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Enzymatic synthesis and modification of high molecular weight DNA using terminal deoxynucleotidyl transferase

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Abstract

The recognition that nucleic acids can be used as polymeric materials led to the blossoming of the field of DNA nanotechnology, with a broad range of applications in biotechnology, biosensors, diagnostics, and drug delivery. These applications require efficient methods to synthesize and chemically modify high molecular weight DNA. Here, we discuss terminal deoxynucleotidyl transferase (TdT)-catalyzed enzymatic polymerization (TcEP) as an alternative to conventional enzymatic and solid-phase DNA synthesis. We describe biochemical requirements for TcEP and provide step-by-step protocols to carry out TcEP in solution and from surfaces.

1. Introduction

Beyond their role as the genetic material of living things, nucleic acids (DNA and RNA) are now being used to create synthetic polymeric materials for use in nanotechnology (Bae, Kocabey, & Liedl, 2019; Liu, Jiang, Wang, & Ding, 2019; Suo et al., 2019; Zhao et al., 2019), biosensing (Abolhasan, Mehdizadeh, Rashidi, Aghebati-Maleki, & Yousefi, 2019; Mason, Tang, Li, Xie, & Li, 2018; Tjong, Yu, Hucknall, Rangarajan, & Chilkoti, 2011) and drug delivery (Liu et al., 2019; Mathur & Medintz, 2019). Nucleic acids are versatile due to their customizable sequences and specific molecular recognition. Applications of DNA and RNA nanotechnology often require chemical and enzymatic polynucleotide modifications such as the incorporation of fluorescent groups, reactive groups including azides and amines, biofunctional groups such as biotin, and sugar modifications such as 2'-O-methyl (2'-OMe) or 2'-deoxy-2'-fluoro-ribonucleotide (2'-F) to bestow RNA with nuclease stability for RNA interference (RNAi) and CRISPR-based genome editing (Yin et al., 2017).

Modifications of DNA can be introduced chemically by using solid-phase synthesis or enzymatically by using a DNA polymerase together with unnatural deoxynucleotidyl triphosphates (dNTPs). Solid-phase synthesis is widely used when large quantities are desired, but is limited by the oligonucleotide length that can be produced (~200 nt) and by the compatibility of functional groups with the chemical reactions used in oligonucleotide synthesis (Rothlisberger & Hollenstein, 2018). Enzymatic DNA synthesis methods such as

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PCR (Rittié & Perbal, 2008; Terpe, 2013; Vosberg, 1989), primer extension (Carey, Peterson, & Smale, 2013) and rolling circle amplification (RCA) (Dean, Nelson, Giesler, & Lasken, 2001) typically use polymerases that have limited tolerance for chemically modified dNTPs (Houlihan, Arangundy-Franklin, & Holliger, 2017) and that require a template to catalyze primer extension. In contrast, terminal deoxynucleotidyl transferase (TdT) is a template-independent polymerase which tolerates chemically modified dNTPs and thus is suitable for the synthesis of chemically modified, high molecular weight DNA. This mechanism is harnessed in TdT catalyzed enzymatic polymerization (TcEP) for the synthesis of single-stranded polynucleotides (Tang, Navarro Jr., Chilkoti, & Zauscher, 2017) (Fig. 1).

TdT is a member of the X family of DNA polymerases, whose members add dNTPs randomly to the 3'-hydroxyl group (3'-OH) of single-stranded DNA (Bollum, 1960). Biologically, TdT contributes to the diversity of immunoglobulins and T cell receptors (TCR) by participating in the rearrangement of variable (V), diversity (D), and joining (J) segments within immunoglobulin and TCR genes, as briefly described next.

The vertebrate adaptive immune system consists of B cells and T cells that express a vast repertoire of antigen receptors (Alberts et al., 2002; Owen, Punt, Stranford, Jones, & Kuby, 2013). Binding of these receptors to antigens on the surfaces of pathogens or foreign bodies triggers a cascade of responses that activates the immune system. Rather than encoding the huge number of antigen-binding receptors with individual genes, cells use a gene rearrangement process called V(D)J recombination. This process is orchestrated by polymerases, nucleases, and ligases. TdT participates in the second stage of the process, following a double-stranded break at a highly conserved recombination signal sequence (RSS) that is induced by the recombination-activating gene (RAG) enzyme and the endonuclease, Artemis (Jung & Alt, 2004; Nick-McElhinny et al., 2005). Cleavage at the RSS by RAG results in the formation of a hairpin structure following transesterification of the 3'-OH of a nicked strand with a phosphorous on the opposite strand. Artemis opens the hairpin and TdT adds ~1–10 random nucleotides (Schatz, Oettinger, & Schlissel, 1992). TdT preferentially adds dGTP and dCTP, resulting in a GC-rich region in these genes. This GC bias promotes efficient annealing of single-stranded DNA in the subsequent ligation.

As we show and discuss in more detail in Section 3, TdT catalyzed enzymatic polymerization can be harnessed for the synthesis of high molecular weight single-stranded homo- and co-polynucleotides in solution and from surfaces (Chow, Lee, Zauscher, & Chilkoti, 2005; Gu, Tang, Aritome, & Zauscher, 2017; Tang et al., 2014; Tang et al., 2017; Tjong et al., 2011). These polyelectrolytes hold great promise for applications ranging from drug delivery to biosensors. Very recently it was shown that TdT can be employed in the synthesis of sequenced polynucleotides, overcoming some of the limitations of current solid phase oligonucleotide synthesis (Lee, Kalhor, Goela, Bolot, & Church, 2019).

