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A Molecular Hero Suit for In Vitro and In Vivo DNA Nanostructures

Megan E. Kizer, Robert J. Linhardt, Arun Richard Chandrasekaran,* and Xing Wang**

Precise control of DNA base pairing has rapidly developed into a field full of diverse nanoscale structures and devices that are capable of automation, performing molecular analyses, mimicking enzymatic cascades, biosensing, and delivering drugs. This DNA-based platform has shown the potential of offering novel therapeutics and biomolecular analysis but will ultimately require clever modification to enrich or achieve the needed "properties" and make it whole. These modifications total what are categorized as the molecular hero suit of DNA nanotechnology. Like a hero, DNA nanostructures have the ability to put on a suit equipped with honing mechanisms, molecular flares, encapsulated cargoes, a protective body armor, and an evasive stealth mode.

1. Introduction

We know of many comic book characters who build themselves a suit with different functionalities, and become superheroes. We introduce the molecular hero here, whose suit is much more than just a body armor. It has a built-in operating system, a global positioning system, oxygen supply, and even missile launchers. What the suit provides our hero is sustenance. Now think of DNA; its uses have been re-imagined in the past three decades—from being a building block to construct nanoscale objects/devices, to being useful in applications ranging from biosensing, biomolecular analysis, molecular computation, and drug delivery.[1] For DNA to be used in vivo, it must be multifunctional; that is, it needs a hero suit (**Figure 1**).

Scientists in the field have, in many earlier reviews, described the features of DNA and why it is advantageous to use DNA as a material.[2–7] The origin story begins with Seeman who proposed turning canonical linear-shaped DNA into designer molecular scaffolds (3D lattices) to organize other macromolecules, mainly proteins, in a long-range ordering fashion for solving their crystal structures.[8] Seeman started the "game" by building a synthetic, branched DNA junction that is stable and would serve as the minimal component unit of DNA-based construction.[9–11] That was the 1980s. Fast forward 35 years and we now have DNA objects (e.g., prisms) that are built from just one or two strands,[12,13] a range of hollow or solid objects varying in size from tens to hundreds of nanometers using the DNA origami strategy,[14–17] and multi-crossover structures that are not just planar, but even have designed curvatures[18] and free-form designs.[19] Distinct from its four-stranded junction origin, scientists have expanded their approach to include algorithms for construction,[20] specific design parameters for component motifs (double crossover (DX),[21] triple crossover (TX),[22] and paranemic crossover (PX)[23] motifs), single-stranded DNA and RNA origami[24] and micrometer scale assemblies from single-stranded tiles using a strategy called DNA bricks.[25,26]

DNA nanostructures have found applications in many fields. Periodic arrays assembled from individual DNA motifs provide control over spatial positioning of functional molecules.[27,28] One of their main uses is to create enzyme cascades,[29–31] in which the reactivity of enzymes of interest was found to be higher when confined within nanostructures compared to those in free solution. Another important application is in biosensing,[32] where DNA nanostructures are designed to undergo stimuli-specific conformational changes and, therefore, provide specific outputs for target biomolecules present in a sample.[33–35] Additionally, DNA origami structures[14] can be designed in many desired 2D/3D shapes. Origami structures have been employed as molecular pegboards in analyzing biomolecular interactions such as nucleosome unwrapping,[36] base-excision repair,[37] RNA kissing complex interaction[38] and G-quadruplex formation.[39] DNA nanostructures are also used in molecular computation, for instances, in computing square roots,[40] multiplying numbers,[41] serving as memory devices,[42,43] or providing logic gated outputs.[44] On the frontier of biology and medicine, the most important and rapidly developing application of DNA nanostructures is in drug delivery.[45,46] In this review, we aim to highlight these prominent applications and the necessary requirements for success in translating these technologies to a wider array of labs as well as the clinical setting.

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2. DNA Nanostructures for Drug Delivery

The aspects of DNA nanostructures in drug delivery can be viewed and emphasized both in a structural and in a functional context. In the previous section, we briefly discussed the structural properties and advantages of DNA for serving as a material to construct different geometries, all of which contribute to its application in drug delivery as a carrier or vehicle. Specifically, the structural properties of DNA nanostructures in drug delivery stem from their underlying chemical properties. First, DNA offers predictive self-assembly through one of the most loyal types of molecular recognition, Watson–Crick base pairing. Thus, one can design specific DNA sequences to construct solid or hollow objects, with exceptional control over dictating the size and geometry of DNA nanostructures. Second, DNA can be chemically modified with other molecules to offer multiple functionalities such as targeting ligands, imaging agents, and stabilizing moieties useful for being directed to in vivo destinations. Third, DNA nanostructures are biocompatible and relatively stable (especially, after they are bound to protection polymers, either covalently[47] or noncovalently),[48,49] under physiological conditions allowing the control of life-time, circulation-time, and drug release rate after administration. DNA nanostructures have all the required characteristics of an effective drug carrier: good safety profile, ease of cargo loading, target specificity, high cellular uptake, intracellular biostability, triggered release of the cargo, and allowance for the introduction of additional functionalities (**Figure 2**). In a functional context, some of the main criteria that DNA nanostructures satisfy as an efficient carrier are targeted delivery, triggered release of the cargo, additional functionalities such as cellular tracking, and most importantly, stability and compatibility with the physiological system.

The design properties of DNA nanostructures allow for encapsulation of a variety of cargoes. For example, small molecules[50–56] and metal complexes[57] intercalated in DNA nanostructures are useful in cancer therapy. Cytosine–phosphate–guanine (CpG) sequences are delivered through nanocarriers to enhance immune response.[58–61] Similarly, antisense[62] and small interfering RNA (siRNA)[63,64] carried by DNA platforms are useful in specific gene knockdown or knockout. DNA nanostructures serve as new platforms for vaccine construction by allowing the assembly of both antigen (e.g., streptavidin) and adjuvant (e.g., CpG oligonucleotides) in the same complex.[65] Photosensitizers are also delivered using DNA nanostructures since they lack efficiency due to poor solubility in aqueous environment and have a tendency to aggregate. Binding to DNA origami reduces aggregation of photosensitizers, leading to enhanced therapeutic efficacy when used in photodynamic therapy.[66,67] Metal nanoparticles and nanorods can be fabricated on DNA nanostructures and delivered to tumor sites for thermal ablation.[68,69] For detailed descriptions of different cargoes hosted by DNA nanostructures and different encapsulation strategies, we refer the readers to other recent and focused in-depth reviews.[45,46,70–72]

