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Design and Application of Multifunctional DNA Nanocarriers for Therapeutic Delivery

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Abstract

The unique programmability of nucleic acids offers versatility and flexibility in the creation of self-assembled DNA nanostructures. To date, many three-dimensional DNA architectures have been precisely formed of varying sizes and shapes. Their biocompatibility, biodegradability, and high intrinsic stability in physiological environments emphasize their emerging use as carriers for drug and gene delivery. Furthermore, DNA nanocarriers have been shown to enter cells efficiently and without the aid of transfection reagents. A key strength of DNA nanocarriers over other delivery systems is their modularity and their ability to control the spatial distribution of cargoes and ligands. Optimizing DNA nanocarrier properties to dictate their localization, uptake, and intracellular trafficking is also possible. In this review, we present design considerations for DNA nanocarriers and examples of their use in the context of therapeutic delivery applications. The assembly of DNA nanocarriers and approaches for loading and releasing cargo are described. The stability and safety of DNA nanocarriers is also discussed, with particular attention to the in vivo physiological environment. Mechanisms of cellular uptake and intracellular trafficking are examined, and we conclude with strategies to enhance the delivery efficiency of DNA nanocarriers.

Keywords

DNA nanotechnology; delivery vehicles; self-assembly

Introduction

For numerous diseases such as muscular dystrophy, cystic fibrosis, and sickle-cell anemia, the genetic basis (and hence therapeutic target) is known [1]. Viruses are an extremely effective approach for gene transfer, but safety concerns continue to motivate the need for alternatives. In other diseases such as cancer, their complex nature remains an open challenge, with combination therapies driving the development of multifunctional delivery systems. Regardless of the specific disease, the potential of any therapeutic intervention hinges on safe and efficient delivery. The field of DNA nanotechnology has now matured to the point where nucleic acid nanostructures are poised to be exploited as multifunctional and modular carriers for drug or gene delivery.

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The structural programmability of DNA via Watson-Crick base pairing allows the formation of discrete nanostructures with high precision and efficiency. The control over formulation and versatility are hallmarks of DNA nanocarriers over other delivery vehicle systems. Since particle size and shape significantly affect the fate of delivery vehicles, DNA nanocarriers can be optimized for specific diseases. Furthermore, biodegradability, biocompatibility, and the ability to load and release cargo further promote interest in utilizing DNA nanocarriers as therapeutic vehicles. To date, DNA nanocarriers have been employed in several applications including imaging, delivery of nucleic acids and drugs, and as vaccine adjuvants [2-9].

In this review we focus on design criteria for DNA nanocarriers and factors that govern the efficiency of these delivery systems. We begin by summarizing the formulation of DNA nanocarriers and approaches for therapeutic cargo loading and release. The ability of DNA nanocarriers to recognize target sites and to be internalized by cells is subsequently discussed. Because of their importance, biological stability and safety are also examined, although there is relatively little data on this emerging topic. We conclude with future perspectives toward the improvement of these systems in delivery applications.

Design and Assembly of DNA nanostructures

A key strength of DNA nanostructures is that the base-pair hybridization underlying their self-assembly process is extremely specific. As a consequence, precisely defined three-dimensional structures are obtained in high yield. It is often difficult to obtain such narrow size distributions with other techniques driven by polyelectrolyte complexation or the hydrophobic effect. Furthermore, a tremendous diversity of nanostructure shapes and sizes can be realized. The smallest structure reported to date is a DNA prism constructed out of a single DNA strand with a characteristic dimension of 3.4 nm [10], which is roughly the size of a 10 kDa globular protein [11]. Separate efforts have focused on building increasingly larger structures, for example, DNA wireframe structures have been created with radii of 50-100 nm [12], DNA “containers” have been created with edges up to 55 nm in length [13], and DNA nanotubes can reach axial dimensions exceeding 10^4 nm (i.e., 10 microns) [14], [15] (Table 1). Besides excellent control over size and shape, DNA nanostructures have tunable mechanical properties: the local rigidity of DNA nanostructures can be attenuated by placement of nicks (defects) or mismatches [16], [17], or it can be increased by the incorporation of crossover motifs [18], [19]. This interplay of rigidity and flexibility can also be deliberately balanced to achieve specific geometries [20].

The assembly of DNA nanostructures begins with a design step using computational tools to generate candidate sequences for a given size and shape [19, 21-24]. These sequences are “assigned” along the oligonucleotide building blocks (Figure 1A) or longer single-stranded DNA (ssDNA) which constitute the nanostructure. Once the candidate ssDNA strands are synthesized or otherwise obtained, the assembly step is performed. Typically, assembly involves simple mixing followed by thermal annealing to yield the final desired structures. It should be noted that the sequences generated in the design step must be sufficiently unique to avoid unwanted interactions among the ssDNA strands during assembly [21]. Differences in various design and assembly strategies can be categorized by the nature of the process (e.g., one-pot, step-wise, scaffolded), or by the structural features (e.g., asymmetric, origami). For further details of design and assembly we refer the interested reader to the relevant reviews [25-28], while emphasizing that there is much ongoing work to create ever more complex and intricate DNA-based nanostructures. For the sake of brevity here, we mention selected assembly shapes in Table 1, along with their sizes, assembly strategies, and characterization methods. Similarly, the examples of DNA forms and nanostructures given in Figure 1 are not meant to be exhaustive, but rather, representative.