2. Biochemical requirements for TdT mediated polymerization

A TdT-catalyzed enzymatic polymerization reaction consists of an oligodeoxynucleotide as the initiator, natural or unnatural dNTPs, TdT, and buffer supplemented with a metal ion

cofactor (Fig. 1) (Bollum, 1960, 2006; Kato, Goncalves, Houts, & Bollum, 1967). This section will describe each of these components in TcEP reactions in more detail.

2.1 Initiators

An oligodeoxynucleotide with a free 3'-hydroxyl terminus is essential for initiation of polymerization by TdT. Generally, TdT prefers either a single-stranded DNA (ssDNA) initiator or double-stranded DNA (dsDNA) with a four-nucleotide 3' overhang (Delarue et al., 2002; Fowler & Suo, 2006). The presence of a secondary structural motif at the 3' terminus can hinder access of the 3'-OH to the catalytic site of the enzyme and limit nucleotide addition (Delarue et al., 2002). Even single-stranded homooligonucleotides such as poly(dC) and poly(dG) can form secondary structures (i-motifs and G-quadruplexes, respectively) that impede polymerization. Even though poly(dA) can form secondary structures, it is an efficient TcEP initiator, as is poly(dT), which does not form secondary structure. The minimum length for efficient initiation by TdT is typically a triodeoxynucleotide (Bollum, 1974; Tjong et al., 2011), though Hayes et al. successfully used a dinucleotide as initiator (Hayes, Mitchell, Ratliff, Schwartz, & Williams, 1966). However, for such short initiators (<4nt), 5' phosphorylation is required. Initiation from blunt-ended dsDNA is also possible. However, in that case, the reaction buffer must be supplemented with Co^{2+} rather than Mg^{2+} (Roychoudhury, Jay, & Wu, 1976). Interestingly, TdT can extend an initiator that has a single ribonucleotide 5'-monophosphate (rNMP) at the 3' terminus, but in this case the reaction rate is reduced and the number of nucleotides added is limited (Roychoudhury, 1972).

2.2 Deoxynucleoside triphosphates (dNTPs)

TdT can incorporate a wide range of natural and unnatural dNTPs (Table 1), where the preferred order of incorporation by TdT is dGTP>dCTP>dTTP>dATP (Berdis & McCutcheon, 2007; Chang et al., 1988; Wan et al., 2013). However, as mentioned above, initiator extension in the presence of only dGTP or dCTP is limited by the formation of secondary structures of oligo(dG) and oligo(dC) (Bollum, 1974). Poly(dA) and poly(dT) ssDNA homopolymers as long as 8 kb have been synthesized by our group using dA₁₀ and dT₁₀ initiators and dATP and dTTP monomers, respectively (Tang et al., 2014; Tang et al., 2017; Tjong et al., 2011). We observed that the ratio of monomer (dNTP) to initiator concentration (M/I ratio) as well as the incubation time provide control over the polynucleotide length (molecular weight) (Tang et al., 2017).

Unnatural nucleotides can be used to endow synthesized DNA with desirable functionalities. A variety of unnatural dNTPs have been used successfully in TdT-mediated polymerization, including dNTPs modified with biotin (Flickinger et al., 1992; Gavrieli et al., 1992), alkyne (Gu et al., 2018; Winz et al., 2015), digoxigenin (Schmitz et al., 1991), or fluorescent dyes (Guerra, 2006; Igloi, 1998; Tjong et al., 2011) as well as amine allyl (Kumar et al., 1988) and dideoxy (Guerra, 2006) dNTPs (Table 1).

The ability of TdT to incorporate these unnatural nucleotides depends on the type and position of the dNTP modification and must be evaluated on a case-by-case basis. DNA polymerases generally tolerate modifications at the C5 position of pyrimidines (T and C)

and at the C7 position of purines (A and G) (Fig. 2) that do not hinder interactions with the enzyme or affect Watson-Crick base pairing (Bergen et al., 2012; Obeid, Baccaro, Welte, Diederichs, & Marx, 2010). However, dNTPs modified with bulky hydrophobic groups such as dibenzylcyclooctyne (DBCO) or large fluorophores such as Cy3 or fluorescein often require a hydrophilic linker such as poly(ethylene glycol) (PEG) to position these groups away from the enzyme's active site. A study of how TdT tolerates modifications at position 2 of dATP found that bulky modifications such as phenyl groups have an inhibitory effect on TdT activity, while smaller modifications such as Cl, NH₂, CH₃, vinyl, and ethynyl groups are readily incorporated (Matyašovský, Perlíková, Malnuit, Pohl, & Hocek, 2016). Interestingly, dNTPs with bulky, hydrophobic modifications impart nuclease stability to the modified ssDNA (Gu et al., 2018). Like most of the DNA polymerases, TdT poorly facilitates the addition of ribonucleotide triphosphates (NTPs). However, substituting ribose with arabinose altogether inhibits polymerization (Hansbury et al., 1970; Roychoudhury et al., 1976).