2.1. Targeting Strategies

For DNA nanostructures to be used in drug delivery, a major required functionality is the ability to target these carriers to specific locations in the body. Recognition elements that can target cell surface receptors include small molecules,[73] peptides,[74] aptamers,[75] antibodies,[76] and proteins.[77–79] These "add-ons" act as homing mechanisms and direct the nanocarriers to cell receptors on the target cells. An often-used DNA nanostructure is a wireframe DNA tetrahedron.[80] This structure can be modified to contain cetuximab antibodies that recognize epidermal growth factor receptors, often overexpressed on the

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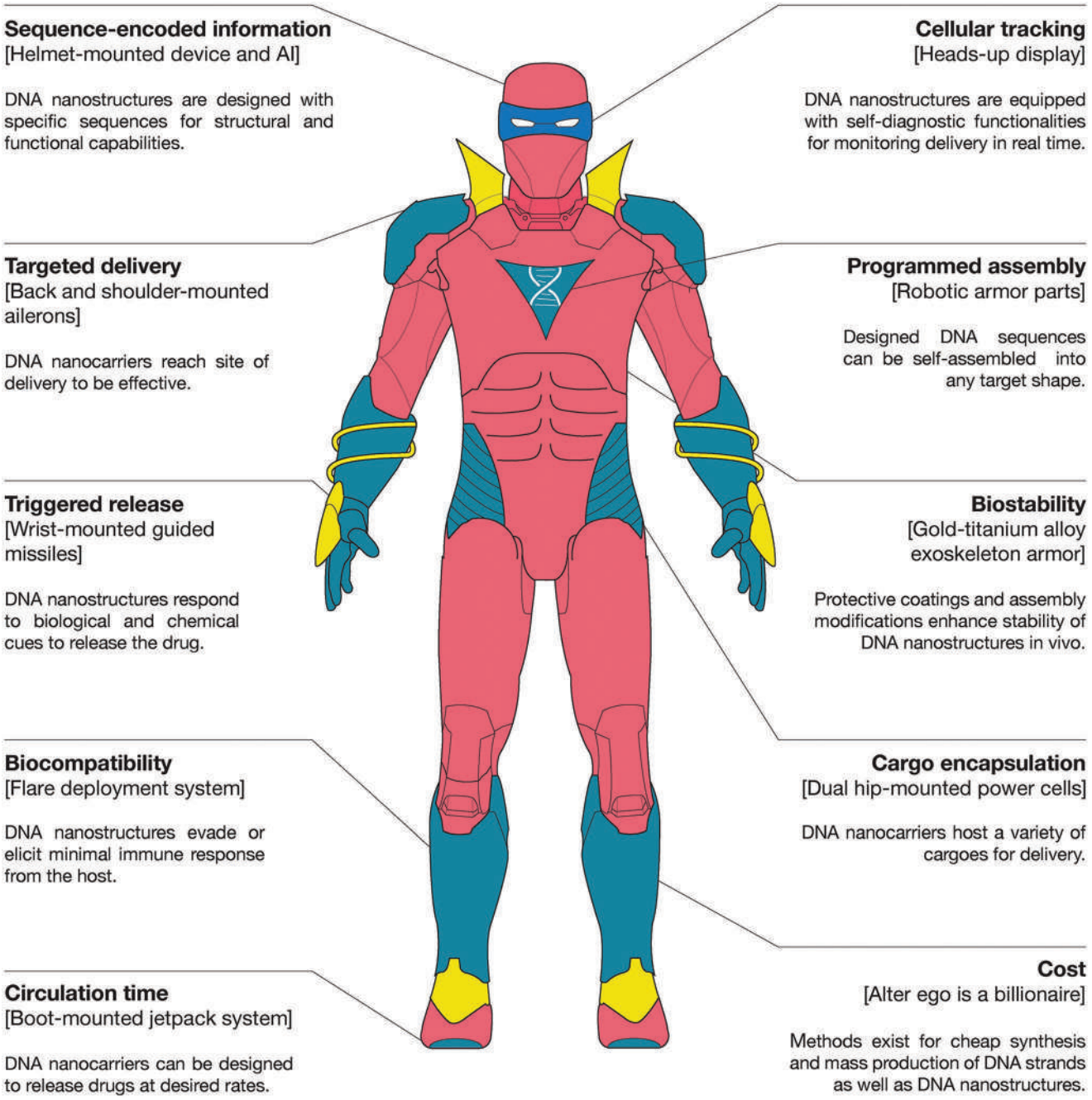

Figure 1. The molecular hero suit for DNA nanostructures. For use as drug delivery vehicles, scientists have functionalized DNA nanostructures with a variety of features, the collection of which comprise the suit for DNA nanostructures. We compare the different attributes of the molecular suit with modifications of DNA nanostructures that aid in translating lab-based research into a clinical setting.

surface of several types of cancerous cells.[76] The same structure, when functionalized with a tumor-penetrating peptide (specific to transmembrane glycoprotein neuropilin-1), showed enhanced uptake in glioblastoma cells.[81] In another example, planar 2D rectangular DNA origami sheets were modified to contain the protein transferrin, which helped in increasing intracellular uptake by 22-fold in tumor cells, when compared to unmodified nanostructures.[82] Depending on the type of target cell and the payload, different DNA nanostructures have been developed. A majority of such structures use multiple single-stranded DNA (ssDNA) as building blocks (hundreds in the case of DNA origami) to construct specific shapes. Moreover, drug loading capacity of these structures is of prime importance. Considering these factors, Zhu et al. developed DNA nanotrains, a long linear DNA nanostructure assembled from two short DNA strands via hybridization chain reaction.[83] This structure was coupled with a sgc8 aptamer that can bind to human protein tyrosine kinase 7 (PTK7), overexpressed on target CEM

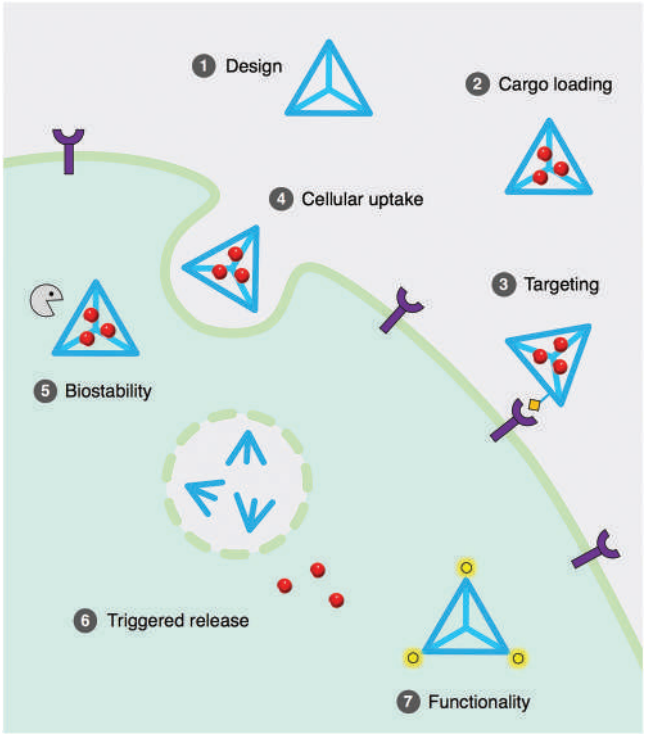

Figure 2. Overview of drug delivery pathway using DNA nanostructures. Designed nanostructures are loaded with cargoes and targeted to specific sites where they are taken up by cells. The design and geometry of the carriers are made to be stable in biological conditions and once triggered, the carriers disassemble or degrade to release the cargo. Nanostructures are also designed to perform other functions such as in vivo imaging.

