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Nanostructured DNA for the delivery of therapeutic agents

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ABSTRACT

DNA and RNA, the nucleic acids found in every living organism, are quite crucial, because not only do they store the genetic information, but also they are used as signals through interaction with various molecules within the body. The nature of nucleic acids, especially DNA, to form double-helix makes it possible to design nucleic acid-based nanostructures with various shapes. Because the shapes as well as the physicochemical properties determine their interaction with proteins or cells, nanostructured DNAs will have different features in the interaction compared with single- or double-stranded DNA. Some of these unique features of nanostructured DNA make ways for efficient delivery of therapeutic agents to specific targets. In this review, we begin with the factors affecting the properties of nanostructured DNA, followed by summarizing the methods for the development of nanostructured DNA. Further, we discuss the characteristics of nanostructured DNA and their applications for the delivery of bioactive compounds.

Contents

1. Introduction

Nucleic acids, *i.e.*, DNA and RNA, are the ones of the most important molecules for life, because they store and transmit the genetic information of all living organisms on the Earth. The genetic information stored in the sequence consists of only four types of nucleobases: adenine, thymine, guanine and cytosine. Accurate base pairing between DNA and DNA, DNA and RNA, and RNA and RNA is quite important for decoding the information. According to Watson-Crick base pairing, adenine binds with thymine or uracil, and guanine binds with cytosine by hydrogen bonding.

Nucleic acids interact with a variety of biological molecules, including proteins and lipids, to exert their functions [1,2]. Fig. 1 summarizes the interaction of nucleic acids with other types molecules: proteins, including transcription factors and Toll-like receptors (TLRs), and

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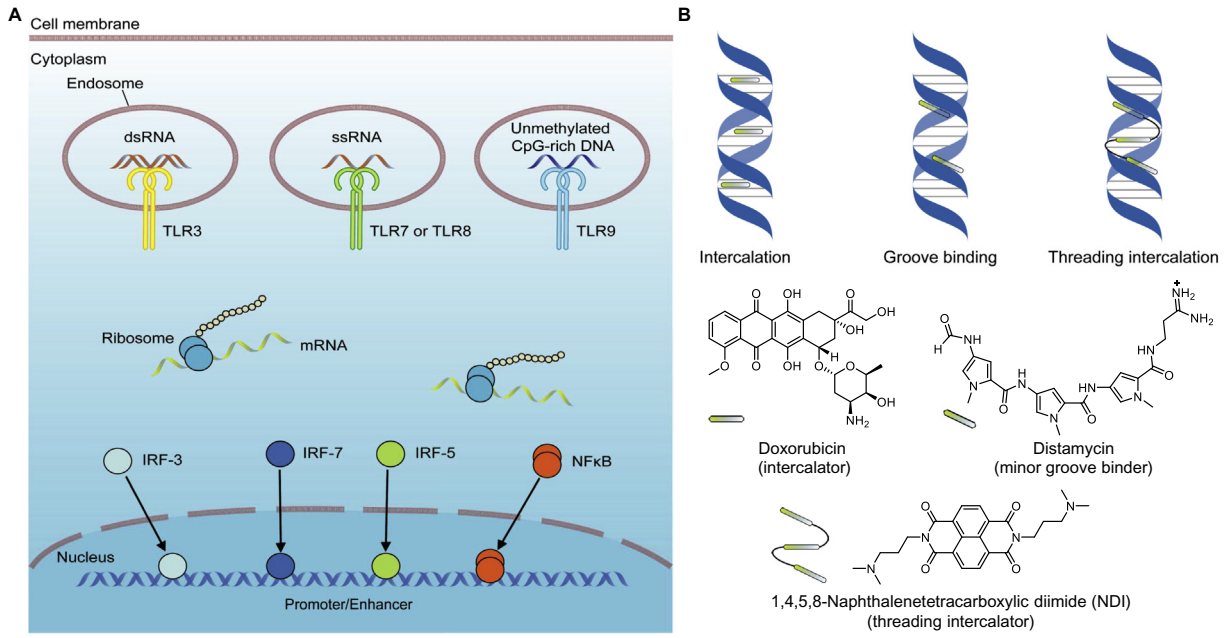

Fig. 1. Interaction of nucleic acids with other types of molecules. (A) Interaction with transcription factors, Toll-like receptors (TLRs), and ribosomal proteins in cells. Exogeneous nucleic acids can bind to TLR3, TLR7/8 or TLR9 in the endosomes after endocytic uptake. Transcription factors, including interferon regulatory factor (IRF)-3, IRF-5, IRF-7, and nuclear factor-κB (NF-κB), bind to the promoter or enhancer on the genomic DNA or synthetic 'decoy' double-stranded DNA in a sequence-specific manner. In the ribosome, ribosomal RNA interacts with ribosomal proteins. dsRNA, double-stranded RNA; ssRNA, single-stranded RNA; CpG, cytosine-phosphate-guanine; mRNA, messenger RNA. (B) Interaction with low-molecular-weight compounds. Various compounds interact with nucleic acids by intercalation, groove binding or threading intercalation. Rectangles indicate the parts of the molecules interacting with nucleic acids. The chemical structures of doxorubicin, distamycin, and 1,4,5,8-naphthalenetetracarboxylic diimide, which are typical examples of such compounds, are also shown.