Given the wide range of sizes and shapes summarized in Table 1, we can restrict this list of candidates by applying considerations relevant to delivery carriers. First, the size of the carrier will dictate its maximum cargo-carrying capacity, and its chemistry will dictate how cargoes can be loaded. As already mentioned, the characteristic dimensions for DNA nanostructures vary from a few nm [10], [29] to tens of nm [30]. Second, there is evidence that shape and mechanical properties can improve carrier performance in vivo [31-33]. Third, while there is substantial evidence supporting a key role for size on cellular uptake and intracellular trafficking routes [34], the role of carrier mechanical properties is an unexplored topic.

Cargo Loading and Release from DNA Nanocarriers

Several approaches have been used to either entrap or attach cargo to DNA nanocarriers. If the cargo is a nucleic acid itself, then the cargo can be directly integrated into the carrier nanostructure during the design stage. Indeed, this approach has already been used to “load” antisense [5], aptamer [3], and CpG sequences [35] into wireframe DNA tetrahedra (listed as “Integrated” in Table 2).

Passive physical entrapment is a simple but inefficient method that is reasonably successful if the cargo is compatible with the assembly process (e.g., thermal annealing) and smaller than the internal space of the carrier. This approach was used by Sleiman and coworkers to load gold nanoparticles into wireframe DNA tubes [36]. However, especially for “open” structures, including specific interactions between cargo and carrier is desirable to minimize cargo escape by diffusion. Church and coworkers passively loaded gold nanoparticle or antibody fragment cargoes into hexagonal DNA barrel structures [37], and to obtain retention of this cargo they incorporated partially hybridized strands (i.e., overhangs, see Figure 1B) on both cargo and carrier (listed as “Overhangs” in Table 2). Depending on the relative sizes of cargo and carrier, this deliberate introduction of overhangs into a DNA nanocarrier can permit direct cargo attachment (if it bears the complementary strand) before assembly, and hence higher loading efficiencies. Other advantages of the overhang approach over synthetic conjugation chemistries include: its natural compatibility with nucleic acid cargoes (e.g., antisense, aptamers, siRNA), no chemical modification of the DNA is required, and the assembly process is generally unchanged. Furthermore, control over the number and placement of overhangs gives exquisite flexibility in regard to the local presentation of cargoes and the valency of interactions with cell receptors, to be discussed in the following sections.

If the desired cargo cannot be readily linked to DNA, the overhang approach cannot be pursued and other chemical approaches are necessary. Such linkages are often common bioconjugation reactions which are realized with modified DNA bases or modified DNA ends [38]. Whether or not the cargo remains attached to the DNA nanostructure (or a DNA strand) can of course influence therapeutic activity. Towards addressing this issue conjugation strategies can be used to enable non-covalent linkages (e.g., coordination complexes, antibody-antigen) (listed as “Non-covalent” in Table 2). Such interactions have the advantage of being modular and reversible, although equilibrium binding affinities need to be considered. Among non-covalent approaches, the metal ion coordination complex hexahistidine-NTA has been used by our group to attach proteins to wireframe DNA tetrahedra [39]. This coordination approach is particularly of interest because of its sensitivity to multiple stimuli (e.g., pH, ion chelators, and temperature). Taking advantage of the strongest known non-covalent interaction, Mao and coworkers showed that streptavidin could be linked to wireframe DNA tetrahedra via displayed biotin groups [40]. Because streptavidin is tetravalent, any remaining free sites could in principle be used to introduce any other biotin-linked cargo (of which there are many). Lastly, the antibody-antigen

interaction has also been explored to stabilize cargo; either as the antibody itself [40] or as an antigen captured by a DNA-bound antibody [37].

DNA nanocarriers typically have aqueous interior compartments, making them naturally suited to carry hydrophilic therapeutic cargoes, such as bioactive nucleic acid motifs (e.g., antisense, DNAzymes, siRNA, or miRNA), peptides, and proteins. Such biomolecules are typically susceptible to enzymatic degradation, and thus encapsulation within an interior aqueous compartment will help preserve bioactivity. It is worthwhile to emphasize that the purpose of encapsulation must be considered in the context of the cargo. For example, many drugs are hydrophobic and sparingly soluble in aqueous solution; therefore encapsulation is needed to attain therapeutic dosages. Such hydrophobic drugs are not amenable to be carried by DNA nanocarriers or other carriers with aqueous compartments. One notable exception is the anti-cancer agent doxorubicin, which by nature of its chemical structure can intercalate within the DNA double helix through pi-pi stacking (see Table 2). It should be noted, however, that intercalating agents affect the mechanical properties of the double helix [41], [42], potentially altering the properties of DNA nanocarriers, such as their uptake and trafficking. Other drugs like cisplatin will react with the DNA nanocarrier itself, and therefore are not likely to be good candidates for either release from the DNA nanocarrier or for therapeutic activity.

Besides the interior space of DNA nanocarriers, their outer surfaces can also be modified with great precision. For example, Huang and coworkers demonstrated controlled placement of aptamer strands on the outer surface of wireframe DNA icosahedra [3]. Anderson and coworkers were able to place folate ligands in various well-defined patterns on the surface of wireframe DNA tetrahedra [9]. In both the above cases, these surface ligands were used to promote cell targeting (see section below). The controlled placement of ligands or functional groups is also important to responsive DNA nanostructures: in “lock-and-key” systems [30], [43], where binding of ligands trigger large conformational changes.