Palluk et al. recently demonstrated the use of covalent TdT-dNTP conjugates for *de novo* (template-free) DNA synthesis (Fig. 3) (Palluk et al., 2018). In template-free DNA synthesis, TdT incorporates the dNTP into a primer and remains bound to the complex, blocking the 3' end of the primer and inhibiting further addition. Excess unbound conjugate is removed and the 3'-OH is activated by cleaving the linker that connects dNTP to TdT. The oligonucleotide can then be subjected to the next round of extension with another TdT-dNTP conjugate to create the desired sequence (Palluk et al., 2018). As TdT can synthesize kilobase-length polynucleotides, this method can be used to overcome the limitations of sequence fidelity and length encountered in conventional chemical syntheses.

2.3 TdT

Terminal deoxynucleotidyl transferase was discovered in 1960 when it was purified from a calf thymus gland (Bollum, 1960). The full length 58kDa protein undergoes proteolytic degradation, generating fragments some of which still show enzymatic activity (Chang, Plevani, & Bollum, 1982). However, this makes the purification of a homogenous, active TdT enzyme from calf thymus tedious. Growing interest in the structure-function relationship of TdT required producing pure enzyme in large quantities, thus other methods to obtain TdT have been explored, including expression of human TdT in human acute lymphoblastic leukemia cell lines or in baculovirus (Chang et al., 1988) and expression of avian TdT in *E.coli* (Yang, Gathy, & Coleman, 1995). Nevertheless, these systems were unable to generate sufficient quantities of active enzyme. Boulé et al. increased the expression level of murine TdT in *E.coli* by lowering the culture temperature and overexpressing a rare arginine tRNA (argU) (Boulé, Johnson, Rougeon, & Papanicolaou, 1998). In an attempt to improve the enzyme's activity for *in vitro* applications, a truncated version of calf thymus TdT with a 20–30 fold increase in activity was expressed in *E.coli* (Mueller et al., 2009).

In 2002, more than 40 years after the discovery of TdT, the first crystal structure of the catalytic core of murine TdT (130–510 amino acid residues) was solved (Delarue et al., 2002). Like in most DNA polymerases, TdT's catalytic core exhibits the typical “thumb,”

“palm,” and “finger” domains (Fig. 4). However, unlike DNA pol β , and other DNA polymerases, TdT has an additional 16 residue loop (loop 1) that assumes a lariat-like conformation and is thought to hinder the accommodation of a template strand (Delarue et al., 2002). Thorough study of the initiator-TdT binary complex suggested that the last four nucleotides at the 3'-OH end interact with the polymerase, irrespective of the nature of the base. At the catalytic site metal ions coordinate with the conserved aspartate residues and the incoming dNTP, positioning its alpha phosphate (see Fig. 2) for nucleophilic attack by the 3'-OH. The absence of specific interactions of the nucleotide bases with TdT explains the lack of specificity toward addition of dNTP (Delarue et al., 2002; Gouge et al., 2013).

2.4 Metal ions as cofactors

Divalent cations coordinate catalytic polymerase residues with the incoming dNTP and thus play an important role as cofactors in the synthesis of polynucleotides by DNA polymerases (Steitz, 1993). Unlike other DNA polymerases, TdT can utilize several different metal ions including Mg^{2+} , Co^{2+} , Mn^{2+} and Zn^{2+} , though its preference for nucleotide incorporation depends on the metal ion used. For example, when the buffer is supplemented with Mg^{2+} , the purine nucleotide incorporation rate is 10-fold higher than that of pyrimidines (Douglas & Morgan, 1976). When the buffer is supplemented with Co^{2+} , the pyrimidine nucleotide incorporation rate is higher (Chang & Bollum, 1990). Interestingly, supplementing Mg^{2+} with sub-millimolar concentrations of Zn^{2+} increases TdT activity 2–4 fold and also rescues TdT inhibition by metal chelators (Chang & Bollum, 1990).

3. Applications

3.1 TdT catalyzed polynucleotide synthesis in solution

Tang et al. demonstrated that TcEP proceeds by a living chain-growth polycondensation mechanism, and thus the molecular weight of the product can be tuned by changing the feed ratio of nucleotide (monomer) to oligonucleotide (initiator) (M/I ratio) (Tang et al., 2017). Taking advantage of the template-free, promiscuous polymerization ability of TdT, single stranded polynucleotides with narrow molecular weight distribution and containing various unnatural nucleotides, including amines, alkynes, azides, and hydrophobes, can be synthesized (Gu et al., 2018). Thus it is easy to functionalize the ssDNA chains to produce stable nucleotide nanoparticles (Fig. 5) (Tang et al., 2014), to synthesize cytostatic polynucleotide drugs and to increase their nuclease resistance (Gu et al., 2018) which potentially helps to increase the half-life of the drug. Interestingly, a recent study by Que. et al. combined the template-independent property of TdT with the high amplification efficiency of RCA to develop a label-free, TdT-induced RCA based method to detect DNA Methyltransferase (MTase) (Que et al., 2019). This method has great potential for DNA MTase-related cancer diagnosis and disease therapeutics.