low-molecular-weight compounds, including anticancer agents. Some interactions are based on the base sequences of nucleic acids. Transcription factors bind to the specific site of the genome DNA depending on the base sequence. TLRs function to detect danger signals, which are the pathogen-associated molecular patterns derived from microorganisms and the damage-associated molecular patterns that are secreted by dying cells. Some TLRs recognize the sequence of nucleic acids for subsequent signal transduction [3]. TLR9 is a well-known TLR that recognizes DNA or oligodeoxynucleotide (ODN) containing unmethylated cytosine-phosphate-guanine (CpG) motif, which is found predominantly in the DNA of microorganisms [4,5]. Besides, TLR3, TLR7, and TLR8 are the TLRs that specifically recognize RNA [6–8].

Except for bases, other parts of nucleic acids are sometimes used for the interaction with other molecules. For example, the nucleosome, in which genomic DNA is stored, consists of DNA and histone proteins [9,10]. The major driving force of this complexation is electrostatic interaction between negatively charged DNA and positively charged histone. The space between base pairs within the double-helical structure of DNA gives chance for the coordination with various molecules, like anticancer agents [11,12]. Many anticancer agents, such as doxorubicin, intercalate into DNA to show their cytotoxic activity. In a word, the interactions of nucleic acids with various molecules are influenced by not only the base sequence, but also the overall structure and electric charge of the molecules.

The nature of nucleic acids to form double-helix can be used to design nucleic acid-based nanostructures with various unique patterns, including loops, junctions, branches and bends. Fig. 2 summarizes the schematic drawings of some nanostructured DNAs with simple shape, some of which have loops, junctions, branches or bends (see below for details). In most cases, DNA, not RNA, has been used for constructing such nanostructures and the technology is generally referred to DNA nanotechnology [13]. Owing to the shape of the nucleic acids, including DNA, is one of the most important factors determining the interaction with other molecules or cells, nanostructured DNAs will have novel and unique features compared with natural single- or double-stranded DNA. Therefore, several nanostructured DNA, in which RNA and nucleic acid derivatives can also be used, have been developed as delivery systems for therapeutic agents as summarized in recent review articles [14,15]. In this review, we first sum up the factors affecting the properties of nanostructured DNA, followed by discussing the methods for the development of nanostructured DNA. Then, we review the characteristics of nanostructured DNA and their applications for the delivery of bioactive compounds.

2. Factors affecting the properties of nanostructured DNA

Prior to the discussion on the characteristics of nanostructured DNA, it is important to summarize the impact of chemical modification on the physicochemical properties of nucleic acids, because chemical modification would affect the properties of DNA as well as nanostructured DNA. As enzymatically unstable property of natural phosphodiester linkage limits further *in vivo* application of DNA and RNA, various chemically modified nucleotides have been developed, some of them have been used in approved nucleic acid drugs. Such modifications could have impact on the interaction of nucleic acids with biological components [16,17], although most nanostructured DNAs are solely made of phosphodiester DNA. Fig. 3 schematically illustrates the factors affecting the properties of nucleic acids.

Except for chemical modification, it is also worth noticing the effects of fluorescent labels or radiolabels. In most experiments using nanostructured DNAs, their interaction with cells and tissue distribution *in vivo* is traced using fluorescently-labeled or radiolabeled DNA. Because the labeling is inevitably associated with chemical modifications, it would affect the properties of nanostructured DNA.

Protein binding, which will affect the blood circulation time as well as cellular uptake of drugs, is also a crucial factor related to the delivery application of nanostructured DNA. Chemically modified nucleotides can have different protein binding properties compared with natural, phosphodiester nucleotides. Besides, electrostatic interaction is one of the most important driving forces to determine the interaction with