cells (human T-cell acute lymphocytic leukemia). This selective binding ability of sgc8 aptamer routes the DNA nanotrains to specifically to their target cells and not other types of cells. DNA nanoflowers[84] are another example of minimal DNA nanostructures that are assembled from long DNA strands generated using rolling circle replication (RCR). These nanoflowers do not rely on Watson–Crick base pairing and are instead created by dense packaging of DNA strands, eliminating the need for complicated DNA sequence design. In addition, these structures are highly resistant to nuclease degradation or denaturation, and when functionalized with specific aptamers (sgc8), deliver doxorubicin specifically to tumor cells and induce target-specific cytotoxicity. Another example is a tubular DNA nanorobot, created by folding a planar DNA origami sheet into a tubular structure. Nucleolin-specific aptamers functionalized on a nanorobot help in targeting the nanostructure to tumor sites, as well as molecular triggering to open the DNA construct to release encapsulated thrombin and activate coagulation.[85] In other instances, DNA nanostructures are modified with folate/folic acid to target the carriers specifically to tumor cells that usually overexpress folate receptors.[63,86]

2.2. Triggered Release

Drug carriers that contain cargoes within should be able to hold the cargo until they reach their destination, so as to be effective in delivery and minimizing the required dosage of the drug. Once at the target destination, the nanocarriers need to be triggered open so they can release the drug molecules and cause the intended effect. For DNA nanostructures to be triggered open, their configurations can be designed to respond to a variety of chemical and biological cues. We discuss representative examples of some of these stimuli here including i) nucleic acids, ii) temperature, iii) pH, iv) light, v) aptamer reconfiguration, and vi) enzyme activity.

The porosity of DNA nanostructures can be altered using specific nucleic acids that bind to component strands of the structure.[87,88] In a similar fashion, pore size of hairpin-containing DNA polyhedra can be controlled by heat; the hairpin stem opens at a higher temperature causing the encapsulated drug to escape.[89] pH changes can also be used as a stimulus by incorporating pH-sensitive elements in the nanostructures. For example, DNA boxes with lids containing i-motif interactions can be closed or opened using pH changes.[90] Another pH-sensitive element is triple helical DNA.[91] Assembly of 3-point-star motifs into a DNA tetrahedron is stabilized by triplex formation at pH 5; the interactions become weaker at pH 8 when the triplex forming strand dissociates, thus breaking open the tetrahedron.[92] Cargo molecules attached to DNA nanostructures through photocleavable crosslinkers can be released by exposure to light of specific wavelengths.[93] In some cases, hollow DNA nanostructures (e.g., hexagonal barrel-shaped DNA origami) are locked by binding an aptamer to its complementary strand. When the structures reach their target site, the aptamers recognize specific cell surface proteins and reconfigure on binding to their target, thus, opening the structure to expose or release the payload.[94] Similar aptamer-containing structures also respond to specific ligands, such as ATP, that cause conformational changes to the drug carrier.[95] In another example, connective regions of DNA icosahedra are designed to include telomerase primer and telomeric repeats. Telomerase activity on these carriers results in primer elongation and destabilization of sticky ends, causing dissociation of the DNA icosahedron and release of the encapsulated platinum nanoparticles.[96]

2.3. Functionalization

Another aspect of DNA nanostructures is the additional functionality they provide.[97] A drug-containing DNA nanostructure can be further modified with fluorescent dyes to track the cellular uptake of nanostructures.[98] For example, DNA nanoflowers decorated with fluorophores for multicolor fluorescence resonance energy transfer imaging aids in cellular imaging and traceable targeted drug delivery.[99] Such functional molecules conjugated to DNA nanostructures are also used to signal the deployment of the cargo.[100,101] These functional groups, in combination with techniques such as single particle tracking[102] and fluorescence microscopy,[103] allow investigation of cell entry pathways of nanostructures. In addition to fluorophores, DNA nanostructures are functionalized with accessories that can aid cellular uptake, enhance tumor destruction, or elicit a higher immune response in case of vaccines. For example, a DNA tetrahedron containing CpG oligonucleotides was functionalized

with streptavidin through biotin modification of component strands.[65] This assembly acted as an antigen–adjuvant complex, causing a strong immune response in vivo by induction of streptavidin-specific memory B cells. Other moieties such as gold nanoparticles or nanorods are attached to DNA nanostructures through single-stranded extensions that are complementary to strands on the nanoparticles.[68,69] Such DNA origami nanostructures with gold nanorods are used in photothermal therapy by thermal ablation of cancer cells.

2.4. Developing Drug Delivery Systems

As discussed above, DNA nanostructures for drug delivery have been widely characterized as specific, potent, and versatile agents, but the design of these vehicles is not trivial. Targeted drug delivery is highly dependent on the cell type or tumor being targeted. An optimal targeting strategy relies on the most prominent and selective receptor that is present in the desired cell. DNA modification is not a limiting factor, as ligands utilized in targeting strategies are easily conjugated to DNA,[73–79] but the repertoire may be expanded through new chemistries. Beyond targeting ligand optimization, the DNA nanostructure's shape and design are of great importance, and are also dependent on the ultimate application. For triggered release, a structure with hinges and cavities is desirable, while payloads that intercalate DNA could benefit from a more densely packed structures (e.g., origami and rolling circle amplification (RCA) nanostructures) for efficient drug loading and less densely packed structures (e.g., wireframe) for efficient drug release. These parameters must also be balanced with desired uptake properties (discussed further in Section 3) where shape seems to play a prominent role in uptake efficiency and therefore drug delivery. Finally, additional functionalities should be considered during drug delivery, and thoughtful consideration of whether single modifications may act in a dual manner (e.g., targeting and tracking), and/or how additional functionalization may interfere with the assembly of the nanocarrier and its drug delivery activity. For example, gold nanoparticles may be a desirable tracking agent, but due to their large size, they may interfere with accessibility of any targeting ligands. We therefore urge new users of DNA platforms to perform a careful review of targeting strategies and uptake profiles for their application, and established DNA nanotechnology researchers to develop generalized design principles (discussed further in Section 5) for these targeted drug delivery systems.