As hinted at earlier in this section, various cargo loading issues need to be considered regardless of the delivery strategy. In a few cases some effort has been made to quantify encapsulation efficiency, which is typically defined as the ratio of loaded cargo mass to initial cargo mass. In two separate studies of doxorubicin loading into DNA nanocarriers, it was found that encapsulation efficiency depends on the initial bulk concentration [3] and incubation times [8]. Other conventions are also used to characterize delivery carriers, such as the weight ratio of cargo to carrier (i.e., “loading”) or the percentage of theoretical capacity [44]. In Table 2 we report cargo loading on a per carrier basis, for simplicity. It is apparent and perhaps expected that small molecules such as drugs and dyes can achieve much higher loading than larger complex biomacromolecules. Interestingly, Church and coworkers found a distribution of antibody fragment and gold nanoparticle loadings within DNA barrels, with mean values of 25-33% of the theoretical capacity [37], suggesting either steric or mass transport limitations.

As is well known in the field of gene and drug delivery, cargo loading is only one part of the challenge; in general, release is also needed for therapeutic activity [34, 45]. Various stimuli can be used to trigger conformational changes in DNA structures and thereby achieve cargo release. Numerous papers have used strand-displacement reactions to switch between different states [46], [47], and in some cases used this switching to release or expose a cargo [36]. However, such strand-displacement approaches are not suited for in vivo applications due to the need to introduce these trigger strands.

An alternative approach for triggering release is to exploit natural intracellular conditions, such as pH or redox potential. Indeed, several DNA objects have been constructed which

use the non-Watson-Crick base pairing between cytosines at low pH [48-50]. Our group recognized the example from nature whereby acidification triggers (viral) dis-assembly, and taking this inspiration, we designed and constructed DNA tetrahedra which dis-assemble at low pH but are assembled at neutral pH [39], enabled by “i-motif” quadruplex structures [51] (see Figure 1C). These DNA tetrahedra were also shown to release protein cargo based on this acidification trigger. A related approach to the above is to use ligands as triggers, specifically in the context of binding to DNA aptamer structures [52],[53] (Figure 1C). Such ligands can further be chosen to be associated with disease states or local sites of inflammation. For example, Church and coworkers used the 41t aptamer to trigger carrier opening in the presence of platelet-derived growth factor (PDGF), and thereby present antibody fragments to cells [37].

Site-Specific Targeting

Early-generation therapeutic delivery carriers were often compromised by poor target selectivity, low internalization, and entrapment within undesired intracellular organelles [54]. To improve the performance of such carriers, various targeting strategies have been explored, which can be divided into passive or active schemes. Passive targeting is a non-selective targeting approach that exploits the unique characters of tumor morphology via the enhanced permeability and retention (EPR) effect [55], which allows nanocarriers smaller than a cutoff size to accumulate and be retained within tumors [56], [57]. A size range between 10 nm to 100 nm has been suggested to be optimal for passive targeting schemes [34], [54], and there are many nanocarrier-based drugs that are commercially available for cancer treatment, mainly in the form of liposomes or as polymer-protein conjugates [58]. From the above discussion, it is clear that DNA nanocarriers are also suitable for passive targeting since they can be assembled such that their size and shape fits the criteria for the EPR effect. Indeed, recent studies have been shown DNA nanocarriers to passively deliver antisense DNA [5] or chemotherapeutic drugs to cancer cells in vitro [3], [59]. However, the passive targeting approach suffers serious limitations. First, certain tumors can have non-uniform permeability, which leads to heterogeneous carrier extravasation and compromises delivery efficiency [60], [58]. Second, the non-selectivity of passive targeting will very likely cause off-target delivery to otherwise healthy cells, generating undesired toxicity and adverse side effects. Therefore, recent studies have put greater emphasis on active targeting schemes where nanocarriers will selectively localize to specific locations via receptor-ligand interactions.

The selection of receptor targets is generally based on their unique expression or overexpression on target tissues while being lacking or minimally present on non-target sites [34]. Subsequently, the ligands to these receptors can be identified and functionalized to nanocarriers for guided delivery. Even at the tissue targeting level, size is an important consideration. For example, microparticles are especially suited for targeting cells along large-size blood vessels due to their efficient localization to the cell wall [61], whereas carriers in nanometer size range are generally suited for tissues that have access to small-size blood vessels (e.g., cancer).

To our knowledge, both small molecules and aptamers have been used to guide DNA nanocarriers to target cells [3, 9, 37, 62]. Folic acid, which is a ligand for the folate receptor (a common cancer biomarker) can be conjugated to DNA nanocarriers via standard bioconjugation chemistry [62], [9]. A group of targeting ligands that are particularly suited for DNA nanocarriers are known as aptamers. Aptamers are short ssDNA strands that can bind antigens or receptors, and this binding ability is typically due to an internally hydrogen-bonded structure such as a quadruplex (Figure 1C). Aptamers can be readily incorporated into the oligonucleotide building blocks of a nanostructure, without the need for any

chemical modifications. Several works have shown that aptamer-functionalized DNA nanocarriers [3, 37] or antibody-functionalized nanocarriers [63, 64] can selectively bind to their receptors on cancer cells with minimal localization to non-target cells.