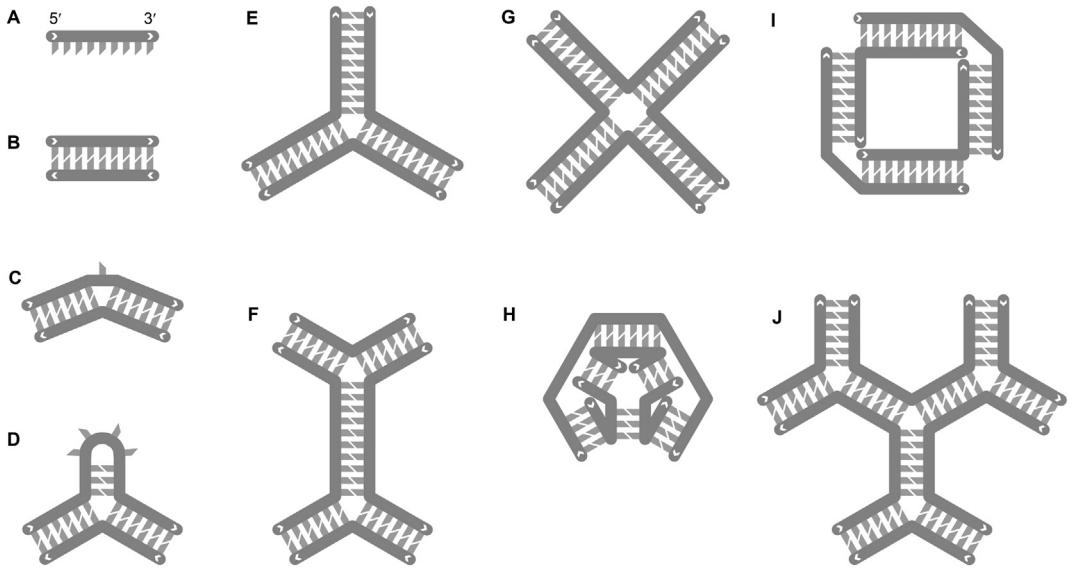

Fig. 2. Schematic drawings of some typical nanostructured DNAs with simple shape. (A) Single-stranded DNA; (B) double-stranded DNA; (C) bend DNA; (D) bulge (or hairpin) DNA; (E) DNA with a three-way junction (tripod-like structured DNA); (F) DNA with two three-way junctions (DNA barcode); (G) DNA with a four-way junction (tetrapod-like structured DNA); (H) tetrahedral DNA; (I) tetragonal DNA; and (J) dendrimer-like DNA (DNA with four three-way junctions). Dark gray bars represent sugars and phosphates, and pale gray rectangles represent nucleobases. The white arrow heads on dark gray bars indicate 5′ to 3′ direction.

various molecules and cells, and the change to the charge of oligonucleotides would greatly alter their interaction with proteins and cells.

2.1. Chemical modification with little change in the electric charge of nucleic acids

Most chemical modifications of nucleic acids and oligonucleotides are intended to increase the resistance against enzymatic degradation. Because the chemically modified oligonucleotides have been generally developed to increase the *in vivo* stability and potency of nucleic acid-based drugs [18–20], their properties are frequently discussed in relation to the therapeutic and/or biological activities.

One of the most primal and frequently used chemical modifications is phosphorothioate linkages of nucleotides, in which a non-bridging oxygen is replaced with sulfur (Fig. 3A) [21–23]. This modification significantly increases the resistance of oligonucleotides against nuclease degradation and greatly increases the affinity of the modified nucleotides to various proteins like serum albumin. Therefore,

