DNA Transformations for Diagnosis and Therapy

So Yeon Ahn, Jin Liu, Srivithya Vellampatti, Yuzhou Wu,* and Soong Ho Um*  

Due to its unique physical and chemical characteristics, DNA, which is known only as genetic information, has been identified and utilized as a new material at an astonishing rate. The role of DNA has increased dramatically with the advent of various DNA derivatives such as DNA–RNA, DNA–metal hybrids, and PNA, which can be organized into 2D or 3D structures by exploiting their complementary recognition. Due to its intrinsic biocompatibility, self-assembly, tunable immunogenicity, structural programmability, long stability, and electron-rich nature, DNA has generated major interest in electronic and catalytic applications. Based on its advantages, DNA and its derivatives are utilized in several fields where the traditional methodologies are ineffective. Here, the present challenges and opportunities of DNA transformations are demonstrated, especially in biomedical applications that include diagnosis and therapy. Natural DNAs previously utilized and transformed into patterns are not found in nature due to lack of multiplexing, resulting in low sensitivity and high error frequency in multi-targeted therapeutics. More recently, new platforms have advanced the diagnostic ability and therapeutic efficacy of DNA in biomedicine. There is confidence that DNA will play a strong role in next-generation clinical technology and can be used in multifaceted applications.

1. Introduction

DNA is famous in biology as the carrier of genetic code written in a four-base code of amino acids. Due to its powerful biological characteristic as a metabolic regulator, it stores and preserves the integrity of the biological information, paving the way for a variety of applications such as forensic analysis, genetic screening, and paternity testing. Branched DNA structures referred to as Holliday junctions are found naturally in living systems when chromosomes exchange information during meiosis. Since the advent of nanotechnology in the 1980s, DNA and its self-complementary nature have been recognized for use as nonbiological material due to its sequence programmability, molecular recognition, reconfigurability, and predictable self-assembly. This technology has enabled DNA and DNA-based materials to produce various supramolecular constructs, which laid a foundation for structural and dynamic DNA nanotechnology by expanding applications in cancer research, biomedicine, and diagnostics. Alongside the structural DNA material evolution, hybrid DNA materials with precise positioning of organic or inorganic materials have come a long way in creating various practical devices through DNA transformations. DNA transformations involve changes in the current state with respect to interactions with other strands or the surroundings. Arguably, DNA nanostructures act as molecular pin boards to attach various biological and chemical hetero-elements. A brief timeline of the milestones in static, dynamic, and hybrid DNA nanotechnology is depicted in Figure 1.

DNA derivatives (or DNA hybrids) have become a comprehensive field of research where DNA acts as a template for anchoring biomaterials (either organic or inorganic nanomaterials) such as haptens, peptides, dyes, gold nanoparticles (AuNRs), polymers, and iron oxides. Hierarchical assembly and patterning of nanomaterials provide multiple binding sites for nucleation as possible synergies to achieve unprecedented accuracy. These structures resemble a planet-satellite-type model, which showcases the potential of DNA nanostructures in various applications such as biosensing, clinical diagnostic devices, and nanomedicine with high sensitivity and efficiency.

Self-assembly of DNA can be controlled with nanometer scale precision, enabling stability and versatility of the formed nanoarchitectures. Since it can integrate with other materials, DNA can be used to achieve new physicochemical properties. To
date, these DNA transformations were predesigned using compact programming techniques theoretically. Such patterns of behavior with clearly defined orientations, stereo-relationships, and maximized interactions with biomolecules contributed to the development of improved materials.[15]

1.1. Evolution of DNA Structural and Dynamic Complexity

Structural DNA technology utilizes the bottom-up approach for the development of complex macroscopic structures at equilibrium with nanoscale precision. The first immobile DNA
nanostructure was introduced by Nadrian Seeman using Holliday junction-like constructs. Shortly after, the branched structures were generalized, leading to the construction of 2D arrays and 3D nanostructures. Inspired by these paradigms, intense progress on the design principles in structural DNA nanotechnology was made in the early 2000s with the milestone of DNA origami (Figure 1).

On the basis of this groundwork in structural DNA nanotechnology, dynamic DNA nanostructures became increasingly important for simulating change in response to an external stimulus. Dynamic DNA nanotechnology focused on mechanizing the DNA nanostructures, which tend to reconfigure autonomous devices with nonequilibrium dynamics. DNA transformations that require energy input have been harnessed as a key component for high-performance biosensors and in healthcare for more than a decade. Reversible transitions between stable and metastable states can be achieved with DNA by utilizing the energy input released from base pairing to change the immobile scaffolds into dynamic machines. Walkers, switches, and logic gates were developed based on the relatively weak free energy of the transitions and had a profound impact on the device behavior. These dynamic devices can endure on-demand changes in their properties with respect to environmental/biological cues or external triggers driven by the thermodynamics of the reaction (Figure 1).

The dynamic nature of programmed DNA devices or containers provided the groundwork for nanoplatforms in diagnosis and therapy. A class of nucleic acid probes called aptamers are single-stranded DNA or RNA structures obtained from a random library of in vitro oligonucleotides known as systematic evolution of ligands by exponential enrichment (SELEX). Aptamers can undergo conformational changes with high affinity and specificity following an induced fit model (from a random coil to an organized conformation) via target-induced structure switching mode. Aptamers are widely used in biosensing systems, diagnostics, and therapeutics because they are easy to modify, small in size, experience reversible denaturation, and have slow degradation kinetics. These ideal characteristics make them more advantageous as recognition units than antibodies. Various promising healthcare nanodevices for disease detection and therapeutics with excellent biocompatibility were engineered using aptasensors and aptazymes.

Mechanisms of reconfiguring DNA nanostructures can be studied using various strategies to develop autonomous molecular motors that are self-sufficient, as discussed below. DNA walkers were well utilized in the field of theragnostics (or termed as theranostics), especially in biosensors such as miRNA detection or in mycotoxin detection, with various walking strategies for high-performance biosensors. Drive motors, which are the major driving forces in mechanical motion of DNA, can be classified into strand displacement reactions, enzymatic cascades, and external stimulus such as light or ions (Figure 2).

1.1. Strand Displacement

Strand displacement reactions, in which one strand is displaced from a double-stranded DNA (dsDNA) mediated by a toehold design, have shown remarkable attuning in terms of structure formation at thermodynamic and kinetic levels. Due to simplicity, strand displacement is the most frequently utilized structural transformation method, and it can be used to switch shapes, open and close cages, and arrest or release components through noncovalent base-pairing. In addition, it can sense specific nucleic acid sequences and induce catalytic reaction cascades in medical theragnostics.

1.1.2. Enzymatic Cascades

In addition to the strand displacement strategy, integrated enzymatic activity that involves a covalent modification which

Figure 2. Design principle of a walking DNA engines. Three elements mainly contribute to make a static DNA into a dynamic machine. They are a driving motor, a walking path, and a walking strand. The drive motor breaks the initial equilibrium and facilitates the conversion of initial input energy into a mechanical energy, which makes the walking strand to move along the walking track. Reproduced with permission. Copyright 2019, Elsevier. The walking tracks can be either a) protein adaptors, or b) helical gold nanorods superstructures with tailored chirality, or c) carbon nanotubes, or d) DNA origami nanovault for controlled enzymatic activity. Reproduced with permission. Copyright 2015, American Chemical Society. Reproduced with permission. Copyright 2012, Dove Medical Press. Reproduced with permission.
enabled the motion of the DNA fragments has been widely studied. Various autonomous DNA walkers were explored through enzymatic cleavage and ligation reactions with the use of DNAzymes and restriction enzymes. DNAzymes have gained attention in the driving mechanisms of DNA walkers, acting as switches and using strands as their inputs and outputs. Strand displacement mechanisms using restriction enzymes such as HincII and polymerases such as Phi29 with endogenous specificity to the target DNA were utilized for rapid and efficient reactions. The sequence-specific HincII effectively creates a nick at the GTTGAC from the original double-stranded target. The primers dissociate from the nicked sites, and these recognition sequences are extended and displaced using polymerases such as Phi29, which are continuously dispatched as amplified products from one or both sides. The HincII recognition sequences in the primers can be replaced by RNA polymerase promoter sequences for creation of transcription templates for target-dependent RNAs.

1.1.3. Photoactuation

A simple and clean renewable energy source without any external disturbances was designed to overcome the factors impeding the molecular motion (e.g., waste from duplexes). The amount of energy required to induce motion can be easily controlled by varying the intensities of the excitation light. The first light-powered autonomous DNA walking device was constructed by incorporating photosensitive moieties inside the DNAzyme. They can trigger the hidden strands and make them hybridize to their complementary strand, enabling a light-driven miniature device by emulating the nature of DNA in motion.

1.2. Theoretical Background for the Design of DNA Transformation

DNA and its variations can be transformed with respect to free energy derived from hybridization of base pairs (enthalpy gain) or dissociation of strands (entropy gain), allowing themselves to be viewed as a reactant from a reaction dynamics perspective. When the system reached equilibrium, the reaction became limited, and no physical work was derived from the system, which is similar to enzymes that are depleted during the course of the reaction. While these systems have been used to achieve considerable technological development, their bio-engineering potential was hampered by some key constraints. A major Achilles heel of this technology is the low yields of self-assembly. The energy landscape in the self-assembly of DNA is often disrupted by local minima, which leads to low yield. Scalability is another current issue since it is necessary to implement practical functionalities in large scale for use in therapeutics, drug delivery, and nanoelectronics. Technical development has begun to address each of these limitations systematically, enabling the application of DNA transformations in the burgeoning field of bioengineering for diagnosis and therapy.