In addition to identity of a targeting ligand, the density and arrangement of such ligands on the carrier surface also plays a role on therapeutic delivery efficiency [9, 35, 65]. Anderson and coworkers demonstrated greater gene knockdown in human cervical cancer cells (HeLa) when treated with siRNA-loaded DNA tetrahedra that displayed at least three folate ligands on the same face or vertex of the nanocarrier [9]. This finding is consistent with the notion that ligand clustering increases binding affinity to receptors through multivalency, and we note that such ligand clustering may influence uptake and intracellular trafficking pathways, which will ultimately affect the efficiency of therapeutic delivery.

Cellular Uptake and Intracellular Trafficking

After being localized to their target sites, nanocarriers should generally be internalized into cells to release their payloads. Due to its hydrophilic and highly anionic nature, DNA is typically poorly transported across cellular membranes. Early work revealed that the penetration ability of oligonucleotides (i.e., linear ssDNA) is inversely proportional to their length [66]. In the years since, formulation of DNA with cationic polymers, lipids, or viral capsid proteins has been widely used to enhance DNA uptake efficiency; nevertheless, these systems can also induce cytotoxicity [67-70]. Alternatively, assembling DNA into three-dimensional nanostructures has been demonstrated to promote DNA intracellular uptake. Wireframe DNA tetrahedra were shown to exhibit greater cellular uptake relative to linear ssDNA and dsDNA forms, presumably due to their rigid and compact structure [71]. Similarly, the arrangement of DNA as wireframe nanotubes or as a dense layer of hollow spheres promoted cellular uptake without transfection reagents [15], [4]. Incorporating targeting moieties on DNA nanocarriers has also been demonstrated to promote DNA uptake [3, 62, 72]. Huang and coworkers observed greater internalization in breast cancer cells of aptamer-bearing wireframe DNA icosahedra relative to bare icosahedra. No uptake difference was found in a non-target cell line, emphasizing that the aptamer ligands facilitate both selectivity and cellular uptake of nanocarriers [3].

The availability of therapeutic agents at their target site (including intracellular locations) is needed for successful treatment. When internalized by cells, nanocarriers can be trafficked via several endocytosis pathways, described in reviews elsewhere [73], [74]. Here only three trafficking pathways relevant for DNA nanocarriers will be discussed. Briefly, clathrin-mediated endocytosis (Figure 2A) is thought to be the receptor-mediated endocytosis pathway mainly responsible for uptake in mammalian cells [73]. Nanocarriers entering cells by this pathway encounter progressive acidification while being routed from early endosomes (pH 6-6.8) to late endosomes and ultimately to lysosomes (pH 5); a final destination intended for degradation [75]. To prevent such cargo degradation, strategies using carriers with proton-buffering cationic polymers or membrane-disruptive agents have been extensively explored [73], [76]. However, any such disruption of endosomal compartments is expected to contribute to cytotoxicity.

Caveolae-mediated endocytosis (Figure 2B), in contrast to the clathrin pathway, is a slow process where nanocarriers are localized into caveosomes and transported to the Golgi and/or endoplasmic reticulum [73], [77] or endosomes [78, 79]. Macropinocytosis is a non-specific endocytosis pathway in which cell membrane ruffles sample the surrounding fluid and substances therein, forming a macropinosome which is internalized (Figure 2C). Depending on the cell type, the macropinosome either recycles its components back to the cell surface or fuses with lysosomes. Macropinosomes are considered leaky relative to other

endosomal compartments, allowing nanocarriers to be released into the cytosol [73]. Among these pathways, caveolae-mediated endocytosis and macropinocytosis hold particular promise for gene delivery, since nanocarriers trafficked along these pathways can bypass lysosome degradation to reach their target site (i.e., the cytosol or nucleus).

Several works have explored the trafficking pathways of DNA into cells. In early studies, the intracellular pathway of oligonucleotides (i.e., linear ssDNA) was demonstrated to be concentration dependent: at low concentration, ssDNA was found to bind to an 80 kDa protein on HL60 cell lines and enter via the clathrin-mediated pathway [66], [80]. As the ssDNA concentration increases and saturates the cell surface, macropinocytosis plays the primary role in uptake [80], [81]. Bijsterbosch and coworkers later showed that phosphorothioate oligonucleotides entered hepatic endothelial cells via the clathrin-mediated pathway by binding to scavenger receptors type AI/AII, which also have a molecular weight of about 80 kDa [82]. Similarly, plasmid DNA was demonstrated to localize into hepatic cells by binding to scavenger receptors [83]. Scavenger receptors (SRs) have been known to be involved in the non-specific cellular uptake of polyanionic ligands such as lipoproteins, polynucleotides, and polysaccharides [82]. Several classes of SRs have been discovered and each class is proposed to interact with different endocytosis pathways [84]. Although SR class A was shown to be involved in clathrin-mediated endocytosis and SR class B could trigger internalization via lipid raft-dependent endocytosis pathways [79, 85], there are cases where SR class A uptake is caveolar/lipid raft-dependent [86, 87].