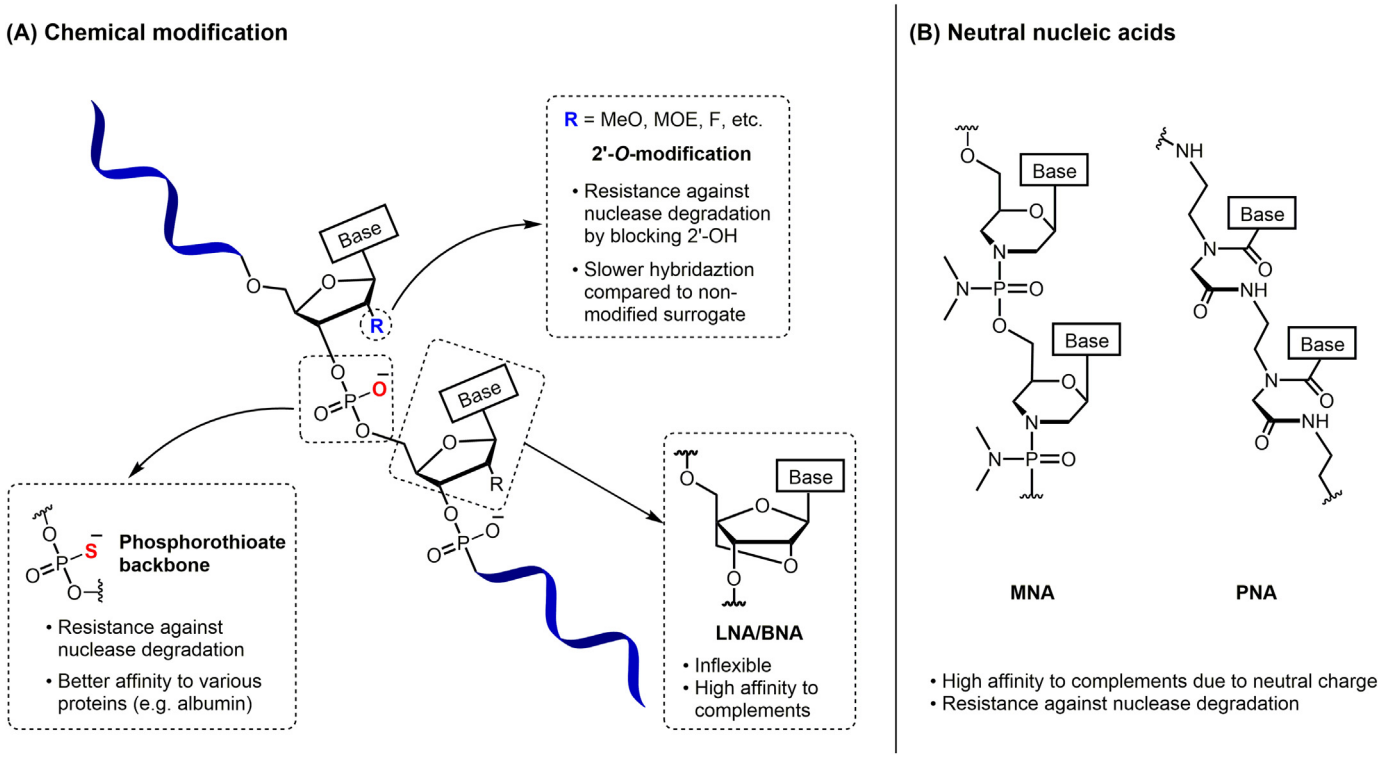

Fig. 3. Factors affecting the properties of nucleic acids. (A) Chemical modifications of the internucleotide linkage or sugar moiety. (B) Neutral nucleic acids. PS, phosphorothioate; OH, hydroxy group; MeO, methoxy group; MOE, methoxyethoxy group; LNA, locked nucleic acid; BNA, bridged nucleic acid; MNA, morpholino nucleic acid; PNA, peptide nucleic acid.

oligonucleotides with phosphorothioate linkages exhibit sustained retention in the systemic circulation after *in vivo* administration because of its high affinity to serum proteins. In addition, the high affinity to proteins also increases the cellular uptake. Fomivirsen is the first antisense drug for the treatment of retinitis cytomegalovirus (CMV) in patients with acquired immune deficiency syndrome (AIDS) [24]. The structure is composed of a 21-mer oligonucleotides with phosphorothioate backbone. Mipomersen, another antisense drug for the treatment of familial hypercholesterolemia, is a 20-mer oligonucleotides with phosphorothioate backbone and has 2′-*O*-(2-methoxyethyl)-modified ends [25]. Nusinersen is also an antisense drug for the treatment of spinal muscular atrophy. It is composed of an 18-mer oligonucleotides with phosphorothioate backbone and contains 2′-*O*-(2-methoxyethyl)-modification on all cytidines [26–28]. These antisense drugs are racemic mixtures, because the individual stereoisomers cannot be synthesized or separated. Recently, Iwamoto et al. have reported a scalable synthetic process that yields therapeutic antisense oligonucleotides having high stereochemical and chemical purity [29]. According to this publication, the phosphorothioate stereochemistry affected various aspects of the oligonucleotides, including protein binding.

Ribose-modified nucleic acids are prepared by the replacement of the 2′-hydroxyl to 2′-*O*-methyl, 2′-*O*-methoxyethyl and 2′-fluoro (Fig. 3A) [30]. The 2′ modification of nucleic acids improves the resistance to nucleases through blocking the nucleophilic 2′-hydroxyl. The thermal melting points of DNA or RNA duplex consisting of 2′-modified nucleic acids are higher than those of nonmodified counterparts [31]. Moreover, 2′-position modified nucleic acids are often used for RNA to improve the stability against RNase H. Therefore, this modification is often used for RNA for improving the stability of RNA. Among these modifications, 2′-fluoro modification shows the highest stability, and 2′-*O*-alkyl modification has the higher stability in smaller substituents than larger ones [32]. On the other hand, the hybridization kinetics of 2′-modified nucleic acids to complementary DNA is slower than that of nonmodified ones [33].

Locked nucleic acid (LNA), which is also known as bridged nucleic acids (BNA), is formed by the linkage of the 2′-oxygen with the 4′-carbon of the ribose through bridging carbons (Fig. 3A) [34,35]. This modification enhances the conformational inflexibility of the sugar moiety in nucleic acids, improving the affinity to complementary nucleic acids. The recognition of complementary nucleic acids by LNA obeys the Watson–Crick base pairing rules. The thermal melting point of LNA and their complementary single-stranded DNA or RNA is generally higher than that of nonmodified DNA or RNA duplex [36]. As for hybridization kinetics, the association rate of DNA-LNA is similar to that of DNA-DNA, whereas its dissociation rate is slower than that of DNA-DNA [37]. Thus, these kinetical characteristics could contribute to the high stability of LNA. LNA can be used as a triplex forming oligonucleotides (TFO), which has high affinity and strong inhibitory effect on double-stranded target gene [38].

