122*, 224–230.
[77] Z. Liu, T. Liu, C. A. Tao, X. Chen, J. Huang, F. Wang, J. Wang, *Anal. Sci.* **2020**, *36*, 835–840.
[78] H. Wang, M. Wang, J. Liu, G. Li, B. Ye, L. Zou, *Anal. Chim. Acta* **2022**, *1211*, 339912.
[79] Z. Liu, K. He, W. Li, X. Liu, X. Xu, Z. Nie, S. Yao, *Methods Mol. Biol.* **2017**, *1500*, 133–151.
[80] Y. Liu, E. Xiong, X. Li, J. Li, X. Zhang, J. Chen, *Biosens. Bioelectron.* **2017**, *87*, 970–975.
[81] K. Abnous, N. M. Danesh, M. A. Nameghi, M. Ramezani, M. Alibolandi, P. Lavaee, S. M. Taghdisi, *Biosens. Bioelectron.* **2019**, *144*, 111674.
[82] Y. Zhao, W. Ma, S. Zou, B. Chen, H. Cheng, X. He, K. Wang, *Talanta* **2019**, *202*, 152–158.
[83] T. Zhao, Q. Chen, Y. Wen, X. Bian, Q. Tao, G. Liu, J. Yan, *Food Chem.* **2022**, *377*, 132072.
[84] D. Wen, M. He, K. Ma, Y. Cui, J. Kong, H. Yang, Q. Liu, *Microchim. Acta* **2018**, *185*, 6–13.
[85] S. Lei, Z. Liu, L. Xu, L. Zou, G. Li, B. Ye, *Anal. Chim. Acta* **2020**, *1100*, 40–46.
[86] K. H. Leung, B. He, C. Yang, C. H. Leung, H. M. D. Wang, D. L. Ma, *ACS Appl. Mater. Interfaces* **2015**, *7*, 24046–24052.
[87] K. Abnous, A. khakshour Abdolabadi, M. Ramezani, M. Alibolandi, M. A. Nameghi, T. S. Zavvar, Z. Khoshbin, P. Lavaee, S. M. Taghdisi, N. M. Danesh, *Talanta* **2022**, *241*, 123276.
[88] J. Ashley, M. A. Shahbazi, K. Kant, V. A. Chidambara, A. Wolff, D. D. Bang, Y. Sun, *Biosens. Bioelectron.* **2017**, *91*, 606–615.
[89] J. Vacacela, A. L. Schaap-Johansen, P. Manikova, P. Marcatili, M. Prado, Y. Sun, J. Ashley, *Angew. Chem. Int. Ed.* **2022**, *61*, e202201061.
[90] C. Klingler, J. Ashley, K. Shi, A. Stiefvater, M. Kyba, M. Sinnreich, H. Aihara, J. Kinter, *FASEB J.* **2020**, *34*, 4573–4590.
[91] R. I. Adeoye, D. S. Osalaye, T. K. Ralebitso-Senior, A. Boddis, A. J. Reid, A. A. Fatokun, A. K. Powell, S. O. Malomo, F. J. Olorunniji, *Catalysts* **2019**, *9*, 1–13.

Manuscript received: September 1, 2022
Revised manuscript received: November 4, 2022
Accepted manuscript online: November 7, 2022
Version of record online: ■■, ■■■

REVIEW

Terminal deoxynucleotidyl transferase (TdT) is a unique polymerase enzyme that does not require a DNA template in order to amplify DNA. The enzyme allows for the stepwise addition of single nucleotides onto the 3′ end of any oligonucleotide sequence. This has made TdT a very exciting molecular tool in biotechnology applications. In this review we explore these applications.



J. Ashley, I. G. Potts, F. J. Olorunniji*

1 – 10

Applications of Terminal Deoxynucleotidyl Transferase Enzyme in Biotechnology