3. Cellular Delivery of DNA Nanostructures

With the plethora of biotechnological and therapeutic applications of DNA nanostructures mentioned in the previous sections, it is imperative that the DNA nanocarrier and their associated cargo are able to be effectively and efficiently delivered into target cells. Established therapeutics such as plasmids, antisense RNA, and gene delivery have traditionally relied on co-carriers including cationic polymers,[104] modified viruses,[105] liposome-based delivery agents,[106] and electroporation.[107] Since DNA nanostructures are made of the same material as plasmids or antisense RNA, similar delivery agents and methods have been investigated and elaborated for DNA nanostructures. Though the delivery of DNA nanostructures is more complex and lacks defined rules, it is important to understand the cellular mechanisms by which internalization occurs and the nanostructure properties that influence uptake efficiency in order to better design the molecular suit components to facilitate this process.

Endocytosis is the primary method of internalization of DNA nanostructures, but the mediation of this endocytosis varies; different cell surface receptors can play roles in promoting the initiation of endocytosis, and some may be targeted by specific ligands on DNA nanostructures.[71] An icosahedral DNA–cargo complex was utilized to identify that the anionic ligand binding receptor (ALBR) in coelomocytes of *Caenorhabditis elegans* is the primary means of uptake and that internalization proceeds through endosomal maturation.[98] The organism, cell type, and nanostructure's size and shape have a large influence on the mechanism and efficiency of uptake.[108,109] Different cell types inherently have different capabilities to endocytose DNA nanostructures: dendritic and immune cells rapidly endocytose foreign material, while endothelial cells typically represent biological barriers. Given the number of variables at play, it is difficult to make absolute correlations and predictions on how well a nanostructure will be internalized by a certain cell type. Recently, Bastings et al. performed a comprehensive investigation on the influence of size and shape of DNA nanostructures on delivery to different cell types.[110] They looked at various origami structures which have the same mass but different shapes and densities. They determined that different cells respond differently to size variations based on their ability to engage with multiple surface receptors that compact shapes (50–80 nm) with low aspect ratio are more efficiently internalized, and that solid DNA nanostructures are preferred over hollow or wire frame structures. This is largely in part due to their distinct folded 3D shape and inherent composition of many nicks and crossover junctions. The kinetics of DNA nanostructure uptake are cell-type dependent, but the mechanism of cell uptake via endocytosis is maintained; immune cells show the highest rate of internalization, which is not surprising as they are specialized for endocytosis of foreign material. With the knowledge of cell internalization mechanism, and utilization of cell specific receptors, there is a large research effort into the in vitro delivery of DNA nanostructures to cells by exploitation of endocytosis mediators as key players to enhance delivery. Additionally, methods that disrupt the lipid membrane have been investigated to bypass such endocytosis mechanisms. As such, the nanostructure delivery effort can be broken into two main methods: endocytosis and transfection.

3.1. Delivery Through Endocytosis

Delivery through endocytosis can be further divided into innate receptor–mediated endocytosis and targeted receptor–mediated endocytosis. In the innate receptor–mediated category, DNA nanostructures are simply incubated with the cells, relying on the cell's previously described natural endocytosis mechanisms to uptake nanostructures (**Figure 3**a). This is effective but has

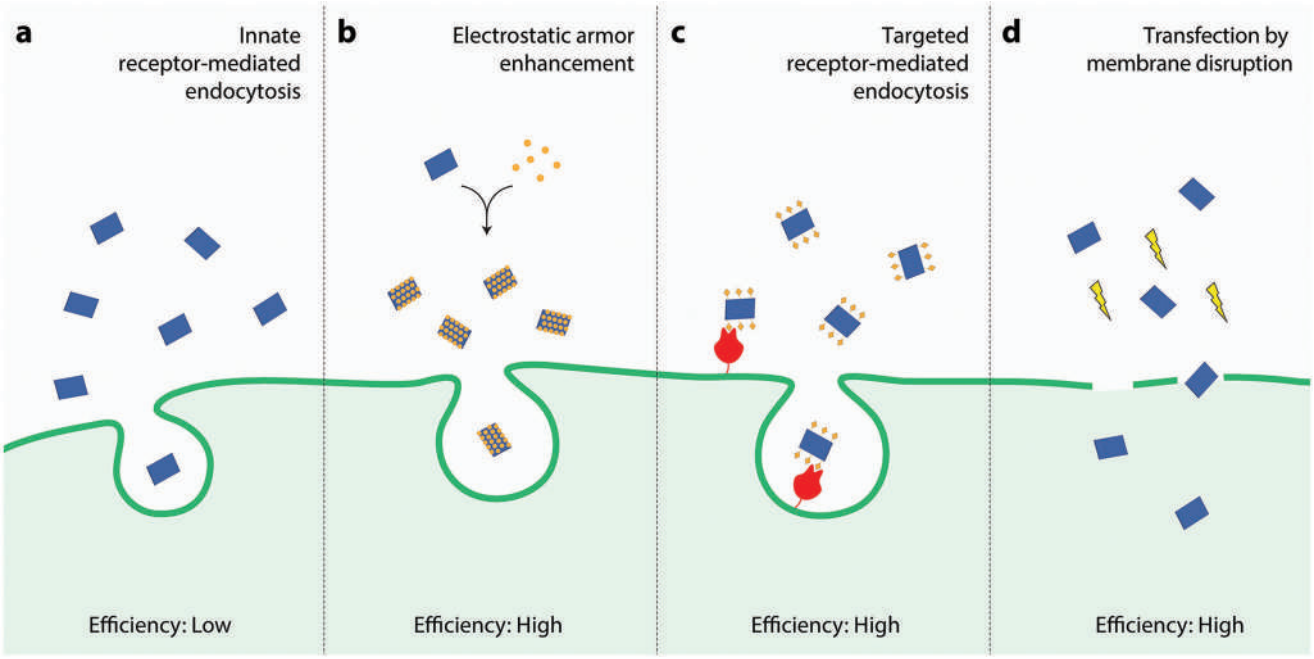

Figure 3. Endocytosis and transfection-based nanostructure internalization mechanisms. a) Uptake of representative DNA nanostructure through innate receptor-mediated endocytosis. b) Electrostatic coating enhancement of cellular internalization during innate endocytosis. c) Targeted receptor-mediated enhancement of cellular internalization. d) Transfection-based delivery of DNA nanostructures, relying on disruption of the cell membrane.