Designer DNA nanostructures based on the molecular properties such as melting temperature, thermodynamics of structure formation, lengths, and sequences are inadequate to design complex architectural structures using NUPACK and IDT DNA analyzers. Today, many DNA nanostructures have been designed using an integrated computational approach for materials research following the rules defined by researchers. Motivated by these developments, various theoretical models and molecular simulation approaches at laboratory timescales have been optimized for rational design of DNA models and include SEQUIN, caDNAno, GIDEON, and CANADA 2.0. Several interactive design tools for nucleic acid nanotechnology have been developed, and the first interactive menu-driven, semi-automatic designing program called SEQUIN was introduced to construct branched junctions in 1990. SEQUIN considers both explicit and implicit assignments by assessing the unwanted complementary aspects that create junction-flanking sequences via branch point expansion. One of the most popular and open-source design tools, caDNAno is an easy-to-use interface that provides tools to introduce anomalies such as forced crossovers in the basic design. It also minimizes error-prone tasks while designing complex and multi-layer DNA structures. GIDEON provides a simple and straightforward approach to study geometrical structures and their conformations by varying the number of nucleotides or repositioning the duplexes. This can be repeated until the required conformation is obtained. A sequence design program using the CANADA package is comprised of software tools that help in converting sequences (in a reverse or complementary way) as required by calculating their thermodynamic factors like melting temperature ($T_m$) or Gibbs free energy ($\Delta G$). Similarity can be measured using the global alignment or disparity of sequence pairs using distances from a simple prediction method.

Although prediction of arbitrary sequences is difficult, the basic premise underlying these algorithms is sequence-symmetry minimization, which prohibits the repeated sequences leading to an unpredictable distortion in the structure. Such minimization reduces the size and complications in obtaining the designed structure. An appropriate DNA motif (crossovers or loops or toe-hold) needs to be nominated as a building block depending on the utility of the construct, either as a static scaffold or a dynamic device capable of structure transitions between their distinct and anticipated adaptations. Programming algorithms for DNA nanostructures require a repository of thermodynamic parameters for precise prediction of their structures and (more recently) their dynamics employing DNA strand-displacement reactions. In addition to the advanced technology of theoretical design, DNA nanostructures can be combined with organic or inorganic materials for user-prescribed applications.

1.3. Functional DNA Hybrid Nanocomplexes

Carbon materials and nanoparticles are widely used in biomedical applications due to their efficient surface modifications, easy conjugation with biomolecules (without altering the activity), small size, and optical and electrochemical properties. Several groups have exhibited strategical assembly of
nanoparticles,[46] quantum dots,[47] and carbon materials[48] on DNA nanostructures for effective detection. DNA nanostructures have been combined with these functional moieties, resulting in complexation.

DNA hybrid nanocomplexes have also found use in nanomedicine as “vehicles” for molecular payloads such as drugs,[49] siRNAs and mRNA therapeutics,[50] and antibodies.[51] These chimeric DNA molecules have tunable retention and clearance rates, which enables the controlled release of drugs, and are biocompatible, making them robust materials for improved discrimination and targeting.[52] Designed DNA nanostructures are capable of monitoring and quantifying the physiological and pathological processes in real-time for early diagnosis and tailored medicine.[53a,b] With respect to the utilization of hybrid DNA nanostructures in diagnostics, fluorescence-based plasmonic DNA origami (as optical antennas) and metallic nanoparticles (as hotspots) were developed for point-of-care-diagnosis of Zika virus. Rapid and accurate real-time detection using DNA-templated synthesis of nanomaterials can be used for various applications like immuno polymerase chain reaction (PCR) for antigen detection, precise patterning of metal and metal oxide nanoparticles for optoelectronics, as a nanoscale reactor in diagnostic applications, in supramolecular polymer growth to induce dynamic reconfiguration or chemical sensing using polyaniline nanowires, and as a sustainable nanoagent in live-cell detection. These DNA-templated organic or inorganic frameworks[53c–54] have been pursued as they can provide essential tools for clinical diagnosis with high sensitivity, as illustrated in Figure 3.

1.4. Intracellular Destinies of DNA Nanostructures

Naturally existing DNA structures and their hybrid-DNA derivatives possess various physicochemical attributes like size, shape, and charge, but their cellular fates also are essential for recognizing their functions in vivo. Myriad branched and structural DNA nanostructures have been produced despite the naked DNA nanostructures, which are electron-rich, cannot penetrate cell membranes. However, on conjugation with

Figure 3. DNA-templated synthesis technology for various applications. a) DNA-templated polymerization by rolling circle amplification (RCA). Reproduced with permission.[53c] Copyright 2010, Royal Society of Chemistry. b) DNA-templated organization of metal nanoparticles. Reproduced with permission.[53d] Copyright 2014, Elsevier. c) DNA-templated nanoreactors. Reproduced with permission.[53e,119] Copyright 2015, Royal Society of Chemistry. d) DNA-templated polymeric nanowires. Reproduced with permission.[53f] Copyright 2014, American Chemical Society. e) DNA-templated inorganic mineralization. Reproduced with permission. [53g] Copyright 2020, Elsevier.
nanomaterials, they gain a potent ability to penetrate a variety of cells, enhancing uptake efficiency. Researchers also proposed the stability of 2D DNA origami in the presence of chaotrophic agents by varying the time, temperature, and concentration of denaturing agents. These materials were stable at room temperature for 24 h and showed less structural damage, indicating them as a potential platform for bio-molecular studies as a structural switch.[52,54] The cellular fate of the DNA nanostructures are elucidated meticulously in Section 3.

Furthermore, DNA transformations with new biochemical properties such as serum stability are summarized (Table 1)[55] and can be used to program complex machines for smart therapeutic systems. Use of DNA and their transformations, construction, and alteration of DNA structures for diagnosis and therapy is the central theme of this review.

### 2. Applications of DNA Transformation in Diagnosis

Growth of DNA materials with exceptional properties has accelerated with development of natural DNA or its modified forms. Advances in the understanding and application of nucleic acids, which began in 1950, resulted in the ability to discover nucleic acids, and accurately read and write them as well. As nucleic acids were recognized as the source of life, their significance was emphasized further reading nucleic acids and the newly acquired physicochemical characteristics of DNA (which are not limited to the natural DNA systems that are challenging). Provoked developments such as isothermal detection methods with sensitivity equivalent to that of PCR, simultaneous multiplexed detection with unlimited probing capacity that have not been experienced in the existing PCR systems were introduced. In this review, we focus on modern alternative strategies based on DNA transformations for the development of the next-generation PCR systems. We also examine the problems of existing PCR-based genetic readouts and describe the innovative efforts to overcome them with use of DNA transformation materials.

#### 2.1. Limitations of Traditional Diagnostic Toolkits and Alternatives

The existing diagnostic technology represented by PCR is theoretically possible only with the use of the target genome, enzymes, and auxiliary factors (such as optical transducers that read it). In reality, the technology reveals chronic problems in each step. Traditional PCR (Figure 4a), which requires a labious analysis process such as gel electrophoresis, has been evolved to modernistic forms like real-time PCR (rtPCR).[60] and digital PCR (dPCR) [61] to emphasize it quantitatively (Table 2). Based on the mechanism of traditional PCR amplification, rtPCR monitors the amplification of a target molecule in real-time through the exponential fluorescence signal curve, which results from the accumulation of amplicons (Figure 4b). However, analysis of the exponential curve in rtPCR provides only the comparative quantification of the target, which requires standard samples for absolute quantification, leaving numerous possibilities for uncertainties.[62] Many attempts in rtPCR were suggested to overcome these incertitudes by reducing the error rate or improving the sensitivity and selectivity.[63] Although various rtPCR methods with improved detection and probe designing mechanisms have improved the sensitivity or selectivity of detection, the chronic error-prone aspect of rtPCR with its limitation in absolute quantification gave rise to the development of a new milestone technology, dPCR. Digital PCR achieved an unprecedented performance by bringing the resolution down to the molecular level with the ability of absolute target quantification.[64] The basic dPCR involves the investigation of amplification in the partitioned compartments or droplets, resulting in complicated guidelines to offset the possible errors and standardize its use, limiting the use in laboratory environments.[59] To overcome these drawbacks, various alternative strategies have been proposed together with the rapid development in nanobiotechnology and the newly acquired physicochemical characteristics of DNA (which are not limited to the natural DNA systems that are challenging). Provoked developments such as isothermal detection methods with sensitivity equivalent to that of PCR, simultaneous multiplexed detection with unlimited probing capacity that have not been experienced in the existing PCR systems were introduced. In this review, we focus on modern alternative strategies based on DNA transformations for the development of the next-generation PCR systems. We also examine the problems of existing PCR-based genetic readouts and describe the innovative efforts to overcome them with use of DNA transformation materials.

| DNA nanostructures | Size [nm] | Conditions | Stable up to [h] | Characterization | Ref. |
|--------------------|----------|------------|-----------------|-----------------|-----|
| Tetrahedron        | 7        | 50% FBS    | 4               | Gel electrophoresis | [55a]|
| Nanoprism          | 10       | 10% FBS    | 2               | Native PAGE      | [55b]|
| Octahedron         | 40       | RPMI media | <6              | Gel electrophoresis | [55c]|
| Cube               | 30       | Cell lysate| Until 24        | Gel electrophoresis | [55d]|