2.2. Neutral or positively charged oligonucleotides

Nucleic acids are negatively charged molecules, and some of their interactions are governed by electrostatic forces. Therefore, neutral oligonucleotides, such as morpholino oligonucleotides, and positively charged oligonucleotide have different properties from natural oligonucleotides. The removal of the negative charge from nucleic acids leads to significant increase in the stability of duplex formation through the elimination of charge repulsion [32]. In addition, the 2′-position modification by addition of a positively charged group such as 2′-*O*-aminoalkyl group leads to the neutralization of the charge of nucleic acids, which improves the hybridization efficiency.

Morpholino nucleic acids (MNAs) and peptide nucleic acids (PNAs) are the major nucleic acids with neutral charge (Fig. 3B). MNAs are not substrates for nucleases, which are used for Eteplirsen, an antisense drug for the treatment of Duchenne muscular dystrophy [39,40]. Compared with other oligonucleotide drugs having phosphorothioate backbone, morpholino oligonucleotides bind poorly to serum proteins. PNAs were prepared by replacing the anionic sugar phosphate backbone of nucleic acids to aminoethylglycine units (Fig. 3B) [41]. The advantages of PNA are not only inherent charge neutrality but also the very high biological and chemical stability. On the other hand, PNA is highly hydrophilic and poorly taken up by cells.

2.3. Fluorescent or radiolabels

Some experiments are performed with oligonucleotides labeled with fluorescent probes or radioisotopes. Such labeling would alter the physicochemical properties of oligonucleotides.

Fluorescent labeling is most frequently used for tracing the movements of oligonucleotides especially in *in vitro* experiments [42]. This labeling method can be classified into two categories: covalent and non-covalent labeling methods. The covalent method is a direct labeling to nucleic acids by chemical reaction. The non-covalent method uses molecules that coordinate with oligonucleotides, such as intercalators and major/minor groove binders.

Radioisotope labeling is often used for the determination of the cellular uptake as well as tissue distribution of oligonucleotides, because it is much more suitable for quantitative analysis than fluorescent labeling. The most common radioisotopes used for labeling are technetium-99 m, phosphorus-32, sulfur-35 and fluorine-18.

Various positions of oligonucleotides, including the 5′- and 3′-ends, are also choices for labeling. As labeling is associated with changes in the structural and/or physicochemical properties of oligonucleotides, it could affect their various aspects, such as the interaction with proteins [43].

3. Methods for the development of nanostructured DNA

Nanostructured DNA can be constructed by several approaches. The most common method is DNA nanotechnology, by which any structure can be designed on the basis of Watson-Crick base pairing (Fig. 2). Noncanonical DNA motifs, which include G-quadruplex and i-motif, can also be used as building blocks or connections in nanostructured DNA. Moreover, enzyme-based rolling circle amplification (RCA) is another method to obtain long DNA with repeated sequences, and this method can also be used to construct nanostructured DNA.

3.1. DNA nanotechnology

DNA nanotechnology has been first developed by Professor Nadrian Seeman in the early 1980s [44–46]. The first reported nanostructured DNAs were DNA junctions and DNA lattices. One of the simplest structures is Y-shaped DNA, which has been used as a component of DNA barcode, DNA dendrimer, and DNA hydrogel [47–49]. Later, Rothemund developed a unique method called ‘DNA origami’, which allows short staple DNAs to fold around a long single stranded circular ‘scaffold’ DNA into a predesigned shape [50]. The DNA origami is a technology to create highly organized, complicated two- and three-dimensional structures. Because of the long length of the scaffold DNA, nanostructured DNAs developed by this method are as large as several tens of nanometers in diameter and much larger than those prepared using only short DNAs. In most cases, excess amounts of staple DNAs over the scaffold are used for the preparation, which may cause concerns about purity of the final product.

Irrespective of the methods used, the basis of DNA nanotechnology-based construction of nanostructured DNAs is the formation of double strands, the essential characteristic of nucleic acids. Because of the strictness of the base pairing, as well as the flexibility and sloppiness, the variations of the nanostructured DNAs that can be developed using DNA nanotechnology will depend on our imagination.

3.2. Rolling circle amplification

RCA is a technology to enzymatically amplify DNA with long and tandem-repeating sequence [51]. In general, a single stranded circular DNA is used as template DNA, and the sequence of this DNA is amplified by Phi29 DNA polymerase, which quickly generates polynucleotides and possesses the functions for strand displacement and proofreading under isothermal conditions.

RCA has been used to develop various types of nanostructured DNAs. Hong et al. made use of a single strand circular DNA that self-assembles to a Y-DNA nanostructure with a three-way junction in a core and three hairpin loops in arms as RCA template to enhance the production of Y-shaped DNA nanostructures [52]. Ultimately, the regular RCA production acquired more than 200-fold increase through Phi 29 DNA polymerase amplification and site-specific restriction cleavage processes. Ouyang et al. illustrated an RCA protocol which employed a relatively short periodic sequences than a traditional DNA origami scaffold and only three types of staples to obtain a large assembled DNA nanoribbon structures which possessed different dimensions and patterns compared with single/double strand DNA as a CpG oligonucleotide delivery agent [53,54].