severe limitations; it is nonspecific for cell type, and the DNA nanostructures are left susceptible to denaturation and degradation, and although probably the easiest method, it is the most time consuming. Still, it is the most common means by which DNA nanostructures are delivered to cells in vitro and can be seen utilized in various studies.[111,112] As the cell membrane is negatively charged, in this innate uptake, DNA nanostructures are likely taken up via nonspecific interaction with cationic cell surface receptors. The greater those interactions, the more readily DNA nanostructures can be internalized. For example, spherically templated DNA oligomers exhibit a higher internalization efficiency to cells, presumably due to the dense availability of oligos that can multivalently interact with cell surface receptors.[113] Additionally, nanotubes generated by RCA and hybridization with triangular rungs showed enhanced uptake in HeLa cells, also likely due to greater number of interactions.[114]

One way to enhance delivery during innate receptor–mediated endocytosis is by adding additional species to the DNA nanostructures, acting as the DNA's body armor to promote entry past the cell membrane barrier. This generally includes electrostatic interactions. While the cell membrane and DNA are both negatively charged, it is intuitive that electrostatic interactions with cationic species would shield these repulsive charges and increase the efficiency of uptake by innate endocytosis. Interactions with cationic proteins and polymers can fully coat the DNA nanostructure, shielding the negative charges and promoting entry into cells (Figure 3b). DNA origami can be coated with viral capsid proteins (CP) before incubation with HEK 293 cells to provide a 13-fold increase in internalization efficiency compared to origami alone and DNA origami, lipofectamine (a common transfection reagent).[115] Efficiency of the CP-coated nanostructure was superior to other coatings, such as CP without a positively charged N-terminal region, avidin (a positively charged reference, which only aggregated and precipitated the origami), and bovine serum albumin (BSA, a negatively charged protein, which does not bind the nanostructure at all). While serum albumin proteins alone are unable to bind DNA nanostructures, dendritic alkyl chains are able to efficiently mediate the interaction. In one example, a 60 helix bundle (60-HB) was coated with a BSA–dendrimer complex.[48] This association allowed for a 2.5-fold increase in uptake efficiency compared to the bare 60-HB. In another example, component strands of a DNA cage were first covalently modified with dendritic alkyl chains, which then interacted with human serum albumin (HSA), promoting uptake by shielding negative charges and directly interacting with cell surface receptor GP60.[116] Polymers are another attractive method of coating DNA nanostructures. Specifically, an oligo-lysine (K_{10})–PEG_{5K} copolymer was found to optimally coat various origami structures without deformation.[117] Preferential uptake of the coated DNA nanostructures upon incubation with bone-marrow-derived dendritic cells was observed in comparison to bare nanostructures. Whether protein or polymer, it is also likely that these coatings confer additional protection against denaturation and nuclease degradation, which will be discussed in a later section.

Covalent modifications of DNA nanostructures can be advantageous for targeted receptor-mediated endocytosis. As we saw in the preceding section, this is also commonly used for targeting of DNA-based drug delivery systems to cells of interest. Covalent modification of DNA nanostructures is relatively easy and can be achieved during or post synthesis on component oligonucleotides. Modifications are typically made so that substrates or sequences in the nanostructure interact with cell surface receptors that facilitate internalization (Figure 3c). Folate

modification promotes endocytosis into cancerous cells where folate receptors are overexpressed. DNA nanotubes produced by origami[118] and tile-based assembly[119] methods that contained folate and fluorescently modified component strands recognized folate receptors on the cell surface, followed by internalization upon binding. This resulted in a tenfold uptake enhancement compared to unmodified origami-based nanotubes. Similarly, folate-modified tetrahedral nanostructures designed to deliver siRNA to HeLa cells showed higher uptake efficiency compared to unmodified tetrahedra or tetrahedra modified with other cancer-targeting ligands.[63] Biotin modification has also been utilized to increase cellular internalization of Ru polypyridyl complex–loaded nanostructures, mediated by cell surface recognition.[57] Strands modified with biotin, in either a DNA tube or a DNA tetrahedron, showed enhanced internalization over a circular plasmid control. DNA tetrahedra showed higher internalization likely due to the compact size compared to nanotubes. Meanwhile, an octahedral DNA nanocage modified with biotin and folate was used to probe two distinct internalization pathways and to show that the selection of cellular receptor target is crucial for ultimate cellular destination and lifetime of the nanostructure.[120]

Component DNA strands can be designed to include additional sequences that increase cellular uptake efficiency. For example, addition of four CpG sequences, which are recognized by the specialized immune system receptor Toll-like receptor 9, to tetrahedra enhanced internalization in immune cells compared to bare or singly modified tetrahedra.[121] Meanwhile, aptamers, oligonucleotides that bind specifically to a target (ligands or receptors),[122,123] have also been utilized to enhance delivery of nanostructures to cells.[83,85] The sgc8 aptamer effectively delivered drug-loaded DNA nanotrains to CEM cancer cells.[83] A nucleolin aptamer on a DNA nanorobot enhanced its internalization into human umbilical vein endothelial cells and facilitated greater delivery of its payload.[85] In both cases, the aptamer recognized its associated target, allowing for coalescence of the nanostructures on the cell surface and promoting internalization. As such, the underlying theme of these covalent DNA modifications is the aim of targeting cell surface receptors to promote endocytosis. As discussed in the previous section, this can allow for targeted delivery, and now we are seeing that it also enhances the internalization efficiency. These types of modifications are doubly advantageous tools of the molecular suit.

3.2. Transfection Methods

Transfection methods introduce DNA to cells by creating transient pores in the cell membrane (Figure 3d). DNA nanostructures in the surrounding solution can then diffuse into the cell before the membrane reseals. Classical DNA transfection methods include electroporation and heat shocking. Both of these methods are incompatible with DNA nanostructures as heat and electricity will affect their structural integrity. As a result, these methods have been largely neglected by the DNA nanotechnology community save for a few examples. The utilization of the cationic polymer spermidine can protect DNA nanostructures against the denaturing effects of electricity required to electroporate Jurkat cells.[124] An approach that bypasses the classical methods' unfavorable conditions is the use of a microfluidic device.[125] The device focuses cells to a T-junction, where pores in the cell membrane are generated upon collision with a small protrusion on the device wall, and introduced nanomaterials can then enter the cell. Under this method, the nanostructures do not have to rely on coatings or modifications and can be effectively delivered in under 2 min. This application is highly advantageous as it does not require any additional functionalities to ensure efficient delivery, but it lacks the ability of targeted cell delivery in multicellular systems and is only applicable to in vitro cell samples. As we have seen, there is an ever-growing effort to enhance the cellular internalization of DNA nanostructures, yet this is all for naught if the structures cannot survive long enough to perform their intended task.