In 1984, the first genetic read-out by Mullis was achieved using a PCR with which the DNA fragments have been identified with several factors such as polymerase and surplus nucleotides.[56] After several technical evolutions (generation of automated devices), the technique can selectively amplify specific DNA fragments from extremely small amounts of template DNA within 2 h in biological fluids. Thus, PCR and various derived technologies play an extremely important role in the overall use of DNA in molecular biology, medical care, criminal investigation, and classification of organisms. Particularly, PCR has contributed significantly to public health by enabling accurate and sensitive diagnosis of fatal diseases such as small lung cancer and COVID-19 with highly specific genetic biomarkers.[57]

PCR has always been the gold standard for genetic sequence determination because of its high sensitivity and accuracy, which attributes to the excellent amplification efficiency of the target. However, there is still room for improvement based on significant problems such as error-prone amplification [58] and the thermal cycle controlling procedure. These drawbacks resulted in complicated guidelines to offset the possible errors and standardize its use, limiting the use in laboratory environments.[59] To overcome these drawbacks, various alternative strategies have been proposed together with the rapid development in nanobiotechnology and the newly acquired physicochemical characteristics of DNA (which are not limited to the natural DNA systems that are challenging). Provoked developments such as isothermal detection methods with sensitivity equivalent to that of PCR, simultaneous multiplexed detection with unlimited probing capacity that have not been experienced in the existing PCR systems were introduced. In this review, we focus on modern alternative strategies based on DNA transformations for the development of the next-generation PCR systems. We also examine the problems of existing PCR-based genetic readouts and describe the innovative efforts to overcome them with use of DNA transformation materials.
in high accuracy and precision by eliminating all the possible sources of error that were previously encountered in rtPCR (Figure 4c). Despite its outstanding performance, dPCR benefit is limited due to the need for costly droplet creation instruments and software, which has not been generalized.

Rapid and direct genome sequencing in the current advent of the point-of-care diagnosis is becoming central in the areas of modern medicine in which the need for accurate, selective, and high-performance detection of disease-specific biomarkers is continuously raising.

Although there are many studies on improving the accuracy and sensitivity of nucleic acid biosensors by qPCR and dPCR, their use is mainly reliant on the laboratory-level thermal cyclers and complex optical devices. Here, we would like to put forward the accurate, sensitive, and portable diagnostics based on DNA transformations developed to eliminate the need for complex laboratory-level devices.

2.2. New Diagnostic Technologies Based on DNA Transformations

DNA design issues associated with improved performance for advanced PCR-based or PCR-free diagnosis are discussed here. We further discussed the design concepts of isothermal amplification and entropy-driven signal amplification for portable yet highly sensitive detection, multiplexed detection, and high-throughput surface-based detections.

2.2.1. Sensitivity Enhancement by Isothermal Amplification

The most widely used quantitative PCR methods, rtPCR, and dPCR are prevalent because they do not require human manipulation during the amplification step and can be automated. However, these nucleic acid detection methods are circumscribed in terms of speed and universalization because they require nucleic acid extraction, thermal cycling, real-time, and complex statistical analysis to evade the false-positives, thus rendering PCR applicable only at the laboratory level. In this regard, systems that enable automated isothermal amplification have been introduced. Isothermal amplification has a wide range of possible applications including point-of-care testing (POCT), genetic testing in low-resource settings (such as in developing countries), rapid testing of food and environmental samples and so on. These techniques enabled a repeated hybridization and dissociation of probes and primers, subsequent
DNA amplification by polymerases without thermal denaturation, and annealing through DNA transformation technology. Techniques such as strand displacement amplification (SDA), loop-mediated isothermal amplification (LAMP), helicase displacement amplification (HDA), rolling circle amplification (RCA), and signal-mediated amplification of RNA technology (SMART) are some of the representative isothermal amplification methods. Their amplification processes involve dissociation of synthesized amplicons through strand displacement or nuclease activity of the specific enzyme used, which leads to the binding of new primer and generation of amplicons iteratively.

Strand Displacement Amplification: SDA, first introduced in 1992,[37] imports an endonuclease to the target amplification process to induce repetitive operation of DNA polymerase without thermal denaturation and annealing and the extended primers provides a amplicons. The endonuclease recognized sites, and the fragment on the 3'-end of the nicked site serves as a new primer for extension while displacing the other fragment on the nicked strand (Figure 5a). The nicking, extension, and displacement is repeated, resulting in accumulation of amplicons, and the displaced fragment serves as a new template for displacement amplification (HDA), rolling circle amplification (RCA), and signal-mediated amplification of RNA technology (SMART) are some of the representative isothermal amplification methods. Their amplification processes involve dissociation of synthesized amplicons through strand displacement or nuclease activity of the specific enzyme used, which leads to the binding of new primer and generation of amplicons iteratively.

Figure 5a. The nicking, extension, and displacement of a target strand.

Table 2. Summary of milestone PCR technologies.[56,60,62b]

| Technology outline | Traditional PCR | rPCR | dPCR |
|--------------------|-----------------|------|------|
|                    | The amount of PCR products accumulated at the end of the PCR cycle is analyzed. | Fluorescence during the PCR amplification is analyzed. | Overall fluorescence trend of nanoliter sized partitioned reaction droplets is analyzed. |
| Quantitation       | Semi-quantitative | Quantitative | Absolutely quantitative |
|                    | Comparison of the gel electrophoretic band intensity of final PCR product to the standard material. | Exponential growth data collected real-time ascertains the quantity of targets. | Absolute and reproducible quantification of target nucleic acids at single-molecule resolution. The fraction of target containing droplets and the number of targets in each droplet is estimated through Poisson statistical algorithm. |
| Applications       | DNA amplification for the following downstream uses: | - Quantitative analysis of pathogens | - Absolute quantification of virus |
|                    | - Genetic sequence analysis | - Microarray verification | - Absolute quantification of nucleic acid standard materials |
|                    | - Genotype analysis | - Quality control and assay verification | - Absolute quantification of next generation sequence analysis library |
|                    | - Cloning | - SNP genotype analysis | - Detection of rare allele |
|                    | - Narrow dynamic range of detection (less than two logs) | It can detect up to double change | - Absolute quantification of gene expression |
| Summary            | Limitations of traditional PCR: | It can detect up to double change | Concentration and separation of mixture |
|                    | - PCR post-processing is required | - Data collection in the exponential growth stage of PCR | Resistant to PCR inhibitors |
|                    | - Non-automatic | - Complex mixtures can be analyzed | Small-scale changes are detectable through linear responses depending on the number of copy number. |
|                    | - Only discrimination according to size is possible | The increase in the reporter fluorescence signal is directly proportional to the number of amplicons generated | |

DNA amplification by polymerases without thermal denaturation, and annealing through DNA transformation technology. Techniques such as strand displacement amplification (SDA), loop-mediated isothermal amplification (LAMP), helicase displacement amplification (HDA), rolling circle amplification (RCA), and signal-mediated amplification of RNA technology (SMART) are some of the representative isothermal amplification methods. Their amplification processes involve dissociation of synthesized amplicons through strand displacement or nuclease activity of the specific enzyme used, which leads to the binding of new primer and generation of amplicons iteratively.

**Loop-Mediated Isothermal Amplification:** Despite this novel approach, use and study of SDA were constrained by its limited range of length and long operation time regardless of their novelty. Instead, the emergence of SDA has inspired many Isothermal amplification methods such as LAMP and HDA. LAMP has achieved superior specificity and sensitivity, with rapid amplification of the target due to the use of four primers recognizing six sequences on the target sequence.[65] Due to its excellent specificity, LAMP is not significantly influenced by the presence of nontarget DNAs.[66] LAMP is simple and easy to perform with four primers, a DNA polymerase, and a thermostat. LAMP requires only a few copies with a detection limit comparable to that of PCR.[66] LAMP can be incorporated into assays for medical investigations, genetic, environmental, and rapid testing of food products, especially in resource-constrained environments. In the LAMP reaction, samples are amplified at a fixed temperature by repeating two types of elongation in the loop regions: self-elongation of the template from the stem loop structure formed at the 3'-terminal and the binding and elongation of new primers in the loop region (Figure 5b). Furthermore, LAMP can be utilized for amplification of a target RNA sequence where, a one-step amplification can be performed (similar to DNA) by simultaneous addition of reverse transcriptase enzyme that also exhibits strand
displacement activity. Simple design of LAMP primers specific for input target sequences is readily available through an automated software (http://primerexplorer.jp/e/). The self-assembling structural approach of DNA transformation led to the introduction of a loop structure in amplicons providing empty sites for primers to bind successively without a dissociation process.

**Helicase-Dependent Amplification**: Thermal shock and consecutive binding of four elaborately designed primers induce the dissociation and elongation of dsDNA through PCR and LAMP, respectively. In contrast, HDA is based on a DNA helicase enzyme that separates the dsDNA and facilitates the repetitive primer annealing and elongation by the polymerase enzyme (Figure 5c). Helicase, enzymatically disrupts the hydrogen bonds of dsDNA and separates them using the energy derived from adenosine triphosphate (ATP) hydrolysis. The first HDA assay was able to amplify 10³ copies of genomic DNA by a factor greater than 10⁶, in addition to being able to directly amplify DNA in human blood without purification or extraction. The performance of HDA was further improved with use of hemilase, a helicase-DNA polymerase fusion enzyme, which increased the length of amplified fragments to 2.3 kb. With coupled use of thermophilic helicase-dependent amplification (tHDA) and reverse-transcription in a single tube, RNA targets were also assayed with amplification.