3.2 TdT catalyzed polynucleotide synthesis on surfaces

In addition to catalyzing nucleotide polymerization in solution, TdT can also be used for the *in situ* synthesis of polynucleotide brushes on surfaces by the “grafting-from” approach. This process was first introduced by Chow et al. who also coined the term “surface-initiated enzymatic polymerization” (SIEP) (Chow et al., 2005). Furthermore, SIEP can be used to

functionalize nanoparticles, including DNA origami (Okholm, Aslan, Besenbacher, Dong, & Kjems, 2015), liposomes (Ruysschaert, Paquereau, Winterhalter, & Fournier, 2006) and gold nanoparticles (Wang et al., 2016), with ssDNA. The template-free SIEP is useful for the fabrication of sensors that can detect nucleases (Chen, Xu, Ji, & He, 2017), DNA (Tjong et al., 2011; Wan et al., 2013; Wan et al., 2014), RNA (Tjong, Yu, Hucknall, & Chilkoti, 2013), metal ions (Mei et al., 2016), tumor biomarkers (Wang et al., 2016) and proteins (Shi, Dou, Yang, Yuan, & Xiang, 2017). An early study by Tjong et al. demonstrated a sensing platform to detect ssDNA by first immobilizing a capture oligonucleotide on a glass surface, followed by SIEP at the 3' end of the detected and hybridized ssDNA (Fig. 6) (Tjong et al., 2011). Efficient initiation (~50%) and narrow polydispersity of the extended product were observed when fluorophore-labeled dNTPs were incorporated. Up to ~50 Cy3-labeled dNTPs per kilo-base could be incorporated into a ssDNA chain using TdT, which translated into a ~45-fold signal amplification compared to the incorporation of a single fluorophore. This led to a detection limit of about 1 pM, and a linear dynamic range of 2 logs.

Other studies used TdT to incorporate biotin-modified-dNTP to take advantage of the biotin-avidin interaction which increases sensor sensitivity. For example, Wang et al. developed an electrochemical biosensor for the analysis of a cancer bio-marker, carcino-embryonic antigen (CEA), by incorporating a pair of aptamers which can specifically bind with CEA (Wang et al., 2016). One of the aptamers, immobilized on a gold electrode, serves as the capture probe, while the other aptamer is linked to a gold nanoparticle (GNP) probe. After the dual aptamer capture of CEA in "sandwich" format, the oligonucleotides on the GNPs are harnessed to initiate SIEP to incorporate biotin-dNTP, which are ultimately used for enhancing the signal (Fig. 7).

SIEP can also be used to produce complex biomolecular structures on flat surfaces with nanoscale resolution (Chow et al., 2005; Khan, Tjong, Chilkoti, & Zharnikov, 2012; Khan, Tjong, Chilkoti, & Zharnikov, 2013). Recently, SIEP was harnessed for the design of a digital information storage medium, in which information is stored in the transitions between non-identical nucleotides of ssDNA (Fig. 8) (Lee et al., 2019).

4. Materials

T₅₀ and 5' end-modified oligonucleotides can be procured from Integrated DNA Technology (IDT). Suppliers including Jena Biosciences, TriLink Biotechnologies, Sigma Aldrich and Thermo Fisher Scientific provide natural dNTPs as well as a range of unnatural dNTPs. Terminal deoxynucleotidyl transferase (TdT) and TdT buffer can be purchased from Promega. Ethylenediaminetetraacetic acid (EDTA) (0.5M, pH 8) solution, sulfuric acid, nitric acid, sodium acetate, sodium chloride, sodium citrate, acetone, ethanol, 10× tris-borate EDTA buffer (pH 8.3) and Tween 20 can be procured from Sigma Aldrich. 0.5 mL Microcon Centrifugal Filter Units can be purchased from Millipore, 5% TBE-PAGE gel from Biorad, Agarose (low EEO) from Thermo Scientific, glass slides from Solulink, and succinimidyl 4-formylbenzoate from VWR. Gold substrates can be fabricated by metal evaporation on a silicon wafer of first a ~10nm titanium (or chromium) adhesion layer followed by a layer of ~100nm gold.

5. Protocol for solution polymerization and characterization

5.1 Protocol

- 1.1 Prepare reaction solution containing 0.5 μM oligo initiator (*e.g.*, T₅₀), natural or unnatural dNTP (the final dNTP concentration depends on the desired length of the polynucleotide), and 1 $\times$ TdT reaction buffer (100mM potassium cacodylate, 1mM CoCl₂, and 0.1 mM DTT, pH6.8). Then add 1.2U/ μL TdT and pipette to mix (DO NOT VORTEX!). If the amount of TdT used is too low, the reaction will yield polydisperse polynucleotides.
- 1.2 Incubate overnight at 37 °C.
- 1.3 Heat the reaction mixture at 90°C for 3min or at 70°C for 10min to inactivate the enzyme. If heat inactivation is unsuitable, add EDTA to a final concentration of 30 mM. Then add an equal volume of water and centrifuge at 16,000 rcf for 3 min to precipitate the enzyme. Collect supernatant by pipetting.
- 1.4 Purify 20–100 μL of the supernatant using a 0.5 mL Microcon Centrifugal Filter Unit (Millipore). Choose the molecular weight cut off (MWCO) of the filter according to the expected molecular weight of the polynucleotide. Perform three consecutive washes with Milli-Q grade water ($>18.2\text{M}\Omega\text{cm}^{-1}$) to remove all the free dNTP. After purification, concentrate the sample using the same filter, and recover the sample into a clean tube, supplied with the Microcon filter.
- 1.5 If a block copolymer is desired, repeat Steps 1–4 with a different dNTP.
- 1.6 Store the polymerization product at –20 °C. The products are stable for up to 2 month.