The uptake and trafficking processes for DNA nanocarriers could be expected to be different than for conventional DNA forms (e.g, oligonucelotides and plasmids) since specific pathways are affected by factors such as size, shape, material, ligand, and of course, cell line [88-91]. Wireframe DNA icosahedra and DNA triangles were shown to enter cells by a SR-mediated pathway and transported to lysosomes of Drosophila haemocytes and C. elegans [2], [92]. However, the specific class of scavenger receptors that mediated DNA nanocarrier uptake was not reported in these studies. Because there are several SR classes that are unique to specific species [85], it is not clear if the results observed in Drosophila haemocytes and C. elegans would reflect behavior in mammalian cells. More recently, Ahn and coworkers observed nanocarrier internalization in human breast cancer cells and found evidence that wireframe DNA tetrahedra were internalized via both caveolae-mediated endocytosis and macropinocytosis pathways [59]. This latter finding suggests that DNA tetrahedra can be potentially used as a nucleic acid/gene carriers, since such pathways avoid lysosomal degradation.

Targeting ligands also play a part in determining the endocytic pathways explored by a nanocarrier. Wireframe DNA icosahedra displaying mucin-1 aptamers were demonstrated to localize into clathrin-coated vesicles and be transported to lysosomal compartments of human breast cancer cells, similar to the mechanism of free mucin-1 aptamers [3], [93]. Remarkably, the arrangement of ligands on nanocarriers can also affect intracellular trafficking. Stellacci and coworkers demonstrated that gold nanoparticles with similar ligand compositions but distinct spatial distributions could penetrate the cell membranes in different manners [65]. Specifically, nanoparticles with a structured ligand distribution entered cells via both endocytosis and non-endocytosis pathways and could escape to the cytosol. On the other hand, nanoparticles with a random ligand distribution inefficiently entered cells and tended to remain trapped in endosomal compartments [65]. Recent work from Anderson and coworkers explored gene silencing in mouse models with a wireframe DNA tetrahedra bearing both folate ligands and siRNA. Administration of these DNA nanocarriers in vivo demonstrated a substantial decrease of the target gene expression and a reduction in tumor volume [9]. Though it was not discussed in that work, the success of this delivery approach could be partially due to trafficking via caveolae-mediated endocytosis,

which is known to involved with folate receptor targeting [94]. The nucleolin-targeting aptamer AS1411 is another example of a ligand relevant to cancer treatment as it was shown to enter cells via the macropinocytosis pathway [95]. Our group is currently exploring AS1411 aptamer-bearing DNA tetrahedra as nanocarriers for combined therapeutic and nucleic acid delivery to cancer cells.

Stability in Physiological Environments

Independent of the spatial obstacles encountered in therapeutic delivery is the degradation of carrier or cargo that occurs in vivo, often catalyzed by enzymes. Although DNA nanocarriers are largely composed of dsDNA, they can also bear regions of circular or linear ssDNA as well as complex crossover structures. Furthermore, a DNA nanocarrier may lack free ends, which bears a topological resemblance to plasmid DNA (i.e., circular dsDNA). Thus it is difficult to predict the stability of DNA nanocarriers in physiological environments, especially with respect to more conventional DNA forms. We first briefly review the stability properties of conventional DNA forms, and then we turn our attention to stability properties of DNA nanocarriers.

Early studies of oligonucleotides (i.e., short linear ssDNA) showed excellent stability under in vitro assays, with no degradation after 2 hours incubation in whole human blood [96]. Stein and coworkers found similar results, reporting a half-life of 2-3 days in 50% human serum [97]. By contrast, oligonucleotides were rapidly cleared following intravenous administration, with initial half-lives of less than 5 min [96, 98, 99]. The length and strandedness of linear DNA also affect its stability in circulation: shorter dsDNA strands are cleared more quickly than longer dsDNA strands, and ssDNA is cleared more quickly than dsDNA [98]. These types of experiments have been described by pharmacokinetic models [100], and suggested that degradation and uptake pathways combine to determine the clearance half-lives: τ (circular dsDNA) > τ (linear dsDNA) > τ (linear ssDNA). The biodistribution of DNA is also sensitive to its structural details, with ssDNA being largely localized to the liver (in a length-dependent manner) and dsDNA being largely found in soft tissues (length-independent). Oligonucleotides showed similar biodistribution to dsDNA, suggesting that dsDNA is ultimately degraded into this form [98]. Consistent with earlier oligonucleotide studies, the in vivo half-life of plasmid DNA tends to be shorter than its in vitro half-life [83].

It seems plausible that the differences between in vitro and in vivo assays are mainly due to cell-mediated internalization, but there are likely other contributions such as protein adsorption, interactions with the extracellular matrix, and circulatory shear stresses. Furthermore, in vivo results should be expected to differ from each other due to variations in: dose, route of administration, animal species, and DNA labeling schemes [99]. For example, DNA delivered by intramuscular or intradermal routes is more stable than when delivered by the intravenous route [101].