**Rolling Circle Amplification**: RCA has been developed based on the rolling circle replication that occurs naturally in many plasmids. RCA involves an amplification process from a circularized template DNA by RNA or DNA polymerase enzymes, resulting in long repetitive ssDNA or ssRNA (Figure 5d). In nucleic acid target sensing, the presence of the target enables the fabrication of a circular template, leading to continuous amplification along the template. On top of its robust amplifying ability, the long and repetitive amplicons of RCA

Figure 5. Isothermal amplification methods for sequence-specific detection. a) Schematics of SDA. The strand synthesized from the extended primer with endonuclease recognition site is displaced by the strand synthesized from the bumper primer. The new cycle of polymerization starts when a nick is formed on the double-stranded amplicon by endonuclease, with the nicked strand serving as a new primer. Reproduced with permission. Copyright 2018, Springer Nature. b) Schematics of LAMP. Extension of the primer with a partial complementary sequence to the synthesized strand (yellow region) establishes hybridization and forms a loop structure. The primers then bind to the loop area, where the single-stranded status is maintained, and polymerization proceeds. The schematics are simplified for clear visualization of loop-mediated primer binding and elongation. Reproduced with permission. Copyright 2008, John Wiley and Sons. c) Schematics of HDA. Helicase autonomously unwinds the dsDNA, facilitating binding of the primer and polymerization. Reproduced with permission. Copyright 2004, John Wiley and Sons. d) Schematics of RCA. The target strand annealed to the padlock probe facilitates circularization of the padlock. The ligated nick on the padlock enables continuous polymerization along the circular template, resulting in a long and repetitive amplicon. Reproduced with permission. Copyright 2017, Springer Nature. e) Schematics of SMART. As the target, extension probe, and template probe assemble, polymerization along the template probe initiates and results in newly synthesized dsDNA with a promoter sequence for RNA polymerase. RNA polymerase recognizes the double-stranded promoter and repetitively synthesizes the RNA amplicon. Reproduced with permission. Copyright 2015, Elsevier.
are capable of providing active sites for multivalent inter and intramolecular interactions, which fosters collective molecular activity for effective signal transduction. This collective molecular behavior is also capable of inducing large colorimetric changes that can be visually confirmed, and it is expected to contribute to POCT.

Signal Mediated Amplification of RNA Technology: SMART amplifies the signal generated by the target-driven cascade reaction rather than direct amplification of the target sequence. Hence, SMART does not dissociate the existing bound target and the probe but utilizes the double stranded (ds) status derived by target-probe hybridization to initiate transcription as a promoter (Figure 5e). Although SMART is not as sensitive as PCR, it is more tolerant to various types of samples such as genomic DNA and total RNA both in Escherichia coli, crude cultures, and nontarget DNA. In addition, the signals generated by this method are the same for all targets, so detection or downstream application of the amplicons can be generalized.

Recombinase polymerase amplification (RPA), multiple cross displacement amplification (MCDA), and nucleic acid sequence-based amplification (NASBA) methods were introduced to amplify the target detection signal based on isothermal use of DNA polymerase with a mild touch of DNA transformation, thus increasing the efficiency of the used polymerase. All these isothermal amplification methods can be combined with various detection methods such as gel electrophoresis, molecular beacons, enzyme-linked oligosorbent assay (ELOSA), and electrochemical sensors to visualize detection and further enhance sensitivity. Introduction of isothermal amplification-based nucleic acid target detection methods are especially useful for POCT due to their inherent simplicity, speed, and precision.

The proposed isothermal amplification method is a way of producing detectable amplicons from low target concentrations through continuous reactions of various polymerases. The next technique to be introduced is not about amplifying the detection material itself through DNA polymerase but it focuses on generating a small amount of target recycle or cascade reactions by repeated hybridization and dissociation of probes and targets through dynamic DNA nanotechnology. Dynamic DNA nanotechnology focuses on the free energy and stability of certain states of nucleic acid transition derived from base-pairing. This approach of balancing and manipulating the energetic stability provides an objective and intuitive view of probe-target interactions and can be used in a process of displacement, recycling, and signal amplification.

### 2.2.2. Sensitivity Enhancement by Entropy-Driven Signal Amplification

Enzyme-free signal amplification method is based on molecular beacon technology that detects a target by quenching and expressing the fluorescence of a fluorophore at the end of hairpin DNA for robust molecular interactions in a solution (Figure 6a). The molecular beacon consists of a single hairpin probe with a simple target, and the complementary sequences generate signals only for targets and probes in a 1:1 response. Thus, target detection and disease diagnosis from a bio-fluid sample with low concentration of a specific target are limited. Therefore, a system is introduced to detect a target more sensitively through touch-mediated strand displacement without participating in a reaction as a reactant or product, and just like a catalyst. The enzyme-free signal amplification method has been developed to facilitate the probe-target strand displacement response and amplify the signal according to the properties of DNA self-assembly and entropy-driven dynamic behavior.

This entropy-driven cascade reaction was improved by considering the probes and targets as reactants colliding with certain energy. Enhancement of colliding frequency by the localization of probes on flexible DNA nanostructures has led to an improvement (384-fold) in limit of detection (LoD) and provided a new approach for the advanced probe design (Figure 6b). 3D DNA nanomachines that are self-powered and triggered by target strands without addition of fuel DNA strands or protein enzymes were introduced to maximize the utilization of these entropy-driven movements of DNA.

A DNA nanomachine with a aqueous basis, facilitating a self-powered movement can achieve an LoD as low as $0.68 \times 10^{-12}$ M of microRNA without use of complex enzymatic procedure or thermal administration (Figure 6c). Apart from the entropy-driven movement of DNA amplifying the target detection, the cascade assembly of DNA origami, initiated by target recognition has enabled a detectable changes in mechanical property that are exempt of labels. The target-triggered cascade reaction of DNA can result in self-assembly of a complex origami-like structures, allowing geometrical rearrangement of inorganic materials such as plasmonic nanoparticles with its distinct optical properties can be converted easily to a detectable colorimetric signal.

In the same vein, the hybridization chain reaction (HCR) was introduced to enable target recognition and signal amplification without external inputs such as, enzymes or energy sources (Figure 6d). Strand displacement of two hairpin species initiated by the recognition of single-stranded targets occurs in chains and is fueled by the potential energy in two hairpins. The resulting nicked double helix can be easily detected physically, optically, or electrochemically. A sensing method based on entropy-driven dynamic properties of DNA does not require DNA-specific enzymes or separate signal detection processes, with only need for required noncovalent binding between DNA probe or even non-nucleic acid molecules. Thus, not only DNA biomarker, mRNA, or miRNA, but also exosomes, cells, proteins, ligands can be detected using the simple DNA construct utilizing aptamer technology. Furthermore, hybridization-based methods differentiate the structural status or maturity of certain targets, thus allowing the differentiation of pre-miRNAs that often co-exist with miRNAs, which would be complicated with the enzymatic amplification methods.

### 2.2.3. Multiplexity

For accurate signal identification and diagnosis, we need collective information such as, comparison of a housekeeping gene and a target, quantitative difference between wild type and a mutation, or overall trends of gene expression. Individual
detection of such targets can result in significant error due to possible subsampling errors. Thus, highly specific technologies are required to practically detect numerous targets in one batch (Figure 7a). For this purpose, the multiplex PCR was introduced to amplify more than one target in a single PCR mixture using multiple sets of primers.\textsuperscript{[94]} Although multiplex PCR is efficient in terms of space, time, and cost, it suffers from the chronic problem of common multiplexed detection, such as, varied cross-complementarity of the probes. However, multiplexed PCR has another aspect to consider, i.e., the $T_m$ and $\Delta G$ of the probe, which is relevant to the thermally controlled amplification of PCR. Furthermore, this problem hinders the amplification process and significantly decreases as the number of targets increase.\textsuperscript{[94]} Here, several examples of DNA transformations were introduced to provide a list of labels or codes for various sequences. In addition, different methods of probe designs have been developed for multivalent labeling. Self-assembling DNA nanotechnology consisting of built-in codes and probes corresponding to plurality of specific targets within a batch allows the system to be constructed simultaneously. The versatility of the DNA nanostructure allows the presence of commonly used fluorophores to be arranged in various forms, combinations, and ratios, in which the targets can be identified in one batch (without multiple runs or probe stripping).\textsuperscript{[40a,95]} In addition, it is important to understand the relative ratio of a housekeeping gene or a wild type gene in the presence of a specific mutation in cancer diagnosis.\textsuperscript{[96]} It is also possible to correct the signal for the negative control by detecting variety of targets through topological transformation in a single unit of a DNA prism (Figure 7b).\textsuperscript{[97]} A statistical analysis and the correction of target detection signals through rtPCR or dPCR are important, but the chronic error rate can be reduced if the relative quantitative and signal corrections account for the negative control at a molecular level using DNA transformation. On the other hand, the structural versatility of DNA transformation and sequence-specific affinity to certain molecules (e.g., aptamer) allow continuous detection of multiple molecules through nanopores without labeling. A technique of designating and reading multiple barcodes via DNA structure and aptamer has been introduced, which enabled direct sensing of various molecules such as protein, antibody, and cocaine\textsuperscript{[98]} (Figure 7c).
2.2.4. High-Throughput Surface-Based Biosensor

In this section, the methods of DNA transformation are described to explain how the target capture capability has been improved to extend it to high-throughput surface-based biosensors. Also, we discussed the design methods for implementing a well-controlled density of probes with improved reactivity. Furthermore, a design method for spatial arrangement of a probe to remove the background noise is introduced.