Tips

- a. Vortex mixing of enzymatic reactions may cause enzyme (protein) denaturation. Therefore, we recommend to mix the reaction by pipette and not by vortexing after addition of TdT.
- b. To obtain a constant polynucleotide molecular weight, maintain the relative ratio of initiator:dNTP:TdT in each reaction.
- c. It is easier to control the polynucleotide molecular weight by adjusting the ratio of dNTP monomer to initiator (M/I ratio) rather than the reaction time.
- d. If a random copolymer rather than a homo-polynucleotide is desired, use a mixture of dNTPs. However, note that nucleotide incorporation efficiency depends on the nucleotide and on the metal ion cofactor used (see Section 2.4).
- e. Use an initiator with a fluorescent tag to facilitate characterization using gel electrophoresis.
- f. Change the filter after 3–4 washes. Be sure to mix the sample on the centrifugal filter surface thoroughly by pipetting between each wash to prevent sample precipitation and loss on the filter. When using filters to remove fluorescent

unnatural nucleotides, the number of wash steps is determined by the fluorescent intensity in the flow-through (wash the sample until no fluorescence is detected in the flow-through).

5.2 Characterization

- 2.1 The weight average and number average molecular weights ($\overline{M}_w$ and $\overline{M}_n$) and the polydispersity index ($PDI = \overline{M}_w / \overline{M}_n$) of the product can be determined by running the sample and the fractions of a molecular weight ladder on a 5% TBE-PAGE gel at 100 V for 40min. The gel should be imaged with a high-resolution gel scanner to best capture the fluorescence intensity distribution. The image can subsequently be analyzed with ImageJ software (freely available at <https://imagej.nih.gov/ij/>) to obtain $\overline{M}_w$, $\overline{M}_n$ and the PDI of the product (Fig. 9), following the procedure developed by (Tang, 2016), pp. 112–123.
- 2.2 A relatively accurate method to determine the molarity of the product is to fluorescently label the initiator and create a standard curve of the fluorophore using a fluorescence spectrometer. Another method to obtain the mass concentration of the product is to use a UV–vis photo-spectrometer (*e.g.*, Nanodrop 1000 UV–Vis spectrometer, Thermo Scientific), and determine the molarity using the molecular weight obtained from gel electrophoresis. Although HPLC is the most accurate way to determine the molecular weight and concentration, it is relatively difficult and time-consuming.

Tips:

- a. To obtain a more accurate molecular weight of a homo-polynucleotide, it is best to synthesize a ladder using the same initiator and oligonucleotide (*e.g.*, phosphorylated T50) used in polynucleotide synthesis, and concatenate these with T4 RNA-ligase in a buffer supplied with ATP.
- b. When using fluorescence to determine the molarity, the standard curve should be made with concentrations that yield a linear fluorescence dependence on concentration, and the sample concentration should be within the range of the standard curve.

6. Protocol for surface-initiated enzymatic polymerization (SIEP) and characterization

6.1 Protocol for SIEP on flat gold substrates using TdT (Chow et al., 2005)

- 1.1 Clean the gold substrate by sequential sonication in Milli-Q water, acetone, and ethanol at 50 °C with intervening rinse steps. Dry the substrate in a stream of dry N₂ and store it in a clean, covered petri dish.
- 1.2 Prepare 10 μM 5'-thiol modified oligonucleotide in 1 × PBS. Drop cast the initiator solution on the gold surface (for reference: a 1 cm² gold surface needs ~100 μL solution for full coverage). Cover the petri-dish and incubate the substrate at room temperature overnight.

- 1.3 Remove the solution by rinsing with Milli-Q water. Sonicate the substrate first in 0.1% Tween 20, and then in water for 5 min at room temperature to remove any remaining free initiator. Dry the substrate in a stream of dry N₂.
- 1.4 Prepare a reaction mixture containing natural and/or unnatural dNTP and 1 × TdT reaction buffer. The choice of reactant concentration depends on the desired polynucleotide length. For random copolymers, a dNTP mixture with different nucleotide ratios can be used. After adding TdT to the solution to a final concentration of 0.5 U/μL, mix the solution by pipetting, and drop cast it immediately onto the initiator-modified substrate surface (as mentioned above, a 1 cm² gold surface needs about 100 μL solution for full coverage). Incubate the substrate in a covered petri dish at room temperature. Brush height (MW) is controlled by the length of the incubation time. However, do not exceed 4h as solvent will evaporate.
- 1.5 Remove the reaction solution by rinsing thoroughly with Milli-Q water. Then sonicate the substrate first in 0.1% Tween 20 and then Milli-Q water for 5 min at room temperature. After sonication, rinse the surface with Milli-Q water and dry the substrate in a stream of dry N₂.
- 1.6 If a *block-co*-polynucleotide is desired, repeat Steps 4–5 with a different dNTP.
- 1.7 Store the DNA functionalized substrates under vacuum until further use.