Our group established that wireframe DNA tetrahedra demonstrate enhanced resistance to nuclease degradation as compared to linear dsDNA [102]. Others subsequently found similar improvements in stability for DNA origami tiles [103] and wireframe DNA nanotubes [15]. Several explanations for this nuclease resistance have been proposed, including mechanical rigidity, steric inhibition, and electrostatic effects. Interestingly, Mirkin and coworkers have shown that gold nanoparticles coated with high densities of short oligonucleotides also demonstrate nuclease resistance. This property of oligonucleotide-decorated gold nanoparticles appears to be mainly due to inhibition of nuclease activity caused by locally high ionic strength [104]. In fact, the gold cores appear to

be unnecessary: their removal leaves behind crosslinked polyvalent shells comprised entirely of oligonucleotides, which retain their nuclease resistant properties [4].

We emphasize that the high intrinsic stability of DNA carriers in physiological environments reduces, and may even eliminate, the need for protection strategies that are common in non-viral gene delivery. For example, chemical modification of the nucleic acid backbone (e.g., phosphorothioates) results in greater stability against nuclease cleavage [38], but can lead to undesirable side effects, toxicity, and a reduction in the intended biological activity [105-107]. Similarly, polycation:nucleic acid complexes, or “polyplexes,” [108, 109] confer protection from nucleases, but releasing the nucleic acid from these polyplexes to achieve to therapeutic effect can be a rate-limiting step [110].

Safety of DNA Nanocarriers

In any delivery carrier system, the balance between therapeutic efficiency and safety is a crucial requirement. Taking gene delivery as an example, although viral formulations were shown to substantially enhance gene transfection relative to other approaches, they are insufficient to offset toxicity concerns. Adenoviral vectors used for gene therapy were reported to cause a fatal systemic inflammatory response and multi-organ failure in a patient with partial ornithine transcarbamylase (OTC) deficiency [111]. This unanticipated clinical outcome led to a temporary suspension of all gene therapy trials in the United States and raised serious concerns regarding the safety of viral vectors [112]. On the other hand, plasmid DNA remains attractive for certain gene therapy applications despite its low transfection efficiency, due to its biocompatibility and safety. Administration of plasmid DNA to critical limb ischemia (CLI) patients in clinical trials showed excellent safety and tolerability, i.e., no serious adverse effects and no innate immune response [113].

This safety evidence of plasmid DNA in animal models and clinical trials sheds light on the potential of DNA nanocarriers as safe and efficient therapeutic vehicles [113], [114]. Nevertheless, it is possible that nanocarriers from similar materials but with different architectures can trigger distinct biological responses [115]; thus, direct investigations of the acute and chronic toxicities of DNA nanocarriers are still needed. Since the application of DNA nanocarriers for therapeutic delivery has recently emerged, there is very limited in vivo toxicity data. Nevertheless, the early signs are promising: in vitro cell viability assays verified that no toxicity was associated with either wireframe DNA tetrahedra or hollow DNA nanospheres [35], [4]. Using a mouse model, Anderson and coworkers measured the in vivo production of interferon-α cytokines, one of the first pathways activated in the innate immune response. They found no change in the level of interferon-α cytokines following in vivo administration wireframe DNA tetrahedra bearing folate and siRNA [9]. Although further studies are needed, these works suggest that DNA nanocarriers will demonstrate excellent biocompatibility, low immunogenicity, and low toxicity.

Conclusions

The ultimate goal of delivery carriers is to safely and efficiently transport therapeutic molecules to their intended location, all while overcoming the daunting set of obstacles presented by the physiological environment (e.g., nucleases, proteases, professional phagocytic cells). We have summarized some of the unique features of self-assembled DNA nanostructures (e.g., excellent control over shape and size) that make them especially well suited as carriers for drug or gene delivery. Their modular nature allows for the simultaneous loading of various cargoes as well as the precise display of targeting ligands. Delivery of bioactive nucleic acids (i.e., antisense, siRNA, miRNA, and aptamers) in particular is of considerable interest since these therapeutics can be readily integrated into

the DNA nanocarriers, without the need for chemical modifications. The nanoscale control of shape, size and rigidity, potentially allows these systems to target specific tissues and intracellular organelles by *design*. Furthermore, while synthetic carriers must address concerns of biodegradation, immunogenicity, and toxicity, all available evidence points to no such concerns for DNA nanocarriers. These attractive features will surely motivate continued efforts in the development of these systems.