The high-throughput surface-based biosensors were presented as an alternative to PCR-based homogeneous assays. The microarrays, which were introduced first for studying the molecular basis of interactions are now serving as powerful tools for both genotyping and diagnosis based on biomarker detection, especially in cancer diagnosis due to its unprecedented high throughput (Figure 8a). In addition, the hybridization-based microarray technology provides high-throughput capability for collective screening of various targets. Despite the high-throughput, the use of microarray technology is often hampered by its high background and limited dynamic range of detection because it relies on simple hybridization between probes and the targets which often require a target amplification process. To be more specific, a 1:1 response of linear probes and targets without high amplification processes, such as PCR cannot obtain sufficient sensitivity. The sensitivities of surface-based sensors are limited by mass transport and crowding effects at the liquid-solid interface. In addition, the sensitivity is also limited by the accessibility of target DNA/RNA molecules to the probes attached to heterogeneous surface, in contrast to probe-target recognition in homogenous solutions. Hence, the sensitivity of surface-based sensors for miRNA usually does not support direct detection of low-abundance target nucleic acids without prior amplification with PCR. Here, the DNA transformation solves the problem of systematic heterogeneity with a DNA nanostructure-based interfacial engineering strategy. The readily programmable DNA nanostructure provides a convenient solution for spatially controlled and enhanced accessibility of probes on the surface. Here, a 3D DNA nanostructure, represented by a tetrahedral DNA nanostructure (TDN), is utilized for improved probe-target recognition properties (Figure 8b). The TDN with pendant probe DNA provides mechanical rigidity, well-controlled spacing, and structural stability for highly ordered upright orientation of probes which is favorable for targets lacking conventional ssDNA probes. In fact, a TDN-based probe system exhibited 250-fold improvement in sensitivity compared to that of an ssDNA probe, achieving an LoD of $1 \times 10^{-12}$ M. In addition, further surface engineering by incorporating tetrahedral...
nanostructures free of probes (to dilute the overall density of DNA probes) minimized the surface crowding effect and increased the target accessibility.\[104\] Furthermore, DNA tetrahedral-modified surfaces are inherently protein resistant, which greatly minimizes the background caused by proteins present in the bio-fluids. This eliminated the need for purification or extraction steps, which prevent portability and reduce the robustness of on-site (or POCT) molecular diagnostics.\[103,105\] Also, protein-resistant surfaces allow the use of high-signal amplification methods such as horseradish peroxidase (HRP), which is the most commonly used Amperometric signal generator.\[106\] To date, TDN-based microarray systems for microRNA analysis have achieved an LoD of a few attomolar (<1000 copies) by introducing various signal transduction techniques; this is a valid range for detection of targets in noninvasive retrieval of biofluids and is comparable to that of rtPCR.\[107\] In addition, due to its versatile bottom-up design, TDN can serve as a protein or ligand detecting probe as an upfront extension of a DNA sensor with the use of aptamer sequences.\[102\] Above all, it is noteworthy that the probe design strategies for solution-based systems, represented by entropy-driven hybridization as discussed above, can also be applied to surface-based probe design by simple extension from surface-anchored nanostructures.\[106\]

3. Application of DNA Transformation in Therapy

3.1. Bottlenecks in Nanomedicine and Opportunities for DNA Nanostructures

Nanomedicine has been considered a promising solution for improving cancer therapy in the past decades. However, despite explosive growth of nanomedicine studies in academic research, clinical translation of nanomedicine faces tremendous bottlenecks. Ideal nanocarriers should deliver therapeutics directly to the targeted site, e.g., tumor, without generating side effects. However, numerous nanocarriers accumulate in the liver, and it was reported that only 0.7% (median) of the administered nanoparticle dose is delivered to a solid tumor.\[108\] Moreover, synthetic nanocarriers, such as polymers and inorganic nanoparticles, were unfavored by clinicians due to their potential nanotoxicity, long time retention in the body, and unclear side effects caused by their degradation products.\[109\] Furthermore, nanomedicine formulations often were achieved by adsorption or encapsulation of drug molecules into nanoparticles, which is difficult for reliable quality control, resulting in poor reproducibility of nanomedicines.\[110\] Therefore, researchers committed to practical application of nanodrugs are facing a high degree of uncertainty, especially characterization of the safety and toxicity of these nanomaterials. Compared with traditional materials, DNA-based nanosystems have many interesting properties. As natural biomacromolecules, DNA nanocarriers were expected to show high biocompatibility and easy degradation without generating harmful byproducts.\[111\] DNA nanostructures can accumulate in solid tumors, indicating their promising potential for tumor-targeted drug delivery.\[112\] More interestingly, in a mouse model without a tumor, DNA origami structures were preferentially distributed in kidneys with minimal liver uptake. Compared to most inorganic nanoparticles and polymer nanoparticles, which are often captured by the reticuloendothelial system (RES) of the liver, this unique feature of DNA origami nanostructures brought exciting new opportunities for nanomedicine.\[113\] Based on this feature, DNA origami could serve as a reactive oxygen species (ROS) scavenger for treating acute kidney injury (AKI) with efficacy similar to clinically used AKI drugs.\[113\] Additionally, DNA nanocarriers could be prepared with highly uniform sizes, shapes, and structures due to the precise
programmability, which also allows easy functionalization at defined positions by sequence-dependent modification. Such a feature significantly improved the reliability and reproducibility of the resulting nanomedicine. Furthermore, smart DNA transformation could lead to greater accurate target recognition and programmable drug release, which might pave the way for next-generation nanomedicine with high target specificity.[114] Various small molecules, drugs, genes, and proteins could be successfully loaded onto DNA structures by absorption, encapsulation, intercalation, and site-specific modification,[115] which further strengthened the potential of DNA nanostructures as a nanomedicine platform. Combined with the above-mentioned potential of DNA sensors, development of theranostic systems using DNA transformation could be envisioned.

3.2. DNA Transformation for Programmable Drug Release

3.2.1. Self-Assembly of DNA Nanocarriers

Assembly of nanostructures with controllable geometry has been realized by DNA self-assembly, which includes tile-based procedures, RCA-derived approaches, and the DNA origami technique.[116] The smallest structure reported to date is a DNA prism constructed of a single DNA chain with a characteristic dimension of 3.4 nm.[117] Sophisticated DNA nanostructures constructed by DNA origami techniques, such as triangles,[112b] rectangles,[118] hexagonal,[119] and hexagonal boxes,[120] could allow a more complex design of nanocarriers with sizes from tens to one hundred nanometers.[121] In addition, periodic assembly of DNA structures could result in larger DNA nanostructures, such as DNA hydrogels,[122] DNA dendrimers,[123] and DNA ribbons,[124] which are also promising candidates for drug delivery (Figure 9). Most of the above types of DNA nanodevices are constructed using the classical B-DNA helix. However, there are many pairing options in addition to the iconic double helix structure, such as the G-quadruplex and i-motif. Each G-quadruplex structure consists of four guanine moieties (which together form a tetrad), whereas the i-motif is rich in cytosine. These secondary structures are formed by folding of single-stranded DNA.[125]

3.2.2. Drug Loading on DNA Carriers

Depending on the drug required, various drug loading strategies could be employed (Figure 10). To attach nucleic acid cargoes such as CpG motif,[126] antisense strand,[127] and siRNA,[128] the simplest route is designing single-stranded sticky ends on the DNA nanocarriers that are complementary to the cargo oligonucleotide. In addition, given that DNA nanostructures are structurally rigid and inherently insensitive to nuclease degradation,[55c,129] the bioactive cargo can be directly integrated into the scaffold of carrier nanostructures in the design stage. Indeed, this approach has been used to “load” antisense,[130] aptamer,[131] and CpG[132] sequences into a wireframe DNA tetrahedral.

Small-molecule chemotherapeutic drugs such as doxorubicin (Dox) and daunorubicin can be effectively loaded on the carrier by directly intercalating them into DNA base pairs. Taking advantage of the intercalation characteristics of drugs, tubular and triangular DNA origami loaded with Dox showed significant cytotoxicity to drug-resistant human breast cancer cells and successfully avoided drug resistance in vitro.[132] Furthermore, three DNA origami nanostructures (triangles, squares, and tubes) possessed enhanced tumor passive targeting and long-lasting accumulation properties in the tumor region. In particular, triangle DNA origami loaded with Dox inhibited tumor growth in vivo without inducing observable systemic toxicity, demonstrating its antitumor efficacy.[128]

In cases where hybridization or intercalation of the cargo to the carrier nanostructure is not feasible, some DNA nanocarriers have aqueous interior compartments, making them naturally suitable for physically encapsulating some nanoparticles or enzyme-sensitive cargoes without compromising cargo functionality. For instance, DNA icosahedrons were utilized for encapsulating biopolymers by trapping them in the cavity of the nanostructure during assembly.[133] Also, RNaseA and cytochrome c were encapsulated by DNA nanoflowers for delivery.[114] To advance the use of DNA nanotechnology for controlled release of bioactive molecules, another strategy is to encapsulate bioactive molecules in DNA nanostructures and use light to release packaged cargoes with high spatiotemporal precision.[115] Using a photolabile crosslinker, cargo molecules are attached to the cavity of DNA nanostructures. They released their cargoes upon brief exposure to light. This technology allows cargo to be released in their unaltered, bioactive state in contrast to existing labile conjugation chemistries.