Tips

- a. The surface needs to be meticulously clean to form an effective initiator monolayer and for subsequent polymerization reactions. If needed, a gold substrate can be first cleaned with plasma or piranha solution (Caruso, Serizawa, Furlong, & Okahata, 1995) before modification.
- b. A set of experiments with different reagent concentrations and reaction times is typically useful to determine optimal reaction conditions to achieve a desired brush height.
- c. Mixed thiol self-assembled monolayers of initiator- and (for example) PEG-modified thiols can be used to adjust the surface graft density of the polynucleotide brush.

6.2 Protocol for SIEP from Hylink™ glass slides using TdT (Tjong et al., 2011)

- 2.1 Conjugate 5'-amine-modified oligonucleotides to succinimidyl 4-formylbenzoate (SFB) linkers by reacting with a 20-fold molar excess of SFB in the modification buffer (100 mM sodium acetate, 150 mM NaCl, pH 7.2) overnight in the dark.
- 2.2 Remove unreacted SFB using Microcon YM-3 spin columns (Millipore). Dilute the concentrated SFB-modified-oligonucleotides in conjugation buffer (100 mM sodium citrate, 150 mM NaCl, pH6.0), and spot onto the hydrazinonicotinamide glass substrates (Hylink Glass slides, Solulink, San Diego, CA) using a noncontact piezoelectric printer (*e.g.*, Piezorray, Perkin Elmer, Inc).

- 2.3 Incubate the spotted slides overnight in a humidified chamber to allow for the covalent immobilization of the oligonucleotides on the glass surface.
- 2.4 Remove unreacted oligonucleotides by rinsing in $1 \times$ SSC buffer (15 mM sodium citrate, 150 mM NaCl, pH7.0) containing 0.1% Tween 20, then rinse with Milli-Q water and spin-dry on a microarray slide centrifuge for 2 min.
- 2.5 Store the DNA functionalized slides under vacuum until further use.

6.3 Characterization

- 3.1 The average dry thickness of the polynucleotide brush can be determined by spectroscopic ellipsometry (Chow & Chilkoti, 2007) or AFM height measurements (Chow et al., 2005).
- 3.2 Surface patterns can be visualized by tapping mode atomic force microscopy (TM-AFM) (Chow et al., 2005; Chow & Chilkoti, 2007). For example, when ssDNA is grown on a gold surface modified with microfluidic channels (Chow & Chilkoti, 2007), a distinct pattern can be observed by AFM imaging (Fig. 10).
- 3.3 The chemical integrity, purity, orientation, and ordering in the polynucleotide brushes can be characterized by using a combination of synchrotron-based X-ray photoelectron spectroscopy (XPS) and angular-resolved near-edge X-ray absorption fine structure (NEXAFS) spectroscopy (Khan et al., 2012, 2013). XPS provides information on the chemical composition of the samples based on the specific core level photoemissions and their chemical shifts (Fig. 11) (Khan et al., 2013). NEXAFS spectroscopy provides information by sampling of unoccupied molecular orbitals rather than the core levels probed by XPS. Furthermore, NEXAFS spectroscopy provides information about molecular orientation (Khan et al., 2013).
- 3.4 The kinetics of TdT-catalyzed DNA extension (Chow et al., 2005) and biosensor function (Chow & Chilkoti, 2007) can be measured in real time by surface plasmon resonance (SPR) spectroscopy. After forming a self-assembled monolayer on the surface, the surface can be mounted in an SPR spectrometer. A solution containing all SIEP reactants (described above) is injected into the spectrometer at a set flow rate (Chow et al., 2005). The SPR response curve shows the growth of ssDNA on the surface over time. After a certain time, the response curve reaches an asymptote, indicating the end of the reaction (Fig. 12).
- 3.5 The behavior of electrochemical sensors can be monitored by using cyclic voltammetry (CV) and differential pulse voltammetry (DPV) (Mei et al., 2016; Shi et al., 2017; Wan et al., 2013). CV measurements can also be used to characterize the stepwise modification process of the sensing electrode. Because of the addition and the elongation of ssDNA sequences on the electrode, the negative charges and steric hindrance are increased, which inhibits the electron transfer and reduces the peak currents (Fig. 13) (Shi et al., 2017).

7. Summary

Template-independent terminal deoxynucleotidyl transferase catalyzed enzymatic polymerization (TcEP) provides an exciting alternative to conventional enzymatic and solid-phase DNA syntheses. Specifically, the tolerance of TdT for unnatural dNTPs can be harnessed to introduce a broad range of functional groups into polynucleotides and thus provides a means to synthesize functionalized polynucleotides for a broad range of applications. In this chapter we provided a brief background on TdT, and described the effect that different reactants used in TdT polymerization have on polynucleotide growth and functionality, with the goal to provide general guidelines for designing the polymerization reactions. Furthermore, we discussed some selected applications of TcEP in solution and on surfaces. Finally, we provided step-by-step protocols to carry out TcEP in solution and initiated from surfaces, paying special attention on how to control the TcEP reaction, and how to purify and characterize the reaction products.