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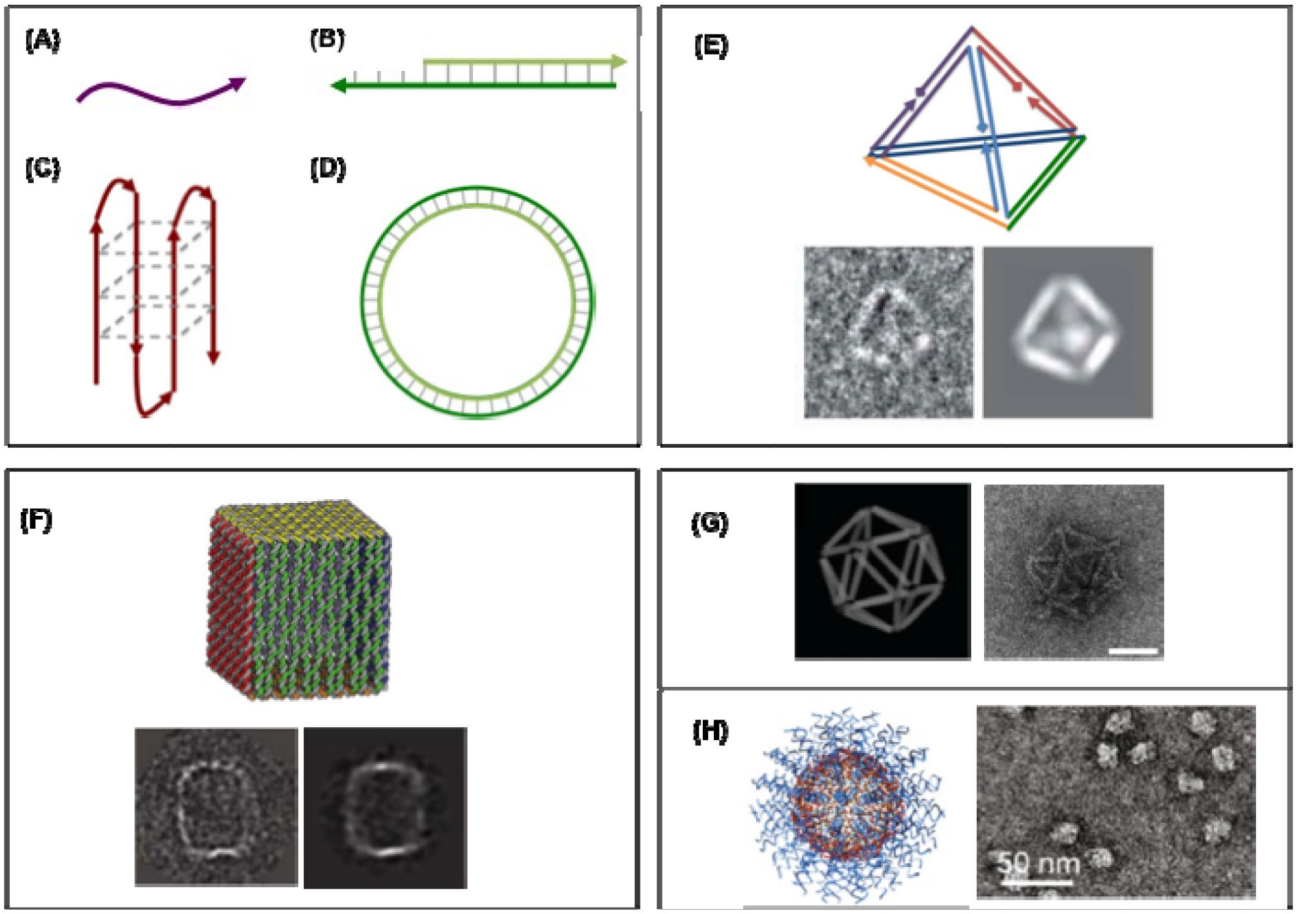

Figure 1.

Various structural forms and nanocarriers of deoxyribonucleic acid (DNA). (A) linear single-stranded DNA (ssDNA or oligonucleotides), (B) linear double-stranded DNA (dsDNA) with an overhang (also referred to as sticky end or toehold), (C) DNA quadruplex (D) circular double-stranded DNA (plasmid DNA), (E) wireframe DNA tetrahedron: image and raw cryo-EM image of individual particle and the corresponding projection of the DNA tetrahedron (Reprinted by permission from Macmillan Publishers Ltd: Nature [116], copyright (2008)), (F) DNA origami box with a controllable lid: a model and cryo-EM image and the corresponding 2D projection (Reprinted by permission from Macmillan Publishers Ltd: Nature [30] , copyright (2009)), (G) Orthographic projection model and TEM data of origami DNA icosahedron. Scale bar is 100 nm. (Reprinted by permission from Macmillan Publishers Ltd: Nature [43], copyright (2009)), (H) Coreless polyvalent nucleic acid spherical nanostructure: a model and TEM data (Reprinted with permission from (Cutler JI, Zhang K, Zheng D, Auyeung E, Prigodich AE, Mirkin CA. Polyvalent nucleic acid nanostructures. J Am Chem Soc 2011;133:9254-9257.) Copyright (2011) American Chemical Society [4]. For clarity, helical features of double-stranded DNA are not depicted and the schematics are not drawn to scale.