Alternatively, chemical conjugation via covalent and noncovalent interactions allows more specific and controlled loading of therapeutic cargoes onto DNA carriers. Reactive groups such as ethynyl groups, azido groups, and thiol groups could be added to oligonucleotides by solid phase DNA synthesis, forming hybridized DNA nanostructures to provide covalent modification handles.[116] Tetrahexedrally DNA nanostructures were successfully conjugated with nuclear localization signal (NLS) peptides through a reaction between azide-modified ssDNAs with NLS peptides containing a propargylglycine and used to modulate the location of DNA nanostructures inside the cells.[137] Additionally, non-covalent binding (e.g., coordination complexes, antibodies-antigens) could be employed to load cargoes with stimuli responsive properties and allow reversibility. For instance, streptavidin can be linked to a wireframe DNA tetrahedral via the displayed biotin group.[138] The antibody-antigen interaction also is utilized to stabilize cargo, either as antibodies themselves[138] or as an antigen captured by a DNA-bound antibody.[118] DNA nanocarriers for different types of cargo including dendrimers,[139] nanotubes,[140] DNA origami rectangle,[141a] and Origami–nanoparticle superstructure[141b] are summarized in Table 3.

3.2.3. Cargo Release from Transformable DNA Nanostructures

In addition to cargo loading, controlled release is another challenge for drug delivery.[142] Since DNA nanostructures could be slowly degraded by nuclease, drug molecules intercalated into DNA strands could be naturally released in vivo.[55c,d,129,143]
Studies have shown that tightly packed DNA nanostructures, such as DNA origami structures, exhibit better stability in blood circulation than coiled DNA strands.\cite{55d,129} Therefore, they can accumulate in solid tumors via both passive and active targeting.\cite{112b} Drug payloads intercalated and encapsulated inside DNA nanostructures could be released inside the tumor cells simply by nuclease degradation. This strategy was often employed by first-generation DNA-based drug carriers.\cite{112b,144} Many successful examples have shown promising therapeutic effects even for multiresistant tumors.\cite{132,144b,145} However, such uncontrolled drug release mechanisms lack target specificity and do not take full advantage of DNA nanotechnology. Dynamic DNA nanostructures with programmable transformation could provide extraordinary opportunities to achieve precisely designed motion control, realizing intelligent drug release responses to multiple stimuli.\cite{146} Several pioneering examples have been demonstrated in vitro and in vivo, which will be the main focus of our discussion here.

**Figure 9.** DNA nanostructures in biomedical applications. a) DNA dendrimer as an efficient nanocarrier of functional nucleic acids for intracellular molecular sensing. Reproduced with permission.\cite{123} Copyright 2014, American Chemical Society. b) DNA hydrogels. Reproduced with permission.\cite{122} Copyright 2014, American Chemical Society. c) DNA flowers that allow highly efficient protein loading while retaining the biological activity of the payloads. Reproduced with permission.\cite{114} Copyright 2017, Wiley-VCH. d) Tetrahedra displaying antisense motifs able to specifically degrade mRNA and inhibit protein expression in vitro. Reproduced with permission.\cite{113} Copyright 2011, Wiley-VCH. e) A synthetic icosahedral DNA-based host–cargo complex for functional in vivo imaging. Reproduced with permission.\cite{113} Copyright 2011, Springer Nature. f) Targeted delivery of rab26 siRNA with precisely tailored triangular prisms for lung cancer therapy. Reproduced with permission.\cite{129} Copyright 2019, Wiley-VCH. g) DNA origami triangles as an in vivo drug delivery vehicle for cancer therapy. Reproduced with permission.\cite{112a} Copyright 2014, American Chemical Society. h) A modular DNA origami tube-based enzyme cascade nanoreactor. Reproduced with permission.\cite{119} Copyright 2015, Royal Society of Chemistry. i) Rectangular DNA origami coated with virus capsid proteins for efficient cellular delivery. Reproduced with permission.\cite{118} Copyright 2014, American Chemical Society. j) Daunorubicin-loaded DNA origami nanorods circumvent drug-resistance mechanisms in a leukemia model. Reproduced with permission.\cite{145} Copyright 2015, Wiley-VCH.

**Figure 10.** The methods of drug loading for DNA nanostructure. a) One-Pot synthesis of multiple protein-encapsulated DNA flowers and their application in intracellular protein delivery. Reproduced with permission.\cite{134} Copyright 2017, Wiley-VCH GmbH & Co. KGaA, Weinheim. b) DNA origami as a carrier for circumvention of drug resistance. Reproduced with permission.\cite{132} Copyright 2012, American Chemical Society. c) Cellular immunostimulation by CpG-sequence-coated DNA origami structures. Reproduced with permission.\cite{126} Copyright 2011, American Chemical Society. d) “Sense-and-Treat” DNA nanodevice for synergetic destruction of circulating tumor cells. Reproduced with permission.\cite{127} Copyright 2016, American Chemical Society. e) Self-assembled multivalent DNA nanostructures for noninvasive intracellular delivery of immunostimulatory CpG oligonucleotides. Reproduced with permission.\cite{55a} Copyright 2011, American Chemical Society.
One representative example of programmable drug release designed by DNA transformation is a logic-gated nanorobot that could recognize the targeted cells and expose molecular payloads specifically to the target. The DNA nanocarrier was designed with two half pieces linked by flexible DNA strands, which could be closed into a hexanol tube with aptamer locks, encapsulating the payloads inside the tube (Figure 11a). When the aptamer locks encounter their targets, the aptamer sequences will undergo structural transformation, resulting in dissociation from the complementary strand and unlocking the structure. For instance, when confronted with human leukocytes with antigens expressed on their surfaces, DNA nanorobots can be unlocked. Internal antibodies encapsulated inside the DNA nanorobot are exposed to cell surface receptors, inhibiting target cell growth or inducing cell signal transduction. Moreover, by designing two aptamer locks to identify different input signals, different logic or gates could be used to differentiate targeted and untargeted cells with high specificity (Figure 11a). Based on the same DNA origami structure, another team realized the dynamic interaction of nanorobots in living animals (Figure 11b). The dsDNA served as a rigid scaffold to position the bimolecular i-motif and G-quadruplex. The G-quadruplex only formed when preceded by assembly of the i-motif, which requires acidic pH and unhindered mobility of the head-motif containing dsDNA nanorings with respect to the central ring to which they are interlocked. These features were employed to convert the structural changes into Boolean operations with fluorescence labeling. The nanostructure behaves as a reversible logic circuit consisting of tandem YES and AND gates. This reversible logic circuit integrated into functional nanodevices may be applied to cascade reactions in biological systems. Furthermore, programmable drug release by nanorobots designed based on the G-quadruplex was achieved by coupling DNA origami with G4-forming AS1411 aptamers and carrying blood coagulation protease thrombin for target-specific action (Figure 11d). Fastening is achieved by six pairs of fastener strands consisting of the AS1411 oligonucleotide and a partially complementary strand. In the absence of nucleolin, a double strand is formed to close the tube. When DNA nanobots recognize tumor cells through aptamers and sense the presence of nucleolin B in tumor cells, DNA nanobots will be deployed to release thrombin to destroy tumor cells and inhibit tumor growth.

| DNA nanostructure      | Cargo   | Drug loading approach | Application              | Ref.      |
|------------------------|---------|-----------------------|--------------------------|-----------|
| Dendrimers             | Doxorubicin | Intercalation         | Cancer therapy           | [139]     |
| DNA hydrogels          | Camptothecin | Encapsulation          | Controlled drug release   | [122]     |
| Porcine insulin        | Doxorubicin | Integration           | Cancer therapy           | [134]     |
| DNA flowers            | RNase A   | Encapsulation          | Protein drug delivery     | [133]     |
| Tetrahedra             | Antisense strand of mRNA | Integration       | Cancer therapy           | [126]     |
|                        | Doxorubicin | Intercalation         | Cancer therapy           | [112]     |
|                        | CpG       | Integration           | Cancer therapy           |           |
| Icosahedra             | Fluorescent biopolymer | Encapsulation     | In vivo imaging           | [131]     |
| Triangular prisms      | SiRNA     | DNA-DNA hybridization | Cancer therapy           | [128]     |
| Nanotubes              | Cy3       | DNA-DNA hybridization | Molecular imaging or preclinical imaging | [140]     |
| DNA origami nanorod    | Daunorubicin | Intercalation        | Cancer therapy           | [145]     |
| DNA origami tube       | CpG       | DNA-DNA hybridization | Immunotherapy            | [118,141a]|
| DNA origami triangle   | Doxorubicin | Intercalation         | Cancer therapy           | [112]     |
| Gold nanoparticles     | DNA-DNA hybridization | Cancer therapy     | Immunotherapy            | [118,141a]|
| DNA origami rectangle  | Thrombin  | DNA-DNA hybridization | Cancer therapy           | [126]     |
|                        | CpG       | DNA-DNA hybridization | Cancer therapy           |           |
| Origami-nanoparticle superstructures | Doxorubicin | Intercalation        | Cancer therapy           | [141b]    |

In addition to conventional DNA double strands formed by Watson-Crick base pairing, some switchable structures involve unusual nucleic acid motifs such as insertion motifs (i-motif) and the G-quadruplex. These unconventional DNA interactions also can be used to control the dynamic structure of DNA nanostructures. For this purpose, a reversible logic circuit built on programmable assembly of a dsDNA pseudocatenane was developed (Figure 11c). The dsDNA served as a rigid scaffold to position the bimolecular i-motif and G-quadruplex. The G-quadruplex only formed when preceded by assembly of the i-motif, which requires acidic pH and unhindered mobility of the head-motif containing dsDNA nanorings with respect to the central ring to which they are interlocked. These features were employed to convert the structural changes into Boolean operations with fluorescence labeling. The nanostructure behaves as a reversible logic circuit consisting of tandem YES and AND gates. This reversible logic circuit integrated into functional nanodevices may be applied to cascade reactions in biological systems.
3.2.4. Surface Decoration of DNA Nanostructures

Although DNA nanocarriers showed great potential for therapeutic applications, their in vivo stability, relatively low cellular uptake, and static physical chemical properties are problematic. The structure of most DNA assemblies is sensitive to the buffer environment. Various ion concentrations and pH levels in physiological fluids often compromise the stability of DNA nanostructures.[150] Moreover, endogenous nuclease could degrade DNA nanostructures, limiting the application of DNA nanorobots and designed DNA transformers.[55b,d,143] In addition, unlike synthetic polymers with tunable physical chemical properties (such as charges and hydrophilicity), the surface charges of DNA nanostructures are highly negative due to the phosphate backbone. This property resulted in their low transfection efficiency into mammalian cells.[151] Therefore, decoration of DNA nanostructures is highly valuable for improving their in vivo stability, modifying their surface properties, and realizing additional environmental responsiveness.