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Fig. 1. Schematic representation of deoxynucleotide addition by terminal deoxynucleotidyl transferase (TdT). This mechanism is harnessed in TdT catalyzed enzymatic polymerization (TcEP). n = number of nucleotides (degree of polymerization).

Fig. 2.
Generic structure of deoxynucleotide triphosphates and nitrogen bases of nucleic acids.
Positions marked in red indicate the location for chemical modifications that are most easily tolerated by DNA polymerases.

Fig. 3.

Schematic for terminal-deoxynucleotidyl transferase (TdT) mediated synthesis of an oligonucleotide with user defined sequence (Palluk et al., 2018). The TdT with dNTP monomer conjugated at the active site *via* a cleavable linker is incubated with ssDNA initiator. After the extension event, treatment of the TdT-ssDNA complex with cleavage reagents (*e.g.*, DTT, peptidase, 365 nm light) releases the ssDNA and makes the 3'-OH available for next extension event. Depending on the intended sequence of oligonucleotide, the user can select the TdT-dNTP conjugate for the next cycle.

Fig. 4.

A 2.6Å resolution crystal structure of murine TdT ternary complex with ssDNA initiator and incoming nucleotide (PDB ID 4I27) (Gouge, Rosario, Romain, Beguin, & Delarue, 2013). The palm, thumb and finger domains are represented in green, red and blue, respectively. Access of a template strand to the active site is restricted by loop1 (yellow). Mg²⁺ ion: cyan; ssDNA and incoming nucleotide: orange.

A (1) Homo-polynucleotide:

(2) Diblock-polynucleotide amphiphile:

Fig. 5.

(A) Schematic showing the enzyme-catalyzed synthesis of diblock polynucleotide amphiphiles. (B) Agarose gel electrophoresis results showing enzymatically synthesized poly(dTTP) with three different MWs (lanes pT1B-pT3B: M/I of 200:1, 500:1, and 1000:1), which were enzymatically end-functionalized with BODIPY-dUTP at a molar ratio of BODIPY-dUTP to poly(dTTP) 10:1. The presence of BODIPY-dUTP was verified by a BODIPY fluorescence scan. (C) AFM Tapping Mode height images of star like micelles formed by amphiphilic diblock polynucleotides. Inset: 3D image of a typical micelle (scale bars: 50 nm XY, 1.5nm Z). Adapted from Tang, L., Tjong, V., Li, N., Yingling, Y. G., Chilkoti, A., & Zauscher, S. (2014). Enzymatic polymerization of high molecular weight DNA amphiphiles that self-assemble into star-like micelles. *Advanced Materials* 26 (19), 3050–3054. doi:10.1002/adma.201306049.

Fig. 6. (A) Schematic showing a single-step, on-chip detection and signal-amplification DNA microarray sensor. (B) Dose-response curve of the sensor shown in a). Adapted with permission from Tjong, V., Yu, H., Hucknall, A., Rangarajan, S., & Chilkoti, A. (2011). Amplified on-chip fluorescence detection of DNA hybridization by surface-initiated enzymatic polymerization. *Analytical Chemistry* 83(13), 5153–5159. doi:10.1021/ac200946t. Copyright 2011 American Chemical Society.

Fig. 7.

Schematic showing the assembly of an electrochemical sensor for the highly sensitive detection of CEA. Aptamer 1 is immobilized on a gold electrode *via* its 3'-thiol modification, and aptamer 2 is immobilized on gold nanoparticles (GNPs) using its 5'-thiol modification to form a nanoprobe. If a target is present, it gets sandwiched between aptamer 1 and the aptamer 2-functionalized nanoprobe. After the incorporation of biotin-dATPs at the free 3'-OH groups of the GNPs using SIEP, numerous, long and biotin-labeled ssDNA strands are generated. This allows for the specific attachment of avidin-HRP complexes which aid in the electrochemical signal amplification. *Reprinted with permission from Wang, P., Wan, Y., Deng, S., Yang, S., Su, Y., Fan, C., ... Zuo, X. (2016). Aptamer-initiated on-particle template-independent enzymatic polymerization (aptamer-OTEP) for electrochemical analysis of tumor biomarkers. Biosensors & Bioelectronics 86, 536–541. doi:10.1016/j.bios.2016.07.025. Copyright 2016 Elsevier.*

Fig. 8.

An enzymatic synthesis strategy for storing information in ssDNA. (A) Schematic depiction of a series of enzymatic synthesis reactions consisting of oligonucleotide initiator (N), TdT and apyrase (AP). The initiator is tethered to a solid support or beads. (B) TBE-urea gel showing the DNA strands synthesized in each consecutive synthesis cycle. (C) Schematic showing the interconversion of DNA sequence to digital information. Raw strands (Strands^R) represent synthesized DNA, while a compressed strand (Strand^C) represents a sequence of transitions between non-identical nucleotides. Transitions between nucleotides are mapped from Strand^C to digital data in ternary digits. If a Strand^C is equivalent to the template sequence, all desired transitions are present and the information stored in DNA is retrieved. (Nucleotides A, T, G, C are colored as red, blue, orange, and dark blue, respectively.) Adapted with permission from Lee, H. H., Kalhor, R., Goela, N., Bolot, J., & Church, G. M. (2019). Terminator-free template-independent enzymatic DNA synthesis for digital information storage. *Nature Communications* 10 (1), 2383. doi:10.1038/s41467-019-10258-1. Copyright 2019 Springer Nature.