Despite the enormous potential of the RCA method, there are few proper techniques to control the size of RCA products. To resolve this issue, Lee et al. incorporated different sequences into the RCA template and demonstrated that the RCA template with secondary structure-forming sequences could generate products with smaller size [55]. Our group used the RCA method and successfully obtained branched DNAs in large quantity [56]. We custom-designed RCA templates by adjusting the sequences reasonably to obtain a highly efficient amplification for polypod-like structured DNA (polypodna) with sticky ends, and subsequently obtained polypodna-based DNA hydrogel by self-assembling. The intermediate RCA products were microflower-like structures observed by an electron microscope, which was required to be decomposed to obtain polypodna. Thus, this RCA technique can be a new approach to prepare DNA nanostructures in high yield at low cost.

4. Structural properties and cellular uptake of nanostructured DNA

Nanostructured DNAs developed thus far greatly vary in terms of size and shape. Some of them are two-dimensional and others are three-dimensional. Structural properties of nanostructured DNAs at least partly determine their interaction with proteins and cells, so that the properties regulate the tissue and intracellular distribution of the nanostructured DNAs. It is deserved to mention that, when the interaction of nanostructured DNA with cells is studied under serum-free or serum protein-free *in vitro* conditions, which are frequently used conditions to study the interaction of nanostructured DNA with cultured cells, the results may not reflect the events occurring serum-containing *in vivo* conditions.

4.1. Structural properties

Because of the versatile nature of nanostructured DNAs, it is quite difficult to briefly summarize their common structural properties. Therefore, we would like to start with Y-shaped DNA, or tripod-like shape structured DNA (tripodna), one of the simplest nanostructured DNAs that can be designed [47].

Y-shaped DNA or tripodna can be constructed by mixing three ODNs with sequences half complementary to the parts of the other two ODNs. It is a branched DNA and has a three-way junction. Formation of tripodna depends on the properties of ODNs used and the experimental conditions, including the concentrations of ODNs and salts in mixing solutions, and temperature. Under optimal conditions, tripodna can be obtained with very high yield by simple mixing or annealing of the ODNs [57]. Atomic force microscopy (AFM) showed that tripodna is in a Y-shape [58]. We attempted to minimize the structure and found that ODNs with 16-mer or longer are needed to construct tripodna under physiological salt and pH conditions (unpublished data). Tripodna migrates in gel electrophoresis much slower than a linear double stranded DNA with the same numbers of nucleotides, because of its branched structure. Luo's group examined the tertial structure of a Y-shaped DNA by using fluorescence resonance energy transfer (FRET) [59]. They found that the Y-shaped DNA was not flat but in a slightly bent structure.

Similar design to tripodna with more ODNs results in the formation of more complicated, more branched nanostructured DNAs. Our group developed polypod-like structured DNA, or polypodna, with three to eight pods: tripodna, tetrapodna, pentapodna, hexapodna and octapodna [58]. Dodecapodna was not obtained under the examined conditions, although another report showed that a 12 branched nanostructured DNA was formed under different conditions [60]. The polypodnas have some common structural properties. First, the migration distance of these DNAs is shorter than that of linear DNA. It was found that the migration distance of these branched DNAs was mainly determined by the total number of nucleotides in one unit [58].

Another important aspect that could be important for the design of nanostructured DNAs is how the ODNs bend to form nanostructured DNAs. We examined this subject by analyzing the bending directions of the ODNs in polypodna when the tertial structure is flattened [61]. Pentapodnas, branched DNAs with five pods, were mainly used and immobilized to a DNA origami-made frame. When two out of the five pods of a tetrapodna were used for the immobilization to the frame, the remaining three pods are in random or orderly patterns. AFM imaging showed that the ODNs in the pentapodna adopted bend-type conformation, not cross-type one, and more than 90% of the pentapodna were in the bend-type conformation. Similar results were obtained with tetrapodna and hexapodna. These data suggest that the ODNs sharply bend at the center to form these nanostructured DNAs.

A clear and important difference observed among these DNAs is the hybridization efficiency. Small-angle X-ray scattering (SAXS) is suitable to obtain polymer chain conformations, micellar structures, and proteins. Our study using SAXS analysis showed that all nucleotides of tripodna and tetrapodna formed base pairs [62]. On the other hand, some nucleotides in the central parts of pentapodna and hexapodna did not form base pairs. This difference would occur because of an increase in steric hindrance as well as electrical repulsion with increasing pod numbers. SAXS analysis also revealed that tetrapodna with some noncomplementary sequences had a larger radius of gyration than one with completely complementary sequences [63].

4.2. Cellular uptake

Cellular uptake is the key process for cell-specific delivery of therapeutic agents using any delivery systems. There are several receptors that recognize nucleic acids, but TLRs, whose characteristics have been extensively studied, are hardly on cell surface, so that these receptors are not involved in the cellular uptake of DNA in any form. Fig. 4 simply compares the cellular uptake mechanisms of phosphodiester or phosphorothioate single-stranded DNA and nanostructured DNA.