To date, strategies including electrostatic interactions,[118,152] strand displacement,[153] DNA hybridization,[154] and conjugation[155] have been developed to decorate DNA nanostructures. Due to the highly negatively charged nature of DNA nanostructures, electrostatic interactions are the most investigated method for fast surface decoration.[118,152,156] For instance, a series of positively charged materials, such as cationic block copolymers and cationic proteins, were utilized to wrap the negatively charged DNA origami via electrostatic interactions to improve its cell uptake efficiency and stability under physiological conditions (Figure 12a).[118,152,156b] Using these strategies, DNA nanostructures could be well protected from enzymatic degradation and even tolerant pH, ionic strength, and temperature changes in a larger range.[156b] The cationic coating could significantly enhance their cellular uptake efficiency, serving as better carriers for drugs and genes.[118] However, electrostatic interactions resulted in a uniform coating of the structure, which is undesired if some surface functional moieties (e.g., receptor recognition sites, responsive transformation linkers, biomarker detection units) have to be exposed to the surrounding medium. Therefore, site-specific decoration by sequence-determined hybridization and chemical conjugation would be preferred in these cases. For instance, polymers could be site specifically decorated on DNA nanostructures by both “grafting from”[106b] and “grafting to” methods. The “grafting from” method allows polymer growth directly on DNA nanostructures, which could result in high-density polymer coating at the desired position. It could provide sufficient protection of DNA structures while keeping the functional moieties actively

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**Figure 11.** a) Schematic of a smart logic-gated DNA origami nanorobot to target cells and subsequently display the molecular payload. Reproduced with permission.[220] Copyright 2012, The American Association for the Advancement of Science. b) Schematic of universal computing by DNA origami robots in a living animal. Reproduced with permission.[47] Copyright 2014, Springer Nature. c) Schematic of interlocked DNA nanostructures controlled by a reversible logic circuit. Reproduced with permission.[149] Copyright 2014, Springer Nature. d) Schematic of targeting nucleolin to obstruct vasculature feeding with an intelligent DNA nanorobot. Reproduced with permission.[141a] Copyright 2018, Wiley-VCH.
available (Figure 12b). This would be highly attractive for developing intelligent nanomedicine with several functional regions. In contrast, the “grafting to” method to conjugate synthetic polymers on DNA strands is favored for decorating a few polymer chains on DNA structures, and it is applicable for more diverse types of polymers. By employing polymers with unique responsive properties, this method could introduce additional stimulus-responsiveness to DNA nanostructures. The resulting dynamic structures triggered by external signals such as temperature, pH, and enzyme were highly attractive as diagnostic tools or drug delivery systems. For instance, by incorporating the thermo-responsive polymer poly(N-isopropylacrylamide) on either side of a flexible hinge, a “DNA origami flexor” could be achieved that reversibly opens or closes with temperature changes of a few Kelvin (Figure 12c). Such a mechanism could be easily incorporated into many DNA nanorobot designs to induce additional stimuli transformation for intelligent drug delivery and bioimaging.

Recently, decoration of DNA nanostructures by inorganic material mineralization was demonstrated. This could further improve the surface properties of DNA nanocarriers. For instance, a silica oxide layer could be formed by mixing DNA nanostructures with prehydrolyzed N-trimethoxysilylpropyl-N,N,N-trimethylammonium chloride (TMAPS) and tetraethyl orthosilicate (TEOS) (Figure 12d). This method achieved a highly defined and uniform coating of silica oxide on sophisticated DNA nanostructures that significantly improved their mechanical strength and stability. When protruding dsDNA were present on the surface of DNA nanostructures, they are preferentially mineralized, allowing site-specific decoration of DNA nanostructures with silica oxide materials. Although these strategies have not been applied for drug delivery, their potential is clear.

3.3. DNA Transformation for Theranostics

Since DNA transformation is highly valuable for both therapy and diagnosis, they are ideal candidates for use in theranostic nanomedicine, which combines therapy and diagnostic functions in one particle. Molecular beacons and different DNA nanostructures combined with DNA aptamers have been developed. The general strategies will be summarized, and the most recent examples will be discussed here. Combination of aptamers with a variety of DNA nanostructures is the most common strategy for theranostics. Drug cargoes and imaging functions can be incorporated by both physical absorption and chemical modifications. For instance, aptamer-based DNA dendritic nanostructures that assemble fluorophores in the inner layer and sgc8 aptamers in the outer layer have been developed. The abundant double-stranded sequences in DNA dendrimers endow them with a high doxorubicin loading capacity and the advantages of DNA
dendrimers and aptamers facilitate tumor targeting and efficient internalization (Figure 13a). These DNA dendrimers could provide cancer cell imaging and targeted anticancer drug delivery simultaneously. In addition, several attractive characteristics, including controllable step-by-step self-assembly, excellent biological stability and biocompatibility, and strong binding affinity, suggest their potential for use in a wide range of applications. Similarly, a dual-targeted DNA tetrahedral nanoprobe for imaging breast cancer cells and targeting drug delivery was also investigated (Figure 13b). The nanoparticle consists of three parts: 1) connection between the functional ligand and the DNA tetrahedral core loaded with doxorubicin, 2) the turn-on probe for Mucin 1 protein (MUC1) detection on the cell membrane based on target-triggered aptamer transformation, and 3) the AS1411 aptamer for nucleolus binding. The MUC1 probe facilitates fluorescence imaging of MUC1-positive cancer cells. Additionally, nucleolus is another cancer marker that is overexpressed in the nucleus and cytoplasm of cancer cells. Because of its bidirectional nuclear localization sequence, excellent biological stability and biocompatibility, and strong binding affinity, suggest their potential for use in a wide range of applications. Similarly, a dual-targeted DNA tetrahedral nanoprobe for imaging breast cancer cells and targeting drug delivery was also investigated (Figure 13b). The nanoparticle consists of three parts: 1) connection between the functional ligand and the DNA tetrahedral core loaded with doxorubicin, 2) the turn-on probe for Mucin 1 protein (MUC1) detection on the cell membrane based on target-triggered aptamer transformation, and 3) the AS1411 aptamer for nucleolus binding. The MUC1 probe facilitates fluorescence imaging of MUC1-positive cancer cells. Additionally, nucleolus is another cancer marker that is overexpressed in the nucleus and cytoplasm of cancer cells. Because of its bidirectional nuclear localization sequence, the nucleolus can be transferred between the nucleus and cytoplasm. Therefore, this dual targeting strategy has great potential in cancer diagnosis and therapy and provides a promising way to avoid side effects and improve the efficiency of treatment.

Due to the properties of inorganic nanoparticles, integration of DNA and inorganic nanoparticles can broaden the application of diagnosis and treatment in many fields. For example, AuNRs have excellent photothermal conversion performance under irradiation of near-infrared light (650–900 nm), allowing wide use in photothermal therapy (PTT) and photodynamic therapy (PDT). Exploiting the photothermal conversion ability of AuNRs, Ding and co-workers combined the characteristics of AuNRs with DNA origami nanostructures to successfully develop self-assembled DNA origami–AuNR composites for bifunctional nanomedical applications (Figure 14a). Compared with naked AuNRs, a DNA origami–AuNR complex significantly enhanced uptake by human MCF7 breast cancer cells. In particular, the triangular DNA origami-AuNR complex showed optimal cell accumulation. Compared with exposed AuNRs, triangular DNA origami–AuNRs complexes showed enhanced photothermal decomposition of tumor cells in vitro and in vivo. The system also provided an appealing platform for two-photon cell imaging, suggesting it as a promising candidate for cancer diagnosis and therapy both in vitro and in vivo. In addition to AuNPs, MnO2 nanoparticles have attracted broad attention in the fields of bioanalysis, cell imaging, and drug delivery for their attractive physical and chemical properties. They could also be combined with DNA transformation systems. For instance, a novel thermosensitive nanoprobe for the nonlabeled fluorescence imaging of Zn2+ and 635 nm red light-triggered PDT was reported (Figure 14b). The probe uses DNA as a template to assemble silver nanoclusters(AgNCs)/porphyrin(P)/MnO2 all in one particle. After entering the cells, MnO2 nanowires were reduced in the tumor microenvironment and released AgNC for label-free Zn2+ fluorescence imaging by hairpin DNA-fueled dynamic self-assembly of three-way DNA junction architectures. The released Mn2+ can be used as an effective magnetic resonance imaging (MRI) contrast agent. In addition, the P-AgNCs–MnO2 nanoprobe decomposed in an acidic H2O2-sufficient environment and released excess singlet oxygen (1O2) under light irradiation, resulting in efficient photodynamic therapy.