Fig. 9. Dependence of the molecular weight distribution on reaction time. Left: Cy5 scan of 5% TBE-PAGE gel images. Right: corresponding relative fluorescent intensity distributions plotted as a function of the degree of polymerization (DP). The resulting polydispersity indices (PDIs) are shown in the plots. *Adapted from Tang, L., Navarro, L. A., Jr., Chilkoti, A., & Zauscher, S. (2017). High-molecular-weight polynucleotides by transferase-catalyzed living chain-growth polycondensation. Angewandte Chemie (International Ed. in English) 56 (24), 6778–6782. doi:10.1002/anie.201700991.*

Fig. 10.

(A) side and (B) top view TM-AFM height images in air of DNA brushes grown by surface-initiated enzymatic polymerization on a micropatterned initiator SAM ($5'$ -SH $(\text{CH}_2)_6$ -dTTP₂₅). (C) Height profile of the line indicated in (B). The dry brush height was about 60nm. Adapted with permission from Chow, D. C., & Chilkoti, A. (2007). *Surface-initiated enzymatic polymerization of DNA*. *Langmuir* 23 (23), 11712–11717. doi:10.1021/la701630g. Copyright 2007 American Chemical Society.

Fig. 11.

(A) C 1s, (B) N 1s, (C) P 2p, and (D) O 1s high resolution XPS spectra of the A25-SH SAM and poly(A) brush with a thickness of 25 nm (open circles). The C 1 s, N 1s, and O 1s spectra are deconvoluted (solid gray lines) into component peaks. The fitted spectral envelopes are drawn by solid black lines. *Adapted with permission from Khan, M. N., Tjong, V, Chilkoti, A, & Zharnikov, M. (2013). Spectroscopic study of a DNA brush synthesized in situ by surface initiated enzymatic polymerization. The Journal of Physical Chemistry B 117 (34), 9929–9938. doi:10.1021/jp404774x. Copyright 2013 American Chemical Society.*

Fig. 12.

Kinetics of TdT-catalyzed DNA extension using four different nucleotides measured by SPR. As a control, the same reaction was performed without the addition of nucleotides to account for the SPR signal generated by the buffer exchange and nonspecific binding of the reaction components to the surface. *Adapted with permission from Chow, D. C., Lee, W. K., Zauscher, S., & Chilkoti, A. (2005). Enzymatic fabrication of DNA nanostructures: Extension of a self-assembled oligonucleotide monolayer on gold arrays. Journal of the American Chemical Society 127 (41), 14122–14123. doi:10.1021/ja052491z. Copyright 2005 American Chemical Society.*

Fig. 13.

CV characterizations of step-by-step modification of a gold electrode (AuE). (A) bare AuE, (B) T-HP/MCH/AuE, (C) (thrombin + S-HP)/T-HP/MCH/AuE and (D) TdT/(thrombin + S-HP)/MCH/T-HP/AuE. T-HP: Thiol-modified hairpin probe; MCH: 6-mercaptophexano; S-HP: signal hairpin probe. Adapted with permission from Shi, K., Dou, B., Yang, J., Yuan, R., & Xiang, Y. (2017). Target-triggered catalytic hairpin assembly and TdT-catalyzed DNA polymerization for amplified electronic detection of thrombin in human serums. *Biosensors & Bioelectronics* 87, 495–500. doi:10.1016/j.bios.2016.08.056. Copyright 2017 Elsevier.

Table 1

Examples of unnatural dNTP substrates for TdT and their applications.

Modification	dNTP	Application
Biotin (Flickinger, Gebeyehu, Buchman, Haces, & Rashtchian, 1992)	Biotin-11-dUTP	Labeling DNA probes <i>In situ</i> labeling of cells (Gavrieli, Sherman, & Ben-Sasson, 1992)
Amine (Kumar, Tchen, Roullet, & Cohen, 1988)	Aminoallyl-dUTP	Tuning nuclease resistance (Gu, Oweida, Yingling, Chilkoti, & Zauscher, 2018)
Azide (Winz, Linder, Andre, Becker, & Jaschke, 2015)	2'-N ₃ -2-dA/C/G/UTP5- <i>PEG</i> N ₃ -dUTP	
Alkyne (Winz et al., 2015)	5-ethynyl-dUTP	
Dibenzocyclooctyne (DBCO) (Gu et al., 2018)	DBCO-dUTP	
Digoxigenin (Schmitz, Walter, Seibl, & Kessler, 1991)	DIG-dUTP	
Dideoxy dNTPs (Guerra, 2006)	Cy TM 3-ddUTP	
Fluorescent dyes (Guerra, 2006; Igloi, 1998; Tjong et al., 2011)	Fluorescein-12-ddUTP Cy5-UTP	
Photocrosslinkers	4-thio-2'-dUTP	Studying DNA structure and interactions with proteins (Taurait , Jakubovska, Dabužinskait , Bratchikov,& Meškys, 2017)