Several cell surface receptors specific for DNA or nucleic acids have been reported, including macrophage scavenger receptor-1 (MSR1, SR-A, and CD204) [64], $\alpha_M\beta_2$ (MAC-1) [65], receptor for advanced glycation endproducts (RAGE) [66,67], membrane-associated nucleic acid-binding protein (MNAB) [68], mannose receptor-1 (MRC1) [69], and lymphocyte antigen 75 (DEC-205) [70] (Table 1). It is worth noticing that the ligands used for the determination of these receptors varied among the studies; which include plasmid DNA, a very long, double stranded DNA, and phosphorothioate ODN. As described above, they would have different properties in the interaction with these DNA receptors.

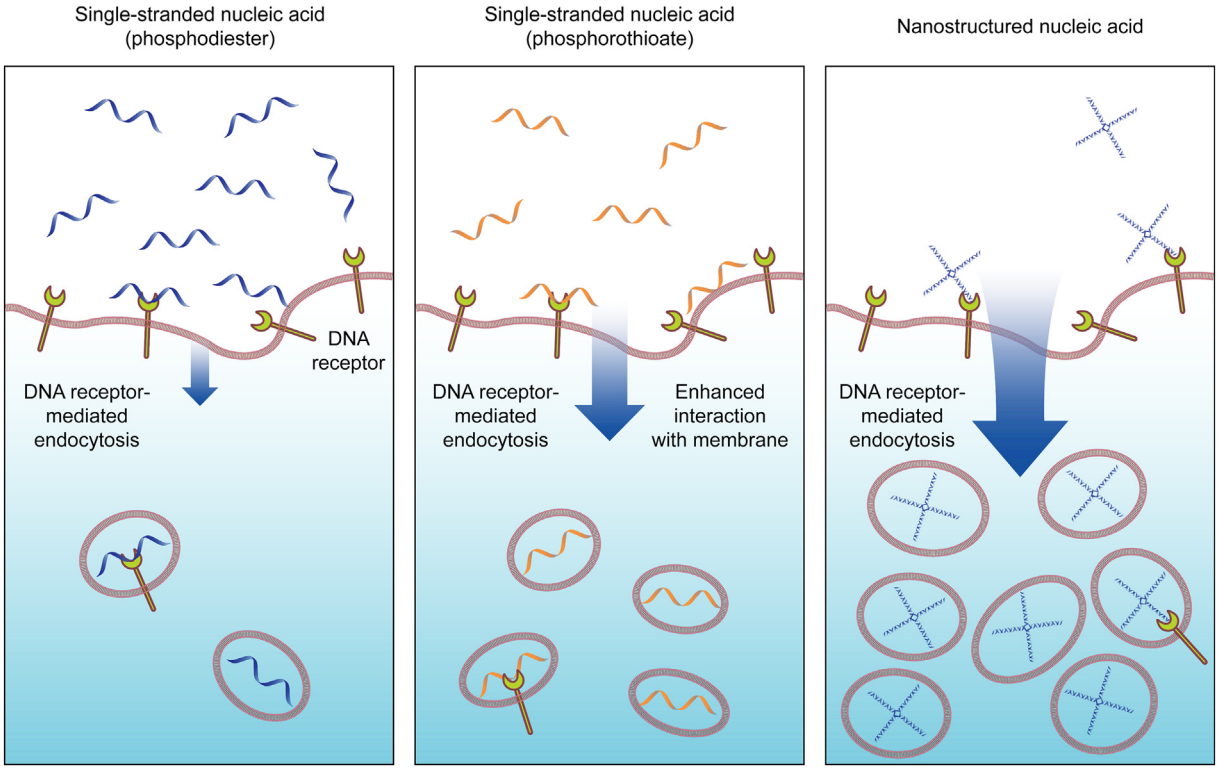

Fig. 4. Proposed cellular uptake mechanisms of phosphodiester or phosphorothioate single-stranded DNA and nanostructured DNA. Natural phosphodiester single-stranded DNA hardly interact with cell membrane, while phosphorothioate one nonspecifically binds to the cell membrane. Nanostructured DNA can efficiently bind to DNA receptors compared to single-stranded DNA.

Scavenger receptors recognize polyanions and the typical ligand is acetylated low-density lipoprotein and polyI:C [71,72]. Among them, MSR1 is expressed on various types of macrophages. Kimura et al. reported that 30-mer single-stranded ODN was taken up by MSR1 [64]. Our group developed an MSR1-expressing cell line using commercially available HEK-Blue hTLR9 cells [73]. HEK-Blue hTLR9/hMSR1 cells efficiently took up CpG2006, which is a phosphorothioate, human-type CpG ODN [74], a single-stranded phosphodiester CpG DNA (ssCpG), and a tetrapod-like structured DNA (tetrapodna) containing ssCpG (tetraCpG). Flow cytometry showed that the uptake of Alexa Fluor 488-labeled DNA samples was significantly higher in HEK-Blue hTLR9/hMSR1 cells than in mock-transfected HEK-Blue hTLR9 cells. In contrast, Alexa Fluor 488-phosphorothiaote CpG2006 was efficiently taken up by the both types of cells. These results suggest that the MSR1 is involved in the uptake of both single stranded DNA and nanostructured DNA.