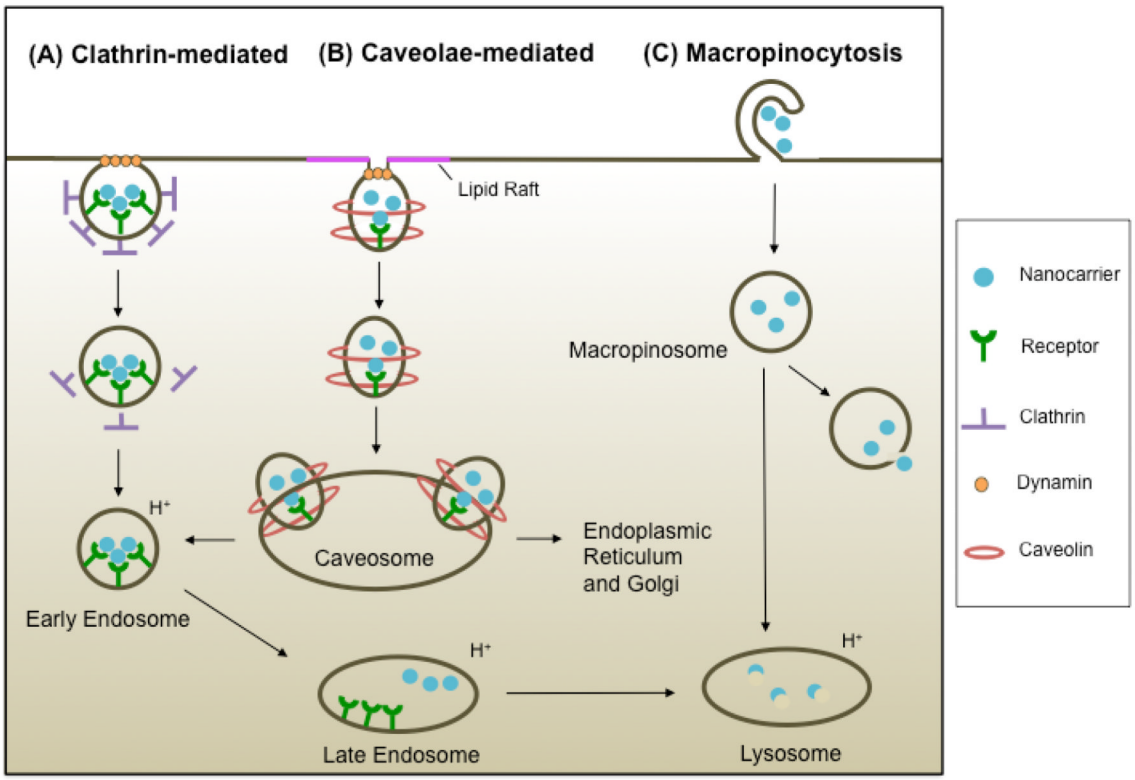

Figure 2.
Endocytic pathways relevant for DNA nanocarriers (adapted from [122]) (A) Clathrin-mediated endocytosis, (B) Caveolae-mediated endocytosis, and (C) Macropinocytosis. For clarity, recycling routes are not shown and the schematic is not drawn to scale.

Table 1

Summary of various DNA nanostructures, organized in order of increasing characteristic size. Abbreviations: WF, wireframe structure; EP, electrophoresis; AFM, atomic force microscopy; DLS, dynamic light scattering; EM, electron microscopy; SAXS, small-angle X-ray scattering.

Shape	Characteristic Size (nm)	Assembly Strategy	Characterization	Citation
WF prism	3.4	Asymmetric	EP, AFM	[10]
WF Tetrahedron	7	Asymmetric	EP, AFM	[29]
WF Tetrahedron	16	Symmetric	DLS, AFM, EM	[116]
WF Dodecahedron	24	Symmetric	DLS, AFM, EM	[116]
Square nuts, Crosses, etc	25	Origami	AFM, EP	[12]
Box	35	Origami	AFM, EM, SAXS	[30]
WF Buckyball	42	Symmetric	DLS, AFM, EM	[116]
Tetrahedron	55	Origami	AFM, EM, DLS	[13]
WF icosahedron	100	Origami	AFM, EP	[12]
WF Tubes	$10 \times (500, 10^3, 10^4)$	Hierarchical	EP, AFM, EM	[36], [117]
Tubes	$25\text{-}40 \times 10^4$	Crossover	AFM, Fluorescence	[14]

Table 2

Various cargoes that have been loaded within, or conjugated to, DNA nanocarriers. Loading is given on a number per carrier basis. Abbreviations: nt,nucleotides; WF, wireframe; cyt c, cytochrome c; EGFP, enhanced green fluorescent protein; Fab', antibody fragment; Dox, doxorubicin; Au NP, gold nanoparticle.

Cargo Type		Size	Loading	Container	Cargo-Container Stabilization	Citation
Nucleic acid						
	CpG	18 nt	1-4	WF Tetrahedron	Integrated	[35]
	CpG	20 nt	up to 62	Origami tube	Overhangs	[118]
	Antisense	20 nt	1	WF Tetrahedron	Integrated	[5]
	Aptamer	25 nt	12	WF Icosahedron	Integrated	[3]
	siRNA	30 nt	1-6	WF Tetrahedron	Overhangs	[9]
Protein						
	cyt c	12.4 kDa	1	WF Tetrahedron	Covalent	[119]
	EGFP	27 kDa	1-4	WF Tetrahedron	Covalent	[120]
	EGFP	27 kDa	1	WF Tetrahedron	Non-covalent	[39]
	Fab'	55 kDa	1-12 (avg 3)	Origami barrel	Overhangs	[37]
	Streptavidin	60 kDa	1-4	WF Tetrahedron	Non-covalent	[40]
	IgG	150 kDa	1	WF Tetrahedron	Non-covalent	[40]
Drugs						
	Dox	0.54 kDa	1200	WF Icosahedron	Intercalation	[3]
	Dox	0.54 kDa	1800*	Origami triangle & tube	Intercalation	[8]
Dyes						
	YOYO-1	1.3 kDa	48	WF Tetrahedron	Intercalation	[121]
Inorganic						
	Au NP	5 nm diam.	1-12 (avg 4)	Origami barrel	Overhangs	[37]
	Au NP	15 nm diam.	many	WF tube	--	[36]

* loading is estimated based on reference [3].