In summary, the programmable structure and inherent heterogeneity of DNA nanostructures promise controlled release of loaded cargo and provide chemical sites for functionalization of a variety of tumor-targeting biomolecules. To further develop a smart nanotheranostic system, DNA nanostructures can serve as the template to integrate multiple functional elements such as photothermal conversion nanoparticles and MRI contrast agents. These result in a multifunctional DNA-based platform that can be used to prepare self-assembled DNA nanostructures for cancer diagnosis and therapy.

4. Challenges for Practical Biomedical Applications

The role of nucleic acid biomarkers for early and fundamental diagnosis of cancerous disease, genetic disease, and infectious disease is increasing with advent of bioinformatics and
accumulation of genetic knowledge. Diagnosis of cancerous diseases or genetic diseases is enabled through detection of established genetic or epigenetic mutations, and diagnosis of infectious diseases is possible through detection of pathogen-specific sequences in the pathogenic genomes.

Among numerous applicable biomarkers for modern genetic diagnosis, detection of RNA biomarkers of the most actively studied pathogenic disease, COVID-19, is discussed. SARS-CoV-2 diagnostic kits are being rapidly developed due to the unprecedented outbreak of pandemic COVID-19. As SARS-CoV-2 is a positive-sense, single-stranded RNA virus, early diagnosis of COVID-19 is standardized for viral RNA detection through reverse transcriptase PCR (RT-PCR). Most of the available RT-PCR tests target regions in viral RNA are nucleocapsid (N), envelope (E), spike (S), and open reading frame (ORF1ab) genes. Among the numerous RT-PCR test kits developed since the earliest SARS-CoV-2 diagnostic panel from the Center for Disease Control and Prevention (CDC), PerkinElmer Inc. released a detection kit with analytical sensitivity down to 8.3 copies mL$^{-1}$ for ORF1ab and 24.9 copies mL$^{-1}$ for N gene assays as confirmed by genetic sequencing. However, the need for higher throughput and sensitive assay kits has persisted since use of high-performance RT-PCR assay kits were limited to laboratory operations with 2 h turnaround times. Qiagen GmbH has released a test kit with impressive multiplexing capabilities, which can target both ORF1b and E genes of SARS-CoV-2 and several other respiratory infections. This kind of multiplexed assay provides a better overall picture of patient conditions and opportunities for mutation tracking and decreases the possibility of false diagnosis.

The main customers of SARS-CoV-2 assay kits targeting viral RNAs introduced above are healthcare providers such as hospitals and urgent care facilities or academic institutions with highly trained healthcare professionals and research technicians. The need for easy-to-use assay kits for COVID-19 point-of-care continues to increase as development of a remedy is delayed and cases of reinfection are being reported. However, the current RT-PCR-based SARS-CoV-2 assay kits are not available for public use. Recently, the U.S. Food and Drug Administration Emergency Use Authorization (FDA EUA) approval of SARS-CoV-2 viral RNA assay kit employing isothermal amplification technology exhibits great potential for emergence of portable nucleic acid test kits. Although currently approved kits require highly specific instruments for signal detection, portable nucleic acid assays have potential to provide significant improvement over high-end PCR assays.

DNA transformers were recently investigated for therapy applications. Their potential as biocompatible carriers with programmable release properties brought new opportunities to the field of nanomedicine. However, plenty of challenges remain before their use in broad clinical applications. First, the stability and bioavailability of these DNA nanostructures under physiological conditions are basic criteria to be considered. Although several decoration approaches have been intensively investigated to address these challenges as discussed above, their value for clinical translation need to be investigated. In addition, most of the decoration methods comprised some attractive features of DNA origami. For instance, coating of DNA nanostructures with a cationic polymer layer might shield the functional modifications on DNA structures and hinder their stimuli responsive transformation. The polymer layer also increases toxicity risks. More diverse decoration strategies are under further development, with the aim to provide more material options (e.g., from polymer to protein and inorganic materials), tunable properties, and better site specificity.

The second challenge recognized in the field is mass production of transformable DNA nanostructures. The high cost of DNA synthesis often limits their large-quantity applications, and well-designed DNA nanorobots often require hundreds of different sequences, which further increases the production cost. Some
biotechnology has been recently developed to produce design oligonucleotides in microbial, which could achieve gram-scale folding of DNA origami.[174] These examples showed the bright future of large-scale DNA applications. However, these strategies often require tedious genetic construction, which hinders their broad applications. Reducing the cost of DNA synthesis and DNA nanostructure products is urgently needed to promote the whole field, and it requires multidisciplinary collaborations of organic chemists, biologists, and automation engineers.

5. Opportunities

Pathogens that cause diseases are diversifying, and we need multi-faceted design-transformable materials to counter them. Design changes are associated with changes in physicochemical properties. The physical properties accompanied by design are modified and applicable to biomedical purposes. For instance, it is possible to achieve precise design concepts such as accurate structure at the nano-level. Since structural characteristics change even with small-scale changes, it is useful to detect small changes in target concentration.

The affinity of DNA transformation with other substances may be introduced in the near future. It is possible to produce various hybrid types of DNA with excellent affinity for use in advanced technologies. For instance, DNA is easily used with SPR or MALDI-TOF and high-performance diagnostic systems, so that increased sensitivity can be expected without use of existing technology, resulting in a huge advantage in terms of cost and labor.

Smart DNA nanorobots could play a more important role in nanomedicine. Assembling DNA nanostructures with a variety of cargos, such as therapeutic drugs and active proteins, will provide drug combinations or cocktail therapies. The program-mability of DNA nanostructures and the stimuli response of conformational transitions make it one of the best candidate structures to control structure-function relationships and achieve more controllable drug release. DNA transformation also could enable more accurate disease diagnosis and better therapy with a deeper understanding of the behavior of DNA materials in vivo and the molecular targets for tumor recognitions. For instance, logic-gated dynamic DNA nanodevices with multiple targeted ligands can “walk” on different platforms. Teaching them to travel to target areas by adhering to cells will enable intelligent drug delivery. Transformable DNA nanomechanical devices also hold great potential for mechanomedicine and self-powdered nanomotors.[175]

Overall, DNA transformations have been developing rapidly and can be broadly applied in biomedical fields represented by sensing and therapy. Their potential has been intensively explored, and the current applications only represent a small piece of the potential uses. We envision that biomedical applications of DNA transformation will evolve and bring significant changes to future therapy and diagnosis technologies.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords

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[13] J. Wang, Biosens. Bioelectron. 2006, 21, 1887.
So Yeon Ahn studied Chemical Engineering at Sungkyunkwan University, Korea; Purdue University, USA; and Nagoya University, Japan. She received her Bachelor’s (2018) and Master’s (2019) degrees as a Brain Korea 21+ scholarship holder at Sungkyunkwan University working with Prof. Soong Ho Um. After completing her Master’s thesis “Sensitivity enhancement of nucleic acid biosensor via contemplation of dynamic molecular interaction,” she started work at Progeneer Inc. in September 2019. Her research focuses on molecular diagnosis and therapeutic proteins based on nucleic acid engineering.

Jin Liu is currently working with Prof. Yuzhou Wu at Huazhong University of Science and Technology, as a post-doctoral associate. He received his Ph.D. in Microbiology from Huazhong Agricultural University in 2017. Since 2017, he has been working in the field of DNA nanotechnology for biomineralization and its application in therapy and diagnosis. His current research focus is on precise organization of manganese oxide nanostructures by DNA origami and in situ biomineralization.

Srivithya Vellampatti is currently working with Prof. Soong Ho Um in Progeneer Inc., as an R&D researcher. She received her Ph.D. in Nanoscience and Engineering from Sungkyunkwan University (SKKU) in 2018. Since 2013, she has been working in the field of DNA nanotechnology by conjugation of drug molecules and metal ions for optoelectronics and biomedical applications. She had also conducted research on microfluidic miRNA detection through PCR techniques at Korea Institute of Science and Technology, South Korea. Her research interests include fabricating nucleic acid architects for therapeutic applications under Prof. Soong Ho Um.

Yuzhou Wu is a professor at the School of Chemistry and Chemical Engineering, Huazhong University of Science and Technology (HUST). She graduated from Zhejiang University and obtained a Master’s degree from the National University of Singapore and a Ph.D. degree from Ulm University in Germany. Before joining HUST, she worked as a project leader at Ulm University and at the Max-Planck Institute for Polymer Research from 2013 to 2016. Her research interests include nano–bio interface engineering, novel chemical methods for functionalization of protein and DNA nanostructures, and nature-inspired nanofabrication techniques for precision nanomedicine.
Soong Ho Um is a professor at the School of Chemical Engineering at Sungkyunkwan University. He earned his M.Sc. and Ph.D. degrees from Cornell University and then joined the department of Materials Science and Engineering in MIT as an associate researcher. Since returning to Korea as a professor in 2009, he has focused more on the design of novel bioinspired nanotechnology for both diagnostics and therapeutics in practical clinical uses. He is now also the chief technical officer (CTO) at Progeneer Inc., which aims to develop a clinically applicable companion theragnostic kit using nucleic acid transformational state-of-the-art technologies.