The detailed mechanism of the interaction of nanostructured DNA with cells needs further investigations, but there have been experimental reports showing that nanostructured DNAs are more efficiently taken up by cells than single- or double-stranded DNA. Polypodnas were efficiently taken up by several types of immune cells, including human cells, depending on the structural complexity [58,75–77]. Other types of nanostructured DNAs were also efficiently taken up by mouse macrophage-like RAW264.7 cells [78]. Our recent study showed that the efficient uptake of nanostructured DNA is relatively specific to immune cells [79]. Interaction of nanostructured DNAs with MSR1 and other DNA receptors can be more efficient than that of single- or double-stranded DNA because of their branched, complicated structures. Then, this would lead to efficient uptake of nanostructured DNAs by these receptor-positive cells. Therefore, these results support the idea that nanostructured DNAs can be used as delivery vehicles to immune cells for therapeutic agents.

5. Delivery of therapeutic agents using nanostructured DNA

The unique properties of nanostructured DNAs can be useful for the delivery of a variety of therapeutic agents. Because DNA as well as RNA is a bioactive compound, nanostructured DNAs have been frequently used for the delivery of such bioactive nucleic acids. A most typical bioactive nucleic acid is DNA containing unmethylated CpG dinucleotide or CpG motif. The DNA, so called CpG DNA, is a ligand for TLR9 expressed on mammalian immune cells, such as dendritic cells, so the delivery of CpG DNA to TLR9-positive cells is useful to efficiently stimulate the innate immunity. As described in the above section, nanostructured

Table 1
Cell-surface receptors that are involved in the cellular uptake of DNA.

DNA receptor	Description	Ligand	Cell	Reference
Msr-1	Macrophage scavenger receptor 1	PO DNA (30-mer)	Macrophages, dendritic cells	[64]
MAC-1	Macrophage antigen-1	PS DNA (15-28mer)	Polymorphonuclear leukocytes, macrophages, natural killer cells	[65]
RAGE	Receptor for advanced glycation endproducts	PS/PO CpG DNA (20-24mer)	Macrophages, endothelial cells	[66,67]
MNAB	Membrane-associated nucleic acid-binding protein	Calf thymus DNA	Macrophages, B cells	[68]
MRC1	Mannose receptor, C type 1	PS CpG DNA (20-22mer)	Macrophages, dendritic cells	[69]
DEC-205	Lymphocyte antigen 75	PS CpG DNA (20-24mer)	Thymic epithelial cells, dendritic cells	[70]

PO, phosphodiester; PS, phosphorothioate.

DNAs are efficiently delivered to immune cells compared with single- or double-stranded DNA, they are good carrier systems for CpG DNA and other immunomodulating nucleic acids. We demonstrated that immunosuppressive ODNs can also be efficiently delivered to TLR9-positive immune cells, and inhibit CpG DNA-mediated immune stimulation [80,81].

Chemically modified bioactive oligonucleotides can also be delivered using nanostructured DNA. One of the most frequently used chemically modified oligonucleotides are ones with phosphorothioate backbone. Oligonucleotides with phosphorothioate backbone were incorporated into nanostructured DNAs [81–83]. Another example is morpholino oligonucleotide, which was efficiently delivered to the liver after intravenous injection into mice [84]. Attention should be paid to the thermal stability of chemically modified oligonucleotides, because tripodna was not formed when three ODNs with phosphorothioate backbone were used [85].

Another class of therapeutic agents that have been delivered using nanostructured DNAs is intercalating agents. Doxorubicin is such an intercalating agent with antitumor activity. Doxobubicin has been incorporated into nanostructured DNA and site-specific and/or sustained delivery of the agent has been reported [86].

In addition to these agents, many different types of therapeutic agents have been delivered using nanostructured DNAs (reviewed in 14,15). Most of these studies, however, were performed with cultured cells and, therefore, it deserves to be mentioned that the *in vivo* stability of these nanostructured DNAs and to their interaction with proteins and cells should be considered.

6. Conclusions and future perspectives

Delivery of therapeutic agents using delivery systems to specific targets depends on the interaction of system with the components of the body [87–89]. As reviewed here, the unique structural properties of nanostructured DNAs can be used for such purpose. Recent advances on DNA nanotechnology have enabled to develop nanostructured DNAs with highly sophisticated structure and functions, so their application to the delivery of therapeutic agents will become more popular in future. Despite detailed information about the interaction of nanostructured DNA with cultured cells, their *in vivo* fate after administration into the body is still greatly limited. Therefore, further progress will largely depend on the understanding of the *in vivo* fate of nanostructured DNA and probably on their improvement in terms of biological stability.

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