4. DNA Nanostructure Stability and Biocompatibility

Whether used through passive or active delivery, the stability of the nanostructure is of utmost importance. If a DNA nanostructure cannot survive in the conditions to which it is exposed, it cannot perform its intended task. The main issues encountered in DNA nanostructure stability in vitro and in vivo are low cation availability and an abundance of nucleases (**Figure 4a**). DNA nanostructures require a high divalent cation concentration ($(5–20) \times 10^{-3}$ M) to successfully form, and this is an order of magnitude higher than that found within a cell or in cell culture media. Nucleases are actively present in cells and cell culturing media, which can result in the rapid degradation of oligonucleotides. Linear oligonucleotides exhibit very short lifetimes, as short as a couple of minutes, in the presence of nucleases.[126] Comparatively, higher order DNA nanostructures exhibit a greater resistance to nucleases, but they are ultimately still susceptible to degradation, especially those structures that contain many nicks or end points. The question and concern of nanostructure stability is definitely an important one, yet the solution is not entirely conclusive; many studies have confounding results,[127] and much of a structure's stability is dependent on the 3D and compact nature (or lack thereof) of a particular nanostructure. As such, there have been investigations specifically in studying the stability of nanostructures under various in vitro and in vivo conditions.

DNA origami structures were shown to be stable for up to 12 h in a prepared cell lysate, though a decrease in recovered origami from 4 °C to room temperature suggests that there is a temperature dependence associated with this stability.[128] A more comprehensive investigation at physiological temperatures revealed that denaturation of nanostructures at low magnesium concentrations is size and shape dependent.[129] A DNA nanotube was the only one of the three structures to survive up to 24 h in only 0.4×10^{-3} M Mg^{2+}, while the other structures studied (octahedron and nanorod) needed at least 6×10^{-3} M magnesium to be stable for 24 h.[129] In media supplemented with 6×10^{-3} M Mg^{2+}, the same structures were shown to be equally susceptible to degradation by nucleases present in 10% fetal bovine serum (FBS) while no significant degradation was seen in 1.25–2% FBS. Interestingly, the age of the FBS played a major role in their lifetimes as the activity of nucleases decreased over time.

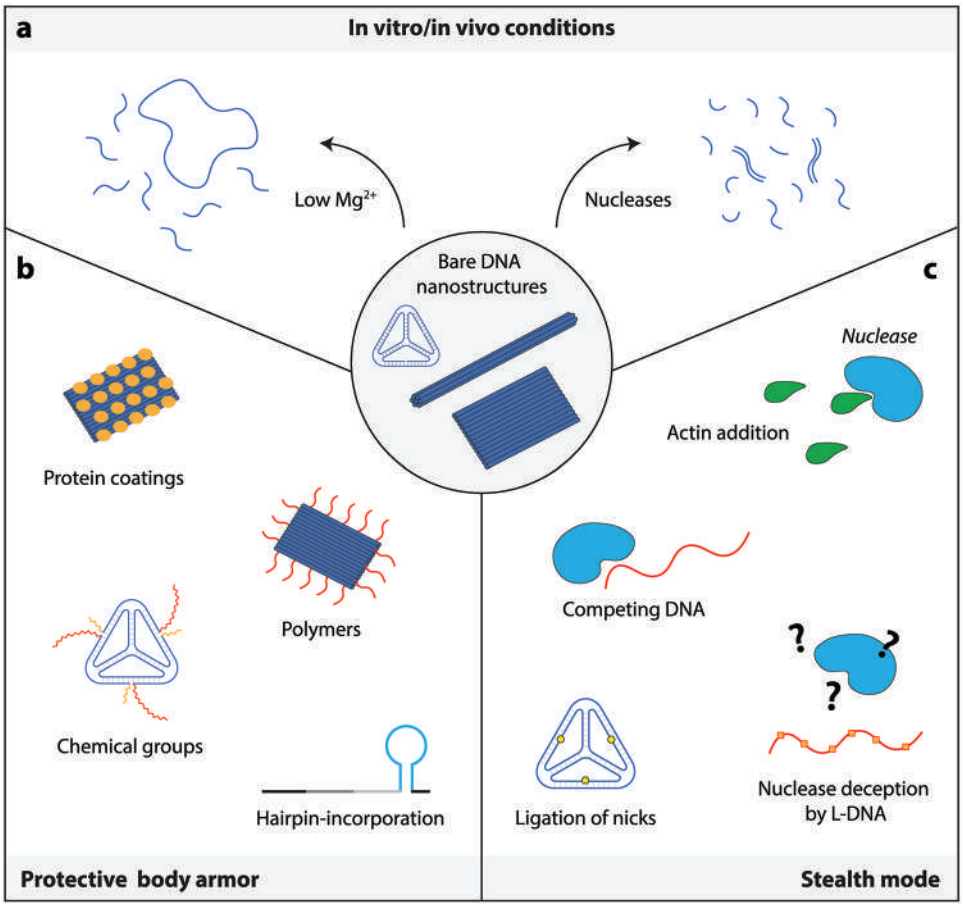

Figure 4. Fates of DNA nanostructures under typical in vitro and in vivo conditions, and methods to protect them in these conditions. a) Low magnesium and nucleases cause denaturation and degradation of DNA nanostructures respectively. b) Protective coatings act as nucleic acid body armor to reduce nuclease action and denaturation by low cation availability. c) Modifications to the DNA or the surrounding media allow nanostructures to stealthily evade destruction.

These studies provide the DNA nanotechnology community with insights into what is required for nanostructures to survive the impractical conditions they must navigate through in vitro. But will this be the case in vivo? A recent study, looking at nanostructures incubated in human plasma, may contradict the current held belief that these DNA nanostructures will be readily degraded in suboptimal culturing conditions, and therefore the same will happen when applied in vivo.[130] The stability and function of two DNA nanomachines were compared in real human blood and in non-heat-inactivated FBS. Due to varying activity levels of the nucleases in animal serums, the nanomachines showed a sixfold increase in lifetime in the human serum (20–44 h) compared to FBS (4–8 h). Human serum contains actin and saline in addition to DNase I, which naturally inhibit the nuclease likely conferring longer lifetimes to the DNA. The tetrahedron, a renowned stable nanostructure, was shown to exhibit even longer lifetimes in human serum. This structure was used to carry ruthenium polypyridyl complexes (RuPOP) to cause reactive oxygen species (ROS)-mediated cell apoptosis. The tetrahedral cage and the cage containing RuPOP complexes remain stable in human plasma for 72 h.[57]

Bare origami-based nanotube[129] and tetrahedron[131] structures exhibit notably greater stability among other nanostructures. Both of these nanostructures are utilized in many drug delivery and imaging systems (see Section 2). Although the topology, compactness, and rigidity of these structures provide some stability, it is important for their applications that their structures remain intact and functional for as long as possible. As a result, there is still a significant effort in development of protective body armor to enhance the stability and longevity of DNA nanostructures in culturing and in vivo conditions.

4.1. Stability by Protective Body Armor

Given the susceptibility of DNA nanostructures to denaturation and degradation, there has been a significant effort in developing a body armor suit to protect the precious DNA and associated cargo. The main avenues of body armor development include the manipulation of the nanostructures without the addition of extra components, or "native body armor generation," and the manipulation of nanostructures with additional components, or "external body armor generation" (Figure 4b). For native body armor, research groups mainly rely on the packing or 2D and 3D structural properties of the DNA.

DNA assemblies have been coupled with polymers to create densely packed DNA–polymer amphiphiles (DPA), where a DNA corona coats a hydrophobic organic polymer core.[132] The high density of negative charge in these structures reduces nuclease activity by steric hindrance. DPAs were stable against a sequence-specific nicking enzyme (*Nt.CviPII*), and somewhat stable in the presence of a nonspecific exonuclease (*Exo III*) where only a few bases on the 3′ end were removed. They also were tested against a more aggressive exonuclease, snake-venom phosphodiesterase, where surprisingly the DPAs showed resistance similar to that of Exo III. In a different vein of native structural modification, Fern and Schulman recently studied the effect of different secondary structures on DNA circuit strands.[133] In Dulbecco's modified Eagle medium media supplemented with 10% FBS and competing ssDNA, a 3′ hairpin domain in the DNA circuit increases lifetime and functionality over a 7 h incubation.

Researchers have added a protective coating to the DNA nanostructures of interest in a variety of ways to generate exterior body armor. For example, CpG modification not only increased the delivery efficiency and specificity, but also conferred stability in a 50% FBS supplemented media for 2 h when compared to ssDNA which rapidly degraded in the same media. DNA prismatic cages containing component strands modified with hexaethylene glycol, hexanediol, and phosphate on 5′/3′ ends have lifetimes of 62 h in serum.[47]

A well-characterized technique for plasmid DNA, protection by liposomes and polymers, has been adapted for the protection of DNA nanostructures. A mixture of poly(ethylene glycol) (PEG)–DOPC (1,2-dioleoyl-*sn*-glycero-3-phosphocholine) and lipid-conjugated oligonucleotides generated a virus-inspired encapsulation of a DNA octahedron, promoting its in vivo stability.[49] The encapsulated DNA octahedron (E-DNO) exhibited decreased renal clearance by 77% compared to nanostructures not viral-encapsulated. Recently, a group of scientists showed that rectangular DNA origami nanostructures have renal-protective properties.[134] DNA origami nanostructures were preferentially accumulated in the kidneys of mice, and were therapeutically efficacious in mice with acute kidney injury. Electrostatic association of a block co-polymer, PEG-PLys (poly(ethylene glycol)-*b*-poly(L-Lysine)), with the negatively charged DNA origamis such as flat rectangle (RO), 24-helix bundle (24-HB), 6-helix bundle (6-HB), and wireframe dodecahedron (D-truss) form polyplex micelles.[135] The DNA origami polyplex micelles (DOPMs) are stable and functional for at least 16 h in DNase I, or RPMI–10%FBS at 37 °C as well as in a buffer containing no Mg^{2+} but 30×10^{-3} M NaCl. The copolymer, K_{10}–PEG_{5K}, which retained structure and increased transfection efficiency, also increased stability in the presence of low divalent cations and nucleases.[117] Similarly, the protein-based coatings mentioned in the previous section have shown to also increase stability, further emphasizing that these body armor components have versatile functions.[48,115,116]

4.2. Stability by Stealth Mode

Another means to protect DNA nanostructures involves putting the structures into "stealth mode" so that they evade dangerous conditions (Figure 4c). The use of L-DNA[136,137] or peptide nucleic acids[138] in nanostructures avoids recognition by nucleases, and ligation[131] increases lifetime as exonucleases can no longer act on free 5′ and 3′ ends. Other strategies include crosslinking helices within nanostructures using click chemistry[139] or photochemical crosslinking.[140,141] Such modifications increase stability at low salt concentrations and high temperatures along with improved resistance to nucleases. Supplementing media with $MgSO_4$ reduces denaturation by low cation availability, and addition of inhibitory agents allows the DNA nanostructures to stealthily evade degradation by nucleases. Supplementing with actin[129] or ssDNA[133] (such as poly-T or salmon sperm DNA) efficiently out compete the more important DNA nanostructure components for nuclease activity. Heat treatment of serum is an effective method of reducing nuclease activity, but it does not support viable cell culturing.[129] Unique formations of the DNA itself can provide enhanced stability against nucleases. RCA appears to promote these special formations as an RCA-made nanotube[114] and nanoflowers[99] showed resistance to degradation in the presence of nucleases. It is likely that the long, non-nicked DNA amplified by RCA, as well as the dense packing of DNA, and extensive inter- and intrastrand weaving conferred added resistance to these formations.

4.3. Biocompatibility

In another way, these DNA nanostructures must be stealthy so that they do not trigger a host immune response when administered in a clinical setting. Although DNA nanostructures are a foreign species, it is generally found that they are non-immunogenic by the lack of significant increase of cytokines such as interleukin (IL)-6, IL-10, tumor necrosis factor-α, and interferon-α.[85,129,137] This confirms that treatment with intact DNA nanostructures results in phenotypically normal immune responses. Interestingly, the BSA-G2 coating of 60-HB further shields the nanostructure from immune response, showing a tenfold decrease in IL-6 production and suggesting that modifications of the nanostructures can also reduce host inflammatory response.[48,142] In another aspect of biocompatibility, the DNA nanostructures used as carriers must also clear out of the body once the payload is delivered. Previous research on nanoparticle-based delivery has implied that nanoparticles not taken up by tumors are degraded through phagocytosis.[143] In a similar fashion, DNA nanorobots containing thrombin as the cargo were shown to accumulate in the liver, possibly via mononuclear phagocyte system.[85] After 24 h, the fluorescence of these nanostructures was minimal in all organs, showing successful degradation and clearance of excessive nanostructures from the organism.

Overall, the body armor components have had beneficial effects in many different areas of nanostructure application, but judicious choice depending on the desired application is paramount. For example, hairpin addition may be beneficial for simpler detection designs, while large polymer coatings would be useful for use of DNA origami structures required to circulate in vitro or in vivo for extended periods of time. Further investigation into other armor types or components is advantageous for translation of these devices to the clinical arena.

5. Conclusion and Outlooks

Since the advent of 3D DNA scaffolds, there have been many studies on designing various DNA nanostructures with increasing complexities and functionalities. This review highlights many of these, their applications, and properties. We focus on prominent applications and solutions to areas of concern that comprise DNA nanostructures-based molecular suit, including advances in drug delivery and tracking, enhancing internalization, and prolonging stability. Aiming to promote the use of DNA nanotechnology in a broader context and other disciplines, we suggest the following areas of improvement and future directions for the DNA nanotechnology field: generalization of design principles and collation of nanostructures, comprehensive characterization of nanostructure cellular uptake and stability, applications in new spaces and translation to the clinical setting.

In recent years, there has been a greater focus on using established DNA nanostructures for specific applications, not just as a proof of concept, but to actually make them work in real-life scenarios in an effort to push the DNA nanotechnology field toward translation to a clinical and applicable setting. The application-focused DNA nanostructures entail complicated design and assembly strategies and techniques that are very specialized to certain labs. Though in some cases, applications are not necessarily dependent on the complexity of the DNA nanostructure. Rather, simple, reconfigurable DNA structures and devices are closer to real-world applications, such as monitoring in vivo pH,[144] and detecting chloride,[145] calcium,[146] or cellular microRNA.[34] In the case of drug delivery, there are a variety of nanocages that can host different drug molecules. While these have been studied individually in different projects, there is lack of general design rules or parameters that can help to create drug delivery vehicles with greater potential.[147] We propose that a generalized design strategy be developed among the DNA nanotechnology community, and a database of currently designed and characterized DNA nanostructures will help to streamline production and applicability in many settings.

Furthermore, a thorough understanding of the cellular uptake of DNA nanostructures of different shapes and sizes is required for these structures to be efficient in targeted delivery during real applications. The biostability of DNA carriers must be addressed as well. That is, every DNA-based system for real applications should be field tested in its optimized molecular suit to maximize uptake and stability. This will further ensure the compatibility of DNA nanostructures in different cell types or animal system. These areas too would benefit from a streamlined process and informative database.

The use of DNA as a nanocarrier hinges on its properties. While these nanostructures provide metrics comparable to existing drug delivery methods, they have additional advantages including functionalities such as cell targeting, triggered release, and monitoring delivery. Adding such functionalities to DNA nanostructures is possible due to the different types of chemistries now available;[148] some of these have even been commercialized. Postassembly functionalization of DNA nanostructures is also possible through sequence specific recognition[91] or click chemistry.[97] Control of these nanostructures by external factors such as light provides spatial and temporal regulation of these drug carriers in living systems.[93] DNA nanorobots are designed to be triggered in living cockroaches even by physiological signals derived from brain activity of a human subject.[149] Development of dynamic DNA nanostructures that can move on DNA tracks[150] is a stepping stone to the creation of in situ theranostics. For such developments to ultimately be useful in a clinical setting, an ease of use and low cost must be addressed. With more and more commercial availability of DNA strands and scaffolds, it is becoming easier to purchase requisite components. But the generation and modification of DNA nanostructures is still prohibitive to outsiders in the field; an ease of assembly would be possible with streamlined procedures or premade kits such as those commercially available through tilibit nanosystems GmbH. Given the ever decreasing cost of DNA,[151] efforts aimed at characterizing the loading capacity, cellular uptake profiles, and efficiency of such a variety of DNA nanostructures should be feasible without being too expensive.

Finally, we encourage developments in disciplines which have not heavily utilized designer DNA nanostructures. A discipline in which we have seen some initial work, but believe can be further developed, is the control of stem cell behavior/differentiation.[152,153] For example, multiple ephrin molecules (a protein ligand) may interact with multiple Eph receptors to induce stem cell differentiation;[154] placing this molecule at different spacing may induce different stem cell behaviors (**Figure 5**). Specific ligands can decorate DNA nanostructures at

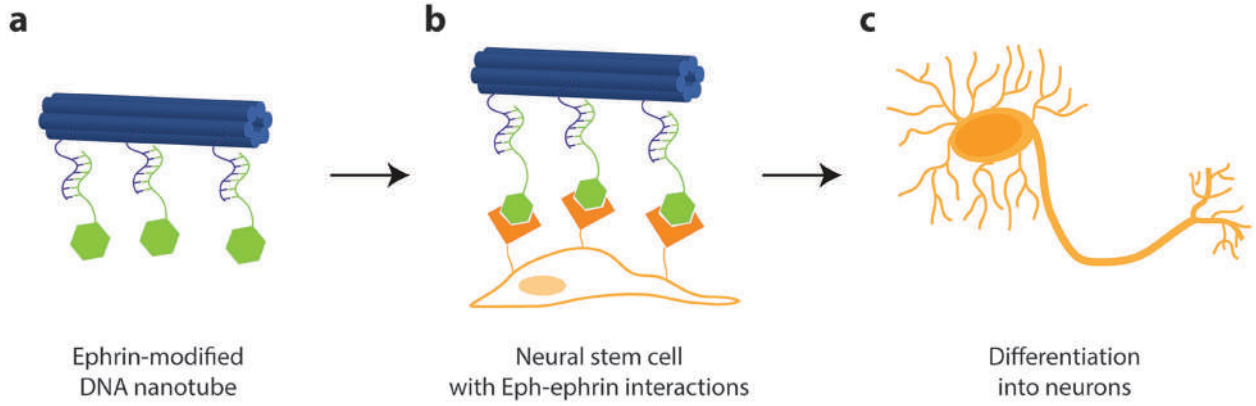

Figure 5. DNA nanotube promotes stem cell differentiation. a) DNA nanotube is decorated with ephrin proteins. b) Ephrin proteins interact with Eph-receptors on the surface of the neural stem cells. c) Differentiation into neurons is controlled through different Eph–ephrin interactions.

different spacing to control the stem cell differentiations. This leaves room for development of DNA scaffolds for a wide range of cell and ligand types. With these challenges solved, DNA nanotechnology is poised to permeate new areas of research and to provide organism- and disease-specific drug delivery with autonomous and dynamic control of multidrug release through biological cues.

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Conflict of Interest

The authors declare no conflict of